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Investigating The Role Of An Azorhizobium caulinodans DNA Binding Protein, AcBBP1, In The Expression Of The Seśbania rostrata Leghemoglobin glb3 Gene

presented by

Susan Yukie Fujimoto

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Major professor

Date 5/20/97

# INVESTIGATING THE ROLE OF AN *AZORHIZOBIUM CAULINODANS* DNA BINDING PROTEIN, ACBBP1, IN THE EXPRESSION OF THE *SESBANIA ROSTRATA* LEGHEMOGLOBIN *GLB3* GENE

By

Susan Yukie Fujimoto

A DISSERTATION

Submitted to Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Program in Genetics

1997

## ABSTRACT

INVESTIGATING THE ROLE OF AN *AZORHIZOBIUM CAULINODANS* DNA BINDING PROTEIN, ACBBP1, IN THE EXPRESSION OF THE *SESBANIA ROSTRATA* LEGHEMOGLOBIN G*LB*3 GENE

By

Susan Yukie Fujimoto

In a screen to identify DNA binding proteins which interact with the *Sesbania rostrata* leghemoglobin g*lb*3 (*Srglb*3) 5' upstream region, a factor originating from the symbiont of *S. rostrata*, *Azorhizobium caulinodans* was identified in extracts of *S. rostrata* nodules (Welters et al., 1993). Characterization and purification of the protein responsible for the binding activity (*A. caulinodans* Bacterial Binding Protein 1; AcBBP1) in addition to the characterization of its target in the *Srglb*3 promoter (Bacterial Binding Site 1; BBS1) using transgenic *Lotus corniculatus* plants harboring chimeric promoter-reporter gene constructs indicated that this interaction may be important for leghemoglobin gene expression. The gene encoding AcBBP1 was isolated, sequenced, characterized and subsequently, an AcBBP1 deficient mutant was constructed. A 20% decrease in nitrogen fixation activity was detected in nodules harboring AcBBP1 deficient bacteria using the acetylene reduction assay. Next, sections of *S. rostrata* stem nodules induced by the wild-type or the AcBBP1 deficient *A. caulinodans* strain were observed by transmission electron microscopy. Nodules harboring the AcBBP1 deficient strain contained a greater population plant cells which were uninfected or which appeared to be slightly delayed in the infection process when compared to its wild-type counterpart.

Immunogold labeling techniques localized AcBBP1 to the bacteroid and peribacteroid membrane. To directly test if BBP1 proteins play a role in *Srglb3* gene expression, transgenic *L. corniculatus* plants harboring the *Srglb3* promoter fused to the *uidA* reporter gene were generated. In addition, the gene encoding the homolog of AcBBP1 in *Rhizobium loti* (RlBBP1), the symbiont of *L. corniculatus*, was isolated, characterized and a *R. loti* RlBBP1 deficient strain was constructed. The *R. loti* wild-type and RlBBP1 mutant strains were inoculated onto the roots of transgenic *L. corniculatus* plants and assayed for reporter gene (GUS) activity to determine what effect this protein may have on *Srglb3* gene expression. No statistically significant differences between *S. rostrata* nodules harboring wild-type or mutant *A. caulinodans* were observed. Therefore, although AcBBP1 and RlBBP1 can bind to a defined region within the *Srglb3* promoter *in vitro*, it appears that these bacterial proteins do not significantly influence *Srglb3* gene expression *in vivo*.

To my parents, for their love and support.

## ACKNOWLEDGMENTS

Thanks are due to the people who contributed to this thesis project and enriched my life during my time at Michigan State University. First, I would like to thank Frans J. de Bruijn for allowing me to conduct independent research on a very interesting topic and for having confidence in me. I also wish to thank my committee members, Thomas Friedman, Sheng Yang He, Jonathan Walton, Lee McIntosh and Natasha Raikhel for support and helpful advice. Research in the lab would not have been nearly as much fun and entertaining without the antics of Krzysztof Szczyglowski, David Silver and Philipp Kapranov. I would like to thank Kurt Stepnitz and Marlene Cameron, who provided photographic assistance throughout my project and the numerous laboratories in the PRL who have helped me along the way.

Finally, I would like to thank all the friends I have made at Michigan State for their support throughout these years, especially the wonderful people in the de Bruijn lab past and present who have made the lab an excellent place to work. In particular, I thank the plant group for all of the memories; Jim Dombrowski and Maria Schneider for their support; Judith Wopereis for her assistance, time, effort and friendship; Chris Vriezen for his encouragement and great meals; Mary Ellen Davey and Kezar for their hospitality, friendship, and the "Ledges"; the Szczyglowski family for their friendship and Caroline Henein for her technical assistance. I would like to especially thank Pauline Bariola who has been a great friend and housemate, and my parents, who have always supported and encouraged me in all of my endeavors.

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# CHAPTER 1

# BACTERIAL-PLANT INTERACTIONS: EVIDENCE FOR TRANSKINGDOM SIGNALING

# INTRODUCTION

Bacteria inhabit almost every niche on this earth. They have found ways to persist where other organisms have failed, ingeniously evolving unique mechanisms to maintain their existence. Some of these mechanisms lead to interactions with eukaryotic organisms.

In plant biology, interactions between plants and bacteria have been studied intensely for different reasons. Plant-pathogen interactions have been studied genetically for quite a long time because of their agronomic impact. Since many bacteria cause disease, which can result in catastrophic crop failure, attempts to unravel the signal transduction pathway which mediates pathogen recognition and the activation of defense responses is of great interest and importance. In contrast, symbiotic interactions between plants and bacteria have somehow evolved a way to escape the defense response (Djordjevic et al., 1987) and to form a relationship with a plant that is mutually beneficial. Understanding the events leading to the establishment of such relationships is important and could have a direct impact on the food supply of the world.

To date, some signals and components of these interactions are known, but much is still a mystery. Uncovering all of the elements involved and determining how everything fits together still eludes us, although significant progress is being made to join the gaps. One important point, which will be discussed further here, is the idea of modulation of plant gene expression by the bacterium. This is an area where currently not much is known. For any relationship between plants and bacteria to exist, signal exchange

between the two must occur. Different types of signals have been implicated in several steps of the interaction between plants and bacteria. Recently, the finding that bacterial proteins are recognized directly within plant cells in a few systems may become a more general means of communication between microbes and plants. I will begin my discussion with the best characterized interaction: *Agrobacterium* with plants.

## *Agrobacterium*

The best studied case of a bacterium modulating plant gene expression comes from *Agrobacterium tumefaciens*, the causative agent of crown gall, a disease which produces tumors on plants. In nature, this tumorous growth occurs at wound sites and leads to the formation of what is known as a "gall" at the soil-air junction of the plant (Kado, 1991; Sheng and Citovsky, 1996). It is noteworthy to mention here that although *Agrobacterium* is considered a pathogen, it does not kill its plant host. Within this structure, specific compounds that *Agrobacterium* can utilize as a sole carbon and nitrogen source are produced, namely the class of compounds called opines (Petit et al., 1978). This results in the creation of a unique environment for the invading bacterium in the plant. In recent years, this tumorous growth was shown to be the result of the expression of genes of bacterial origin that are transferred, stably integrated and expressed in the plant genome (see reviews by Zupan and Zambryski, 1995; Sheng and Citovsky, 1996). This knowledge is the foundation of plant transformation and has been used extensively to construct transgenic plants for applied and basic research. To date, this is

the only known example of stable DNA transfer between kingdoms in nature and is a unique mechanism to modulate plant gene expression.

**VirD2 Protein**

The VirD2 protein is essential for generating the T-strand, a piece of single-stranded DNA (T-DNA, Transferred DNA) that is transferred to the plant cell and integrated into the plant genome (Stachel et al., 1986). VirD2, in concert with VirD1, is essential in this process and is responsible for cleaving the bottom strand of the T-DNA at the border sequences which flank it (Filichkin and Gelvin, 1993). After generation of the single-stranded T-strand, the VirD2 protein remains tightly associated with the 5' end of the T-DNA giving it a polar nature for transport. In addition, another protein, VirE2, binds tightly and cooperatively to this naked DNA, covering the strand to aid in protecting it from degradation from nucleases. The T-DNA plus VirD2 and VirE2, is termed the T-complex which is exported from the bacterium and transfered to the plant nucleus (Howard et al., 1990; Howard et al., 1992; Zupan and Zambryski, 1995). The VirD2 protein plays an important role not only in formation of the T-strand, but probably also aids in importing the T-strand into the plant nucleus. Only the N-terminal half of the VirD2 protein is required for nicking and T-strand formation (Yanofsky et al., 1986; De Vos and Zambryski, 1989). Additionally, *Agrobacterium* strains containing only the N-terminal portion of VirD2 were unable to form tumors on its host (Stachel and Nester, 1986; Steck et al., 1990; Wang et al., 1990). Therefore it was postulated that the C-terminal portion of VirD2 was involved in movement of the T-complex into the plant

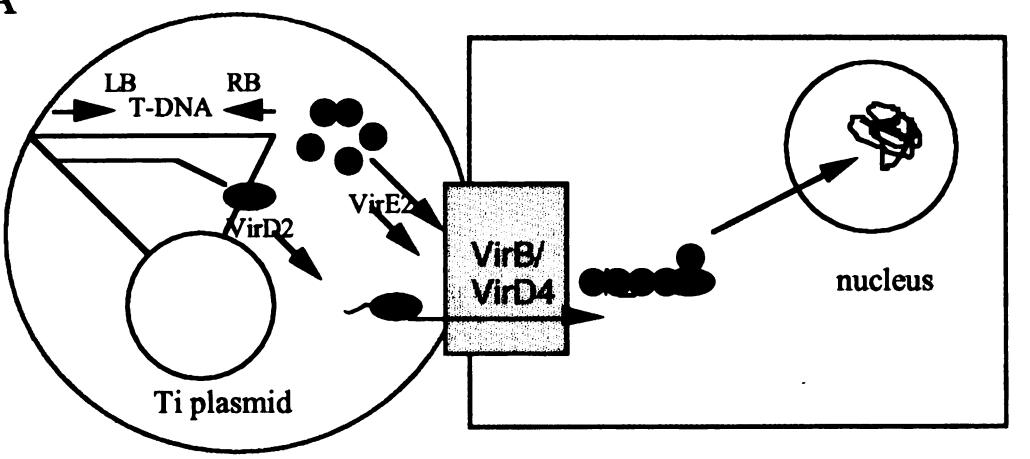
**Figure 1-1. Models for directly influencing plant gene expression by bacterial proteins or DNA.**

(A) *Agrobacterium*-plant interaction. The bacterial derived T-DNA is transferred to the plant nucleus by VirD2 and VirE2 through a secretory apparatus and/or pilus structure and integrated into the plant genome.

(B) *Xanthomonas*-plant interaction. The AvrBs3 protein is postulated to get into the plant cell via a Hrp dependent secretion apparatus and/or a pilus-like structure. The AvrBs3 protein may interact directly or indirectly with an unknown target in the plant cytoplasm or nucleus. Eventually AvrBs3 can enter the plant nucleus and affect plant gene expression.

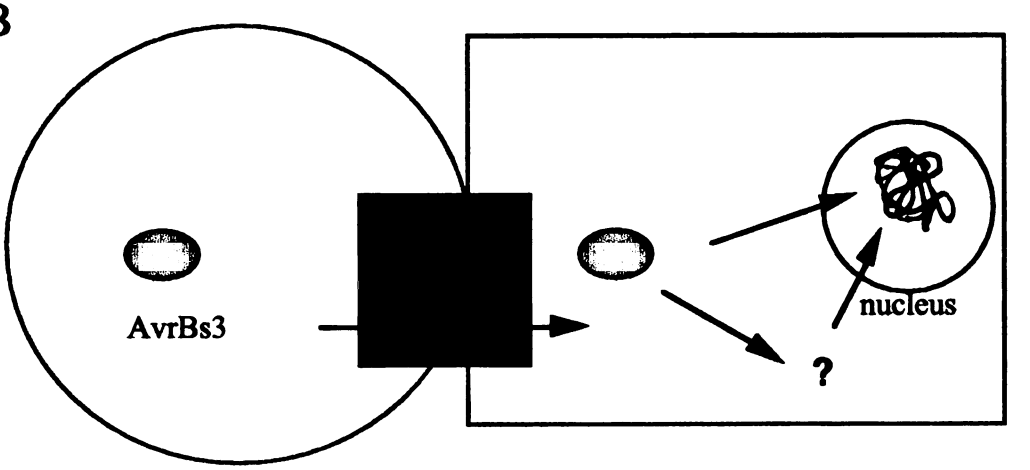
(C) *Rhizobium*-plant interaction. The AcBBP1 protein gets into the plant cytoplasm by an unknown mechanism and is somehow directed to the plant nucleus. The AcBBP1 protein is postulated to modulate plant gene expression by directly affecting transcription by binding to the 5' upstream region of the *Srglb3* gene.

A



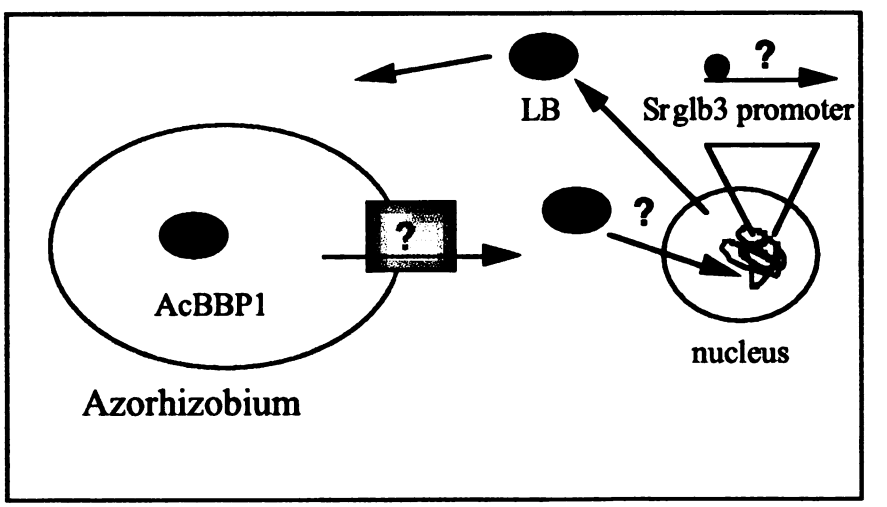

Agrobacterium plant cell

B




Xanthomonas plant cell

C




plant cell

cell. This idea was tested and it was found that VirD2 is capable of directing a reporter protein (β-glucuronidase, GUS) to the plant nucleus of tobacco or maize (Howard et al., 1992; Citovsky et al, 1994).

VirD2 contains three stretches of basic amino acids that may act as nuclear localization signals. It was found by deletion analysis that the two regions found at the C-terminus could direct chimeric reporter proteins to the plant nucleus of tobacco protoplasts (Howard et al., 1992). These regions of basic amino acids also have similarity to known nuclear localization signals (NLSs) found in many nuclear localized proteins (Chelsky et al., 1989). In addition, each of these regions was mutated and tested for their ability to direct chimeric reporter proteins to the nucleus. Both regions are necessary for full nuclear localization (Howard et al., 1992). The absence of one of the regions leads to partial nuclear localization of the reporter protein while the presence of both lead to strict nuclear localization identical to fusing the full length VirD2 protein or the C-terminal half to a reporter protein (Howard et al., 1992). Hence, VirD2 contains a bipartite NLS which can direct it to the plant cell nucleus.

**VirE2 protein**

Since VirD2, the protein located at the 5' end of the T-complex, possesses a bipartite NLS and can deliver fusion proteins to the plant nucleus, the other component of the T-complex, VirE2, was also tested for its ability to aid in nuclear uptake. As mentioned earlier, VirE2 participates in forming the T-complex by coating the naked

single stranded T-DNA, thus protecting it from degradation on its way from the bacterium to the plant cell (Citovsky et al., 1989). This idea was spawned by the fact that the T-complex is a large protein-DNA structure (estimated to be 50,000 kDa, Howard et al. 1992) and a single VirD2 protein may not be sufficient to direct this complex to the nucleus.

Two regions similar to the bipartite NLS of *Xenopus* nucleoplasmin were identified in VirE2 (Citovsky et al., 1992). Additionally, these sequences are conserved among VirE2 proteins from many *Agrobacterium* strains identified (Hirooka et al., 1987; Winans et al., 1987). They were tested for their role in nuclear localization. Using deletion analysis and chimeric reporter gene constructs (β-glucuronidase, GUS), VirE2 was found to direct fusion proteins to the nucleus of tobacco protoplasts. Akin to what was found with VirD2, these two regions act as a bipartite NLS since constructs lacking one of the regions does not fully localize chimeric proteins to the nucleus. Only with both sequences present is efficient nuclear localization observed (Citovsky et al., 1992). Additionally, the use of transgenic tobacco plants supports the biological role of VirE2 in tumor formation. Transgenic tobacco plants expressing the VirE2 protein are able to "complement" an *Agrobacterium virE2* mutant strain with regard to tumorigenicity. Inoculation of this mutant strain on transgenic plants expressing the full VirE2 protein cause tumors while inoculation of the same strain on untransformed plants do not elicit any symptoms. Furthermore, transgenic tobacco plants expressing modified VirE2 proteins display phenotypes which positively correlated the presence of an intact

bipartite NLS and tumor formation when inoculated with the *virE2* mutant. The removal of one portion of the bipartite NLS leads to reduced tumorigenicity while deletion of both regions previously identified to be essential for nuclear localization completely blocks tumor formation.

In summary, *Agrobacterium* represents a unique mode of transkingdom signaling as a piece of DNA originating from the bacterium is transported to the plant nucleus and integrated into the genome. The VirD2 and VirE2 proteins not only protect the T-strand on its journey from the bacterial cell to the plant cell but are essential in directing the the T-complex to the plant nucleus. Thus, this bacterium has evolved a novel mechanism to modulate plant gene expression by stably integrating foreign genes into its host genome. The expression of these new genes in the plant cell create and maintain an environment suitable for its survival.

## *Xanthomonas*

*Xanthomonas* comprises a genus of bacteria which cause various types of disease on many different plants. How one pathogen can cause disease in one plant and not another is an intensely researched subject. Recognition of the pathogen is key and crucial to defense. When a pathogen attacks a plant, the host tries to protect itself by eliciting defense responses which halt further invasion of the pathogen. In many instances, this correlates with the hypersensitive response (HR) in which localized induced cell death occurs in the host plant (Keen, 1990). It is generally believed that recognition of the pathogen involves a gene found in the bacterium, referred to as an avirulence gene (*avr*), that interacts with a resistance gene (R) found in the plant. Alternately, if no

corresponding R gene is found in the host plant, or no corresponding *avr* gene is found in the bacterium, the pathogen will not be recognized, leading to disease of the plant. This so-called "gene-for-gene" relationship defines the basis of resistance to a specific pathogen by a particular plant (Baron and Zambryski, 1995; Staskawicz et al., 1995).

How bacterial avirulence genes confer specificity for certain hosts has remained a mystery since the isolation of the first *avr* gene over 10 years ago (Staskawicz et al., 1984). Now over 30 bacterial *avr* genes have been isolated but only the mode of action of the *avrD* gene of *Pseudomonas syringae* pathovar (pv.) *tomato* has been described. This *avr* gene encodes an enzyme which mediates the production of a low molecular weight compound termed syringolide (Keen et al., 1990). This molecule is the specific elicitor that is recognized by soybean plants harboring the corresponding R gene *Rpg4* (Keen and Buzzell, 1991).

In *Xanthomonas*, a large family of related *avr* genes have been identified. Recent findings by Yang and Gabriel (1995) and Van den Ackerveken et al. (1996) suggest that *Xanthomonas avr* gene products interact with components within the plant cell directly and therefore supports the hypothesis that the *avr* gene product directly binds to the R gene product or the R gene itself (Ellingboe, 1982). Structural characteristics identified in *Xanthomonas avr* gene products provide more evidence for this idea. Furthermore, Gopalan et al. (1996) showed that *avr* genes from *Pseudomonas syringae* are recognized when expressed in plant cells. This was tested by expressing the *avrB* gene of *Pseudomonas syringae* pv. *glycinea* in *Arabidopsis* plants harboring the *RPMI* resistance gene. Plants expressing the AvrB protein exhibited HR in a *RPMI* depedent fashion . This

provides additional support for the direct recognition of bacterial proteins in the plant cell. The interaction of bacterial *avr* gene products and a currently unknown target in the plant cell illustrates another example of transkingdom signaling. In this system, host gene expression is modulated by the direct interaction of bacterial proteins with a plant component within the host cell. This recognition event induces a cascade of events which trigger a defense response in the plant.

**PthA and Avrb6 proteins**

The *pthA* (pth, pathogenicity) gene of *X. citri* and the *avrb6* gene from *X. campestris* are members of a large family of *avr/pth* genes found in *Xanthomonas.* To date, this family includes almost all *avr* genes described in this bacterium. All members are very similar (at least 93%) at the nucleotide level and possess three interesting motifs within their deduced amino acid sequence: 1) a number of tandemly arrayed leucine-rich repeats which determine *avr* gene specificity, 2) a series of heptad repeats which are similar to leucine zippers and may be involved in DNA-protein or protein-protein interactions and 3) three putative NLSs which could direct this protein to the plant nucleus (Herbers et al, 1992; Yang and Gabriel, 1995). The *pthA* and *avrb6* genes are interesting because isogenic strains carrying either of these genes elicit different response phenotypes on plants. Most members of the *avr/pth* family confer the ability to elicit the HR on many plants but the *pthA* gene and *avrb6* genes can specifically confer the ability to produce hyperplasias on citrus or to elicit water-soaking in cotton, respectively (Swarup et al., 1992; Yang et al., 1994). It was determined that the leucine-rich repeats are

responsible for the three phenotypes observed and that the regions flanking the leucine-rich repeats are functionally interchangeable (Yang et al., 1994). However, the function of the heptad repeats or the ability of these gene products to localize to the nucleus was not tested.

Using chimeric reporter proteins (β-glucuronidase, GUS), Yang and Gabriel (1995) determined that the C-terminal portion of the PthA and Avrb6 proteins could act as a functional NLS(s) in plant cells. As previously mentioned, the C-terminal region of these proteins contain three putative NLSs. However, the NLSs were not precisely defined in this investigation nor was the presence of intact NLSs correlated with a pathogenic phenotype. Nonetheless, these results indicate that bacterial *avr/pth* gene products could act directly in the plant cell to influence plant gene expression.

**AvrBs3**

The AvrBs3 protein is also a member of the *avr/pth* family in *Xanthomonas.* Unlike *pthA* and *avrb6* described above, the *avrBs3* gene of *X. campestris* pv. *vesicatoria* (Bonas et al, 1989) specifically mediates resistance to lines of pepper plants of the genotype *Bs3*. In this interaction, pathogen recognition results in the hypersensitive response (HR, Klemet, 1982) which is characterized by localized cell death (Mittler and Lam, 1996) that develops into a distinct area of necrosis.

Genetic data indicate that recognition of the *avrBs3* gene product (Herbers et al., 1992; Bonas et al., 1993) by resistant pepper plants is direct. As mentioned previously,

the leucine-rich repeats found in AvrBs3 in addition to other *avr* gene products in this family determine avirulence gene specificity. Herbers et al. (1992) showed that the precise removal of some of the leucine-rich repeats changes host specificity. In this investigation, a four-repeat deletion in the *avrBs3* gene product abolishes the ability of pepper plants with genotype *Bs3* to recognize this strain as a pathogen. Instead these bacteria are now recognized by *bs3* plants (Herbers et al., 1992). Because changes in the leucine-repeat region cause changes in specificity of pepper plant genotype, the AvrBs3 protein is postulated to be the actual elicitor molecule that is recognized by the plant. However, the AvrBs3 protein does not seem to be secreted from the bacterium nor does the AvrBs3 protein induce the HR when infiltrated into the intercellular spaces of pepper leaves (Van den Ackerveken et al., 1996). Immunogold localization of the AvrBs3 protein show that it is only present in the cytoplasm of the bacterium in culture or when the bacteria were infiltrated into pepper leaves (Brown et al., 1993). No labeling was found in the plant cell.

Recently, Van den Ackerveken et al. (1996), tested the idea that recognition of AvrBs3 occurs in the plant cell and investigated what role putative NLSs found in the C-terminal end of AvrBs3 plays in this interaction. They found that when the C-terminal end of the protein was fused to β-glucuronidase (GUS) and tested for its ability to direct this chimeric construct to the nucleus using particle bombardment of onion epidermal cells, the reporter construct was able to localize to the nucleus. Additionally, the second and third NLS in the C-terminal end of AvrBs3 seemed to be more important for AvrBs3 activity than the first NLS.

The different deletion/mutation constructs were tested for their ability to elicit the HR. There was a strong correlation with nuclear localization and HR. Constructs with mutations that did not affect AvrBs3 activity were localized to the nucleus while mutations that resulted in a total loss or reduction of AvrBs3 activity were not able to efficiently direct reporter gene constructs to the nucleus (Van den Ackerveken et al., 1996).

These observations suggest that some *avr* genes from *Xanthomonas* are probably directly recognized in the plant cell. PthA, Avrb6 and AvrBs3 are members of the same gene family and have similar structural characteristics and all can direct chimeric reporter proteins to the plant nucleus. These results support the idea that *avr* gene products may act directly on an R gene product or the R gene itself and may facilitate the identification of interacting factors and their locations. These results indicate that recognition of bacterial proteins directly within plant cells are involved in pathogen recognition (Figure 1-1B).

## *Rhizobium*

Symbiotic nitrogen fixation is a unique example of a complex and fine tuned interaction in which Gram-negative bacteria belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium* interact with legume plants to form a novel structure, the nodule (Mylona et al, 1995; Long, 1996). Multiple signals from both the bacterium and the plant are exchanged, which induces sets of genes in both symbiotic partners (Nap and Bisseling, 1990; de Bruijn and Downie, 1991; Mylona, 1995; Long,

1996). Plant genes which are specifically induced and expressed during symbiosis are termed nodulin genes and are classified as "early" or "late" depending on their time of induction (van Kammen, 1984). Early nodulin genes are associated with the infection process and nodule ontogeny while late nodulin genes correlate with nodule function and maintenance.

Leghemoglobins (Lbs) are late nodulins which carry oxygen to the actively respiring, nitrogen-fixing bacteroids within the infected cells (Appleby, 1984). Leghemoglobin (*lb*) gene expression in legumes is confined to the infected tissues of the nodule and appears to be localized to the infected cells. In plants which produce indeterminate nodules such as those elicited on alfalfa by *Rhizobium meliloti*, *lb* gene expression has been detected in a single cell layer directly adjacent to the nitrogen fixation zone. In addition, *lb* transcripts appear to be expressed only in infected cells of the nodule. Furthermore, *lb* gene expression seems to be absent in nodules formed spontaneously on specific alfalfa lines (Truchet et al., 1989) or induced by auxin transport inhibitors (Hirsch et al., 1989) or by certain mutant bacterial strains (Dickstein et al, 1988; de Bruijn et al., 1989). Since these nodules are devoid of bacteria, these findings suggest that the presence of a bacterial signal may be important for the expression of *lb* genes (Nap and Bisseling, de Billy et al., 1991; de Bruijn and Schell, 1992).

In determinate nodules, such as those elicited on soybean by *Bradyrhizobium japonicum*, the exact location of *lb* transcripts and Lb proteins have not been clearly determined. VandenBosch and Newcomb (1988) detected the Lb apoprotein in both the

infected and uninfected cells using immunochemical techniques. Further support of this idea came from Kouchi et al. (1989) who found that although *lb* transcripts are mainly detected within the infected cells of soybean nodules, low but significant amounts appear to be present in the uninfected cells.

Chimeric *lb*-reporter gene constructs in transgenic legume plants have been used successfully to study *lb* gene expression. The promoter regions of the soybean *lbc*3 gene and the *Sesbania rostrata glb*3 gene have been studied in the most detail. Both promoter regions can direct a nodule-specific expression pattern on reporter genes in transgenic alfalfa (de Bruijn et al., 1989) and in nodules induced on *Lotus corniculaus* (Stougaard et al., 1986, 1987, 1990; Szabados et al., 1990). Additionally, the work of Szabados et al. (1990), Lauridsen et al. (1993) and Szczyglowski et al. (1994) strongly suggest that *lb* promoter activity is confined to the infected cells of the nodule.

Considerable progress has been made in elucidating the signals which direct early morphogenic events in the legume host. The plant produces a signal, usually a flavonoid, which induces a set of nodulation (*nod*) genes in the bacterium. Subsequently, the bacterium synthesizes a host specific signal, a lipochitooligosaccharide (nod factor), which induces a plant developmental program leading to nodule formation (Hirsch, 1992; Mylona et al., 1995; Long, 1996). Although research focused on the early stages of nodulation has progressed rapidly, the signals and molecular events important for bacterial colonization or nodule functioning and maintenance remain largely unknown (de Bruijn and Schell, 1992).

Although no signals have been identified in the later stages of nodule development, much indirect evidence supports the existence of a bacterium-derived signal in the induction of *lb* gene expression. It has been hypothesized that a signal originating from the bacterium is important for the induction of *lb* gene expression. This idea can be traced back to Bruening and Wullstein (1972) who postulated that leghemoglobin synthesis could be initiated by the transfer of a bacterially produced inducer. Likewise, Truchet et al. (1980) proposed the existence of a *Rhizobium* signal involved in the differentiation of the central infected tissue of the developing nodule. de Billy et al. (1991) also suggested that this factor may play a role in the infected cell-specific expression of the *lb* gene in alfalfa.

Recent findings suggest that transkingdom signaling in symbiosis may occur. Jabbouri et al. (1996) have identified a novel gene in *Rhizobium* NGR234 whose gene product has a putative NLS. In addition, Welters et al. (1993) observed that a factor orginating from the symbiont of *S. rostrata*, *Azorhizobium caulinodans*, can bind to the *S. rostrata* leghemoglobin *glb*3 (*Srglb*3) 5' upstream region. These two pieces of evidence are intriguing and may illustrate the conservation of signaling mechanisms in plant-microbe interactions in which bacterial proteins function directly in the plant cell.

**FixF**

Recently, a gene *fixF*, was isolated from the broad host range *Rhizobium* NGR234. Mutations in this gene abolish the production of a novel, rhamnose-rich lipopolysaccharide that is produced under flavonoid-induced conditions and result in a Fix$^-$ (unable to fix nitrogen) phenotype (Jabourri et al., 1996). Upon inspection of its

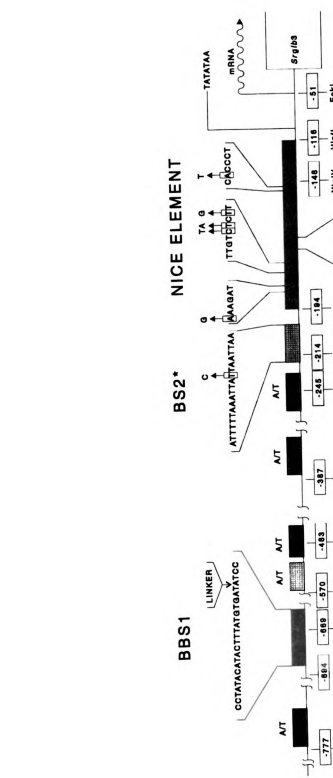
deduced amino acid sequence, a putative NLS was found. The ability of this putative NLS to direct a reporter protein to the plant nucleus was tested and found to localize to this compartment (Jabbouri et al., 1996). In addition, the *fixF* gene appears to be regulated by the alternate sigma factor σ54. This sigma factor has been shown to regulate nitrogen fixation (*nif/fix)* genes in the bacterium (Stitger et al., 1993). It will be interesting to determine the role of this lipopolysaccharide in nitrogen fixation and to ascertain if nuclear localization of FixF is important for nitrogen fixation.

**AcBBP1**

In an attempt to elucidate the signal transduction pathway leading to leghemoglobin (*lb*) gene induction, the identification of *cis*-acting elements within the promoter regions of *lb* genes and *trans*-acting factors interacting with the 5' upstream region was pursued (Figure 1-1). In analyzing the *Srglb3* promoter, a tissue-specific element termed NICE (nodule infected cell-specific element) was identified. This *cis*-acting element confers the tissue specificity exhibited by this promoter and can direct a normally root enhanced promoter to become nodule infected cell specific when expressed in transgenic *L. corniculatus* (Szczyglowski et al., 1994).

In addition, A/T rich elements upstream of NICE were identified. These regions, called ATRE-BS2* elements, are found in multiple copies in the *Srglb3* promoter, are also highly conserved in the *lbc3* and N23 promoters from soybean and in the glnγ 5' upstream region of French bean, and were found to interact specifically with DNA

**Figure 1-2. Structure of the *Sesbania rostrata glb3* gene 5' upstream region** (Taken from Szczyglowski et al., 1994).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LINKER
CCTATACGATACTTTATGTGATATCG
A/T
A/T
A/T
A/T
-777
DraI
-884
SspI
-869
EcoRV
-370
HinfI
-488
BspI
-387
EcoRI
BS2*
NICE ELEMENT
C
ATTTTTAAATTATTAATTAA
A/T
-245
BspI
-214
DraI
G
AAAGAT
-194
DraI
TA G
TTGTGTTTGTT
T
CACCCT
TAATGTCA
ACG
-148
NlaIV
-119
HinfI
TATATAA
mRNA
Srg/b3
-51
FokI
ATG

binding proteins from nodules, roots, and leaves (Jensen et al., 1988; Metz et al., 1988; Forde et al., 1990; Jacobsen et al., 1990). A/T rich elements have been identified in the promoters of other plant genes and in some cases have been shown to act as positive regulatory elements (Forde, 1994), although the function of these elements in the nodulin gene promoter regions is not clear (de Bruijn and Schell, 1992; Forde, 1994). In the *Srglb*3 promoter, this element appears to act as a positive element (Szabados et al., 1990) but is not essential for nodule-specific expression (Szczyglowski et al., 1994). Mutations in ATRE-BS2* result in diverse affects on reporter gene activity and may reflect alterations in the interaction with specific *trans*-acting factors. This result is supported by Jacobsen et al. (1990) who showed that mutations in oligonucleotides corresponding to the A/T rich elements in the soybean *lbc*3 promoter can change binding patterns and affinities of three proteins NAT1, NAT2 and LAT1. NAT1 and LAT1 are HMG I-like proteins and these class of proteins have been implicated in modulating chromatin structure (Jacobsen et al., 1990). A/T rich regions are known to serve as matrix attachment regions or scaffolding attachment regions based on functional assays and may be important for the expression of the *Srglb*3 gene in higher order chromatin structures (Pinaev and de Bruijn, unpublished results).

A factor present in extracts of stem or root nodules of *S. rostrata* was found to bind specifically to a region in the *Srglb*3 promoter using the gel mobility shift assay. It was demonstrated by Welters et al. (1993) that the binding activity is due to the presence of a factor originating from *Azorhizobium caulinodans*, the symbiont of *S. rostrata*. This factor binds to a region in the *Srglb*3 promoter now designated the Bacterial Binding Site 1

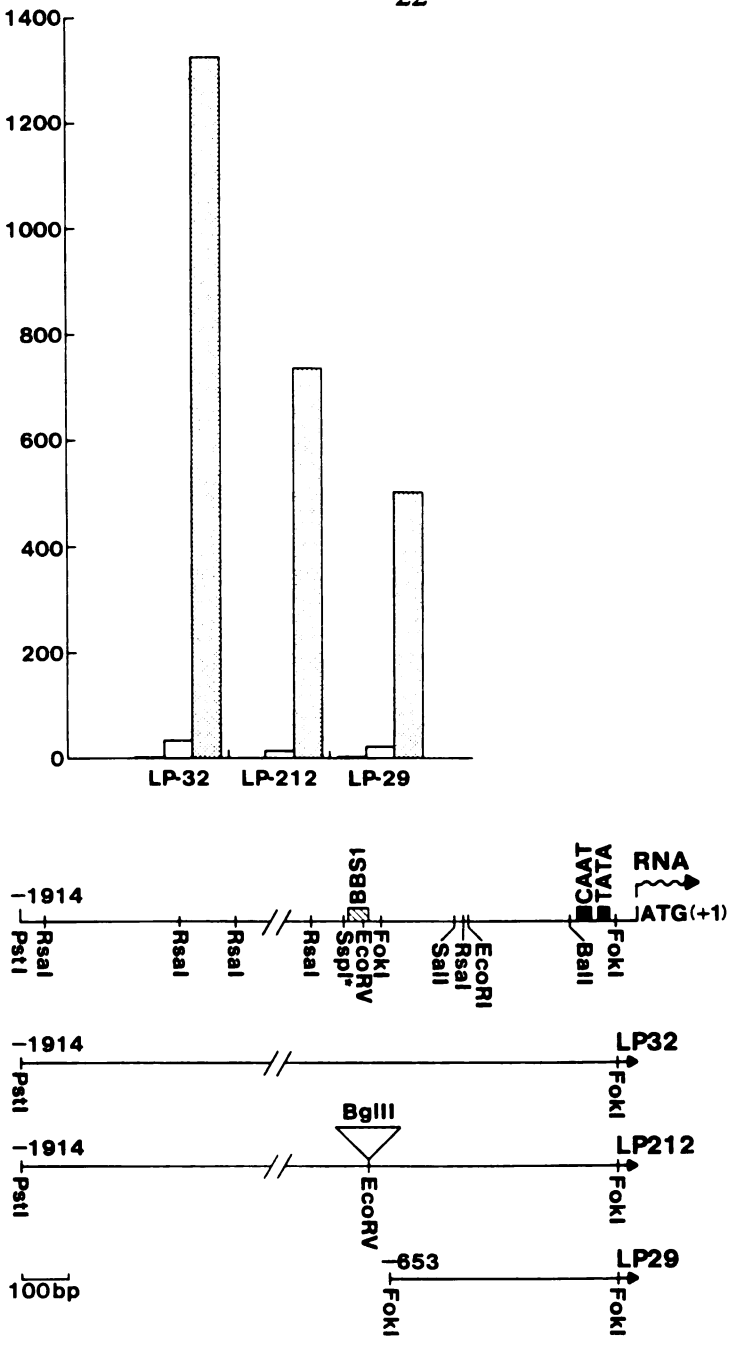


**Figure 1-3. Role of the BBS1 region in *Srglb3* promoter activity**. The top panel shows the Gus activity (expressed in pmol of 4-methylumbelliferone produced $min^{-1}$ $mg^{-1}$ of protein) of chimeric *Srglb3* promoter-*gus* genes in transgenic *L. corniculatus* plants. The shaded columns represent Gus expression in nodules, the open columns represent Gus expression in roots. The structure of the chimeric constructs used is shown under the box and the position 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 BBS1 site is indicated by an open triangle on line 3 (Figure was taken from Welters et al., 1993).

(BBS1; Welters et al., 1993; Szczyglowski et al., 1994) which is located upstream of NICE and is flanked by several A/T rich regions. A detailed characterization of the binding activity was carried out and the developmental appearance of this complex and the presence of Lb were positively correlated (Welters et al., 1993). The protein responsible for this activity was purified and the N-terminal amino acid sequence determined. Transgenic *Lotus corniculatus* plants harboring chimeric *Srglb3* promoter-reporter gene fusions suggest that the BBS1 may modulate *Srglb3* gene expression as disruption or deletion of this site reduces reporter gene activity (Figure 1-2; Welters et al, 1993).

**Intercellular transport**

In order for a microbe to enter a symbiotic or pathogenic interaction with a plant, signal exchange between the two must occur. In *Agrobacterium* and *Xanthomonas*, bacterial proteins can be directly recognized in the plant cell (Howard et al., 1992; Citovsky et al., 1992; Yang and Gabriel, 1995; Van den Ackerveken et al., 1996) as these proteins have the ability to localize to the plant nucleus. Furthermore, pathogenic phenotypes such as tumor formation or the HR correlate positively with functional NLSs. In symbiotic interactions, bacterial proteins also have the ability to localize to the plant nucleus (Jabourri et al., 1996). The finding that a bacterial protein can bind to a plant promoter is consistent with this idea (Welters et al., 1993). One central question that has eluded scientists for some time concerns the mechanism by which these proteins could enter the plant cells.

How proteins or complexes originating from the bacterium could traverse the bacterial membranes and cell wall in addition to the plant cell wall and plasma membrane to get into the plant cell has been enigmatic for a long time. Recently, research in this area has uncovered some interesting findings. In *Agrobacterium*, the idea that conjugation and T-DNA transfer were similiar processes was first proposed following the discovery of the T-strand (Stachel and Zambryski, 1986; Stachel et al., 1986). More recently, Lessl and Lanka (1994) extended this idea by comparing characteristics of the RP4 plasmid and T-DNA transfer. Significant similarities in function, amino acid sequences, gene organization and physical properties of the *trans*-acting enzymes were identified. Since then, Fullner et al. (1996) has shown that *Agrobacterium* produce pili that are proposed to function in the conjugal transfer of the T-DNA to the plant cell. Moreover, a pilus structure was recently identified on the surface of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Roine et al., 1997) under conditions important for pathogenic bacteria to elicit the HR in resistant plants. These two findings suggest that a pilus structure may be one mechanism by which bacterial proteins are delivered to the plant cell.

A secretion apparatus involved in intercellular transport has also been investigated. The genes encoded by the *virB* locus encode proteins that could form a membrane associated export system (reviewed by Zambryski, 1992). The *virB* operon appears to be closely related to the *Ptl* operon of *Bordetella pertussis* which encodes products responsible for the export of the pertussis toxin protein (Weiss, 1993). In plant-pathogen interactions the *hrc* genes are conserved in the bacterial pathogens *P. syringae*

pathovars, *Erwinia*, *Xanthomonas*, and *Ralstonia* (Lidell et al, 1994; Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogandove et al., 1996) and share sequence similarity to a set of genes found in *Yersinia* spp., *Shigella* and *Salmonella* (for a review see Van Gijesgem et al., 1993, He, 1996). All of these genes are important for the secretion of proteins required for pathogenesis (for a review see Van Gijsegem et al., 1993, He, 1996) and are predicted to form components of the type III secretion apparatus. The formation of a secretion pathway illustrates another mechanism by which virulence factors can be exported by the bacterial cell.

With the recent discovery of pilus formation on the surface of *Agrobacterium* and *P. syringae* pv. *tomato* DC3000 and the identification of genes with similarity to components of secretion systems, one can imagine how bacterial proteins could get into the plant cell. One model for intercellular transport involves the export of virulence factors via the secretion system alone. However, the plant cell wall would provide a barrier for these extracellularly localized pathogenic bacteria. Therefore, the involvement of pili in combination with a secretory system may work together to deliver the bacterial protein to the plant cell.

In symbiosis, bacteria have the advantage of already being present in the plant cell. However, thet have an additional membrane, the peribacteroid membrane, which presents another barrier that must be crossed. For the most part, not much is known about the proteins involved in transporting metabolites from the bacteroid to the plant cell. A dicarboxylic acid uptake mechanism exists in bacteroids (Ronson et al., 1987; Werner et al., 1992). To date, no specialized secretion system has been identified to selectively

export rhizobial proteins from the bacteroid or through the peribacteroid membrane to the plant cell cytoplasm.

## Conclusions

Bacterial proteins which are recognized within the plant cell have been identified and studied in two types of plant-pathogenic interactions and have been implicated in symbiotic interactions. The mechanisms by which bacteria modulate host gene expression appears to fall into two categories: the transfer of DNA to the host genome and the use of bacterial proteins to influence host gene expression.

*Agrobacterium* is the only known example to date in which DNA originating from a bacterium is transferred to the plant cell nucleus (Kado, 1991; Sheng and Citovsky, 1996). In this unique example, new genes are introduced into the host genome and expressed. This transfer of DNA can be thought of as altering host gene expression as auxin and cytokinin biosynthetic genes, in addition to genes which encode factors that produce the opines, are located on the T-DNA (Kado, 1991; Zupan and Zambryski, 1995). When these genes are expressed in the host, changes in the plant developmental program occur which ultimately lead to the formation of a gall. (Kado, 1991; Zupan and Zambryski, 1995).

In contrast, *Xanthomonas* proteins (*avr* gene products) and rhizobial proteins (Fix F, AcBBP1) are recognized in the plant cell and may interact with plant proteins or DNA directly. Avr proteins, AcBBP1 and the Fix F protein could act as plant transcription factors or repressors to turn on or turn off gene expression. It is known that *avr* gene

products contain heptad repeats which can mediate protein-DNA interactions (Yang and Gabriel, 1995). The AcBBP1 protein contains a functional DNA binding domain (Welters et al., 1993). Alternatively, the *avr*or *fixF* gene products may participate in protein-protein interactions by interacting with a receptor yet to be identified in the plant cell. Avr proteins from *Xanthomonas* contain heptad repeats and leucine-rich repeats, which can mediate protein-protein interactions (Yang and Gabriel, 1995). AcBBP1 binds DNA and is similar to regulatory proteins from different systems. Transgenic plants carrying chimeric *Srglb3* promoter-reporter gene constructs indicate that the BBS1 is important for *Srglb3* gene expression as mutation or deletion of this *cis*-element reduces promoter strength (Welters et al., 1993). Hence, this protein may act to enhance *Srglb3* gene expression under specific conditions. AcBBP1 is also unique in that its target is already identified. For the *Xanthomonas* Avr proteins, the target within the plant cell is unknown.

*Agrobacterium*, *Xanthomonas*, and *Rhizobium* produce proteins which have the ability to localize to the plant nucleus. In the plant pathogens, nuclear localization of these proteins positively correlates with a specific pathogenic response indicating a probable biological role. As mentioned previously, the targets of most of these proteins are not known. In the case of *avr* gene products, the target may be the R gene itself or the R gene product with interaction occurring in the plant cytoplasm or perhaps in the plant nucleus. In symbiosis, the target for AcBBP1 is known to be a non-A/T rich region in the 5' upstream region of the *Srglb3* gene called BBS1. The biological role of this protein in symbiosis is the subject of my thesis.

Bacterial interactions with plants presents an interesting system to study. Establishment and persistence of these relationship requires a cascade of signals which lead to the formation of an interaction which can be detrimental or beneficial to the plant. We are just now uncovering signals originating from the bacteria which are recognized in the plant cell that modulate plant gene expression. As introduced in this chapter, bacterial proteins appear to mediate plant gene expression. For the most part, this occurs through the interaction of a bacterial protein signal in the plant cell.

The identification of AcBBP1, a protein produced by *A. caulinodans* that can bind *in vitro* to the *Srglb3* promoter, is intriguing and warrants further investigation. As mentioned earlier in this chapter, bacterium-derived signals have been postulated to be important for *lb* gene expression as many pieces of indirect evidence have been collected over the years. My thesis work is focused on investigating the biological role of the AcBBP1 protein in the symbiosis between *S. rostrata* and *A. caulinodans*. With the recent finding that bacterial proteins play a role in plant-pathogen interactions, perhaps signaling of this type is more general in nature.

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# CHAPTER 2

## IDENTIFICATION AND CHARACTERIZATION OF THE GENE ENCODING THE *AZORHIZOBIUM CAULINODANS* DNA BINDING PROTEIN, AcBBP1

Portions of this chapter will be submitted as an article to the journal Molecular Plant-Microbe Interactions.

## ABSTRACT

Previously, *cis*-acting elements in and *trans*-acting factors interacting with the 5' upstream region of the *Sesbania rostrata* leghemoglobin *glb3* (*Srglb3*) gene were identified (Metz et al., 1988; Szabados et al., 1990; Welters et al., 1993; Szczyglowski et al., 1994). A Nodule Infected Cell Expression (NICE) *cis*-acting element important for expression in infected cells of the nodule was delimited (Szczyglowski et al., 1994). DNA binding proteins from nodule, leaf and root extracts that interacted specifically with regions in the *Srglb3* 5' upstream region were analyzed (Metz et al., 1988; Welters et al., 1993). Interestingly, a protein originating from the infecting bacterium *Azorhizobium caulinodans* (AcBBP1), could specifically interact with a *cis*-acting element upstream of the NICE element in the *Srglb3* 5' region and its binding activity was characterized (Welters et al., 1993). The gene encoding AcBBP1 was isolated and the role AcBBP1 may play in symbiotic nitrogen fixation was investigated. The deduced amino acid sequence of the AcBBP1 protein consists of 72 amino acids and features a helix-turn-helix DNA binding motif. Homology searches revealed significant similarities to other DNA binding proteins such as the C proteins found in type II restriction-modification systems, immunity proteins from phage $\phi$ 105 and *E. coli*, and a regulator of vegetative replication and conjugative transfer of the RK2 plasmid. An AcBBP1 deficient mutant was created via reverse genetics and used to nodulate *S. rostrata* plants. This strain was found to be nodulation proficient (Nod+), but *S. rostrata* nodules induced by this strain were

observed to have a small reduction in nitrogen fixation activity. Total leghemoglobin steady state mRNA levels were identical in *S. rostrata* nodules harboring wild-type or AcBBP1 deficient bacteria.

# INTRODUCTION

The induction of nitrogen fixing stem or root nodules on legume plants by bacteria belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Rhizobium* involves the carefully orchestrated exchange of signals involved in the induction of sets of genes in both the plant and the bacterium (Nap and Bisseling, 1990; de Bruijn and Downie; 1991, Verma, 1992; Mylona et al., 1995; Long, 1996). The genes that are expressed in this newly formed plant organ are referred to as nodulin genes and are usually divided into two classes, early and late, depending on their time of induction. Early nodulin genes correlate with the infection process and nodule formation while the late nodulin genes encode proteins that are involved in the functioning and maintenance of the nodule (Nap and Bisseling, 1990; Vance and Heichel, 1991; Hirsch, 1992, Mylona et al., 1995). The latter class includes genes encoding enzymes involved in specific biochemical pathways such as the $\gamma$ subunit of glutamine synthetase involved in ammonia assimilation (Lara et al., 1983, Bennett et al., 1989, Boron and Legocki, 1993), sucrose synthase (Thummler and Verma, 1987), peribacteroid membrane proteins (Fortin et al., 1985, 1987; Miao and Verma, 1993) and leghemoglobins (Brisson et al., 1982).

Leghemoglobin (Lb), an abundant and essential protein within the nodule, is a late nodulin involved in facilitating oxygen diffusion to the actively respiring bacteroids within the infected tissue of the nodule. For the most part, leghemoglobin (*lb*) gene expression has been shown to be nodule-specific and mainly confined to the infected cells of the

nodule (Stougaard et al., 1986, 1987, 1990; de Billy et al., 1991; de Bruijn and Schell, 1992; Szczyglowski et al., 1994). Alfalfa nodules formed spontaneously on roots of specific alfalfa lines (Truchet et al., 1989) and those induced by auxin transport inhibitors (Hirsch et al., 1989) or by specific mutant rhizobial strains (Dickstein et al., 1988; de Bruijn et al., 1989; de Bruijn and Schell, 1992) do not express *lb* or other late nodulin genes, and contrasts the expression of some early nodulin genes such as *Enod2* and *Enod12* which are clearly induced.

Chimeric *lb*-reporter gene constructs and transgenic plants have been extensively used to identify the *cis*-acting elements responsible for the infected cell-specific expression of *lb* genes in the nodule. The promoter regions of the *lbc*3 gene from soybean and the *glb*3 gene from *Sesbania rostrata* have been shown to be responsible for nodule-specific expression in indeterminate nodules induced on transgenic alfalfa (de Bruijn et al., 1989) and in determinate nodules induced on *Lotus corniculatus* (Stougaard et al., 1986, 1987, 1990; Szabados et al., 1990; Szczyglowski et al., 1994). More specifically, *lb* promoter activity has been reported to be predominantly localized to the infected cells (Szabados et al., 1990; Lauridsen et al., 1993; Szczyglowski et al., 1994). Generally, nodules or nodule-like structures that are devoid of intracellular rhizobia appear to be lacking *lb* gene expression, suggesting that the physical presence of rhizobia in the plant cell cytoplasm may be required for late nodulin gene induction (de Bruijn et al., 1989; de Bruijn and Schell, 1992).

*Cis*-acting DNA elements within the *lb* promoter regions of the soybean *lbc3* and *S. rostrata* *glb*3 genes have been delimited and characterized by mutation analyses. These

investigations lead to the identification of the organ-specific element (OSE) in the *lbc3* and the nodule infected cell expression (NICE) element in the *Srglb3* 5' upstream regions of these genes which are responsible for the observed infected cell specific gene expression patterns (Stougaard et al., 1987; de Bruijn et al., 1989; Stougaard et al., 1990; Szabados et al., 1990; Ramlov et al., 1993; Szczyglowski et al., 1994). In order to identify components of the signal transduction pathway responsible for this infected cell-specific expression, gel retardation assays were used to identify DNA binding proteins which interact with the 5' upstream regions of *lb* genes (Jensen et al., 1988; Metz et al., 1988; Jacobsen et al., 1990; Forde et al., 1990; Jensen et al., 1991; Welters et al., 1993). In the *Srglb3* promoter region, a factor derived from the infecting bacterium *Azorhizobium caulinodans* strain ORS 571 (AcBBP1) was identified which could bind specifically to a region about 700 bp upstream from the start of transcription of the *Srglb3* gene (Bacterial Binding Site 1; BBS1; Welters et al., 1993). A detailed characterization of the BBS1 and complex formation was carried out. The protein responsible for the major peak of DNA binding activity was purified and its N terminal amino acid sequence was determined (AcBBP1; Welters et al., 1993).

The gene encoding AcBBP1 was cloned, its deduced gene product was characterized, an *AcBBP1* mutant strain was constructed and the effect of this mutation on symbiotic nitrogen fixation in *S. rostrata* nodules was analyzed.

# MATERIALS AND METHODS

## Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are described in Table 1.

## Polymerase Chain Reaction

Degenerate oligonucleotide primers were designed to amplify an 80-bp stretch of DNA which encoded the first 27 amino acids of the AcBBP1 protein that were previously determined by N-terminal sequencing (Welters et al., 1993). Primer 1 was designed to correspond to the N terminal portion of AcBBP1 (5' ATGGA$^{C}/_{T}$ATG$^{C}/_{G}$GIAA$^{A}/_{G}{}^{C}/_{T}$TIGT 3'). In addition, two degenerate primers were designed to correspond to amino acids 20-27 of the AcBBP1 peptide sequence: Primer 2; (5' GTCTG$^{C}/_{G}$AC$^{A}/_{G}$TC$^{C}/_{T}$TCTG$^{C}/_{G}$GT 3'); Primer 3; (5' GT$^{C}/_{T}$TGIAC$^{A}/_{G}$TC$^{C}/_{T}$TC$^{G}/_{T}$IGT 3'). The reaction mixture contained (final concentrations): 1x Taq DNA polymerase buffer (Promega; Madison, WI), 2.5 units Taq DNA polymerase (Promega), 2.5 mM dNTPs, 50 pmol of primers, 100 ng of *A. caulinodans* genomic DNA in a final volume of 25 µl. The PCR reaction was carried out using a DNA Thermocycler (model 430, Perkin Elmer, Foster City, CA). The following PCR conditions were used: 94°C , 2 min; 31°C, 1 min; 45°C, 3 min with 35 cycles followed by a 10 min extension cycle at 45°C. The resulting fragments were blunt-ended using the Klenow fragment of DNA polymerase I (Boehringer Mannheim; Indianapolis,

IN) and purified through an 8% acrylamide gel. The purified fragments were cloned into the *Sma*I-digested vector pK18 (Pridmore, 1987).

**Southern Blot Analysis**

Total bacterial genomic DNA was isolated as described by Meade et al. (1989). Ten micrograms of *A. caulinodans* total genomic DNA was completely digested with restriction enzymes, separated on a 0.8% agarose gel, and transferred to a nylon filter (Hybond-N; Amersham, Arlington Heights, IL) according to standard procedures (Sambrook, 1989). Membranes were prehybridized and hybridized in a solution containing 4 X SSC, 5 X Denhardt's solution (Sambrook, 1989), 0.5% (w/v) SDS and 100 µg/ml denatured sheared salmon sperm DNA at 65°C. The membranes were washed once for 15 min in 4 X SSC, 0.1% (w/v) SDS; twice for 15 min in 1 X SSC, 0.1% (w/v) SDS, and twice for 15 min in 0.5 X SSC, 0.1% (w/v) SDS at 65°C. The 80-bp PCR amplified probe was end labeled with [$\gamma^{32}$P]ATP (Sambrook et al., 1989), while the full length AcBBP1 gene probe was labeled with [$\alpha$-$^{32}$P]dATP using a random primer kit (Boehringer Mannheim) following the manufacturer's instructions.

**Construction of *A. caulinodans* Partial Genomic Libraries**

Total genomic DNA from *A. caulinodans* ORS 571 was digested with *Eco*RI or *Pst*I and 10 µg of DNA were separated on a 0.8% preparative agarose gel. A one centimeter area in the size range of the fragments found to hybridize with the AcBBP1

PCR probe was excised and the DNA isolated using a phenol freeze-thaw method. The purified fragments were cloned into pK18 (Pridmore, 1987) digested with *Eco*RI or *Pst*I. Positive colonies were identified using standard colony hybridization methods (Sambrook et al. 1989) using the 80-bp PCR fragment as a probe (see above).

**DNA Sequencing and Computer Analyses**

A plasmid carrying a 6-kb *Eco*RI fragment hybridizing with the AcBBP1 probe was mapped using single or multiple enzyme digests. The smallest fragment containing the AcBBP1 gene was cloned and the nucleotide sequence determined (pSF27). DNA sequencing was performed using a Sequenase 7-Deaza-dGTP DNA sequencing kit (United States Biochemical, Cleveland, OH), according to the manufacturer's instructions. Computer analysis of DNA sequences was carried out using Sequencher 2.1 (Gene Codes Corp., Ann Arbor, MI) and SeqEd (Applied Biosystems) software. Multiple sequence alignment was done using pile up in the GCG (Genetics Computer Group, Madison, WI) program and SeqVu 1.01 (Garvan Institute of Medical Research; Sydney, Australia). Analysis of predicted protein sequences was performed using PHDsec (Rost and Sander, 1993; 1994). Homology searches were performed using the BLAST algorithm (Altschul et al., 1990).

**Construction of the AcBBP1 Deficient Strain**

A plasmid carrying a 14-kb *Pst*I fragment hybridizing with the AcBBP1 probe was mapped using single or multiple enzyme digests. The location of the *AcBBP1* gene

was delimited using Southern blot analysis. A 2.4-kb *Sac*II fragment harboring the *AcBBP1* locus was cloned into the Bluescript SK⁻ vector (pSF354) and used for gene replacement. An internal *Sty*I fragment which contained the entire AcBBP1 coding region in addition to 74 bp (upstream) and 176 bp (downstream) of flanking DNA sequences (see Figure 2-1A) was excised and rendered blunt-ended using the Klenow fragment of DNA polymerase I (Boehringer Mannheim). A blunt-ended *Pst*I fragment carrying the kanamycin cassette from pUC4K (Pharmacia Biotech; Uppsala, Sweden) replaced the native *Sty*I fragment creating a deletion/insertion mutation (pSF354del). The entire pSF354del plasmid was cloned into the *Pst*I site of the broad host range vector pLAFR 3 (Staskawicz et al, 1987) and mobilized from *E. coli* to *A. caulinodans* ORS 571 using the helper plasmid pRK2013 (Ditta et al., 1980). Transconjugants were selected as described by Pawlowski et al. (1987) on plates supplemented with tetracycline (Tc) 10 μg/ml, carbenicillin (Cb) 500 μg/ml, and kanamycin (Km) 200 μg/ml. A plasmid (pPH1JI) incompatible with the donor plasmid was introduced to selected transconjugants to force replacement of the mutated AcBBP1 gene (de Bruijn, 1987).

**Gel Mobility Shift Assay**

Extracts of *A. caulinodans* ORS 571 and the mutant ORS 571-425 strains were prepared as described by Welters et al. (1993). An 80-bp fragment of the *Srglb3* promoter containing the BBS1 (fragment 5'203) was used in all reactions under conditions

previously described (Welters et al., 1993). All reaction mixtures were resolved on 8% acrylamide gels.

**Plant Growth and Nodulation**

*S. rostrata* seeds were surface-sterilized and germinated as described by Pawlowski et al. (1987). Seedlings were grown in autoclaved soil (sand:vermiculite:MetroMix, 5:3:1) in growth chambers with 75% RH, 18-hr light, 28°C/6-hr dark, 22°C regime for 5 weeks. For *in vitro* plant nodulation assays, the sterilized seeds were put directly onto B+D medium slants solidified with 1.5% agar (Broughton and Dilworth, 1971) and the tubes were maintained under artificial light at 21°C (16 hr day). Plants grown in soil or on agar slants were inoculated with a two-day old culture of *A. caulinodans* ORS 571 or ORS 571-425 grown in the presence of antibiotics but washed with sterile water to remove antibiotics and residual medium. The pelleted cells were resuspended in sterile water and one ml of this suspension was inoculated onto the roots of one-week old *S. rostrata* seedlings. For stem inoculations, the cultures were generated as described above and applied to five-week old stems of *S. rostrata* plants using a sterile cotton applicator.

**Acetylene Reduction Assays**

To determine the nitrogen fixation activity in *S. rostrata* root or stem nodules the acetylene reduction assay was performed. Root or stem nodules induced on *S. rostrata* plants were excised, placed into vaccutainer tubes and sealed. One milliliter of acetylene

was injected into the tubes (~8% of the final gas phase) as described by Pawlowski et al. (1987) with the following modifications. Samples were drawn every 15 min and injected into a gas chromatograph (Varian model 3700, Sunnyvale, CA). The data were expressed as nmol of ethylene produced/g of fresh weight.

**Northern Analysis**

Total RNA from 5-week old nodules was isolated using the hot phenol method (Verwoerd et al., 1989) except that the extraction buffer was as described by Hall et al. (1978). Ten μg of total RNA was separated through 1.2% agarose-formaldehyde gels in MOPS buffer, as described in Sambrook et al. (1989) and transferred to 0.22μm nitrocellulose membranes (NitroPlus, Micron Separations, Inc.; Westboro, MA). Prehybridization and hybridization reactions were carried out using a solution composed of 0.5M phosphate buffer pH 7.2, 7% (w/v) SDS and 1% (w/v) BSA (Fraction V; Sigma, St. Louis, MO) at 65°C, according to Church and Gilbert (1984). Filters were washed twice for 15 min in 1 X SSC, 0.1% SDS and once for 15 min in 0.5 X SSC, 0.1% SDS at 65°C. Probes were labeled using the random primer kit (Boehringer Mannheim), according to the manufacturer's instructions.

# RESULTS

## Identification and Cloning of the *A. caulinodans AcBBP1* Gene

Using a reverse genetics approach, the gene encoding AcBBP1 from *A. caulinodans* strain ORS 571 was isolated. Degenerate oligonucleotide primers corresponding to the N terminal amino acid sequence of AcBBP1 previously determined (Welters et al., 1993), were used to amplify a DNA fragment encoding this 27 amino acid region using the polymerase chain reaction (PCR). The DNA sequence of the resulting 80-bp PCR product was determined and found to encode the expected 27 amino acid peptide. The PCR fragment was used as a probe to determine the *AcBBP1* gene copy number in ORS 571. Southern blot analysis of *A. caulinodans* genomic DNA digested with *Eco*RI, *Bam*HI, *Hind*III, and *Pst*I revealed single hybridizing bands, suggesting that the AcBBP1 gene is not reiterated (Figure 2-1). This analysis revealed that the *AcBBP1* gene was located on a 6-kb *Eco*RI fragment. To isolate the complete *AcBBP1* locus, a partial *Eco*RI library of ORS 571 genomic DNA was constructed, enriching for fragments of ~6 kb and screened via colony hybridization using the 80-bp *AcBBP1* PCR product as the probe. The recombinant plasmids from several hybridizing colonies were isolated, mapped with several restriction enzymes and re-hybridized with the *AcBBP1* probe. These analyses revealed that the *AcBBP1* gene was located near the border of the 6-kb fragment and would not be suitable for future experiments (e.g. gene replacement). Therefore, a partial *Pst*I library of ORS 571 genomic DNA was constructed, enriching for fragments ~14 kb and screened using the method previously described. A plasmid

**Table 1**. Strains and plasmids used in this study

| | Relevant characteristics | Source or reference |
|---|---|---|
| **Strains** | | |
| *Escherichia coli* | | |
| DH5α | *recA* strain for cloning experiments | Sambrook et al. (1989) |
| INVαF' | *recA* strain for cloning experiments | Invitrogen (Carlsbad, CA) |
| *Azorhizobium caulinodans* | | |
| ORS 571 | wild-type, $Nod^+$, $Fix^+$, $Cb^r$ | Dreyfus et al. (1988) |
| ORS 571-425 | *AcBBP1*, $Nod^+$, $Fix^+$, Nod(stem) delayed, $Cb^r$, $Km^r$ | This work |
| **Plasmids** | | |
| pBluescript II KS/SK (+/-) | Cloning and sequencing vector, $Ap^r$ | Stratagene (La Jolla, CA) |
| pK18 | Cloning and sequencing vector, $Km^r$ | Pridmore (1987) |
| pLAFR3 | cos, $Mob^+$, $Tra^-$, IncP; $Tc^r$ | Staskawicz et al. (1987) |
| pRK2013 | $Tra^+$, $Mob^+$, IncP, $Km^r$, helper plasmid in triparental conjugations | Ditta et al. (1980) |
| pPH1JI | $Tra^+$, IncP, $Cm^r$, $Sm^r$, $Sp^r$, $Gm^r$, for gene replacement | Beringer et al. (1978) |
| p*Srclb*1 | *S. rostrata lb* cDNA clone, $Ap^r$ | Metz et al. (1988) |
| pUC4k | Origin of the $Km^r$ cassette | Pharmacia Biotech (Uppsala, Sweden) |
| pSF27 | 6.0 kb EcoRI fragment of ORS 571 genomic DNA containing AcBBP1 in pK18, $Km^r$ | This work |
| pSF318 | 14 kb *Pst*I fragment of ORS571 genomic DNA containing *AcBBP1* in pK18, $Km^r$ | This work |
| pSF354 | 2.5 kb *Sac*II fragment of pSF318 in pBluescript II SK, $Ap^r$ | This work |
| pSty7L | 470 bp StyI fragment of pSF 354 in pK18, $Km^r$ | This work |
| pSF354del | pSF354 carrying a deletion of the 470 bp*Sty*I fragment and insertion of the $Km^r$ cassette at the *Sty*I site, $Km^r$, $Ap^r$, | This work |

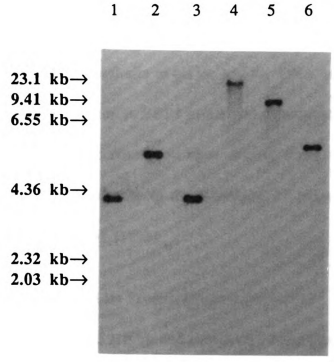



**Figure 2-1. Identification of the *AcBBP1* gene in the *A. caulinodans* genome.** Single hybridizing bands were detected when a genomic Southern blot was probed with the 80-bp *AcBBP1* PCR fragment. Ten micrograms of total genomic DNA from *A. caulinodans* was digested with: **Lane 1**, *Eco*RI; **Lane 2**, *Bam*HI; **Lane 3** *Eco*RI+*Bam*HI; **Lane 4**, *Hind*III; **Lane 5**, *Pst*I; **Lane 6**, *Hind*III+*Pst*I.

(pSF318) containing the AcBBP1 gene within the 14-kb fragment was restriction mapped. Within this 14-kb *Pst*I fragment, a 2.4-kb *Sac*II fragment that contained the *AcBBP1* gene centrally located was identified, subsequently subcloned into the *Sac*II site of pBluescript KS$^-$ (pSF354), and used in further studies. Based on the restriction map of pSF354, an internal 470 bp *Sty*I fragment contained the *AcBBP1* gene. The *Sty*I fragment was blunt-ended, cloned into the *Sma*I site of pK18, and the DNA sequence of several inserts showing hybridization with the AcBBP1 probe was determined. Plasmid pSty7L with a 470 bp insert was found to contain an open reading frame (ORF) encoding a polypeptide with 100% sequence identity to the N-terminal 27 amino acid sequence of AcBBP1 determined by Welters et al. (1993).

**Analysis of the *AcBBP1* Gene and Its Protein Product (AcBBP1)**

The *AcBBP1* gene consists of 210 bp and encodes a 72 amino acid protein with a predicted molecular mass of 9 kDa (Figure 2-2A) and a pI of about 9.5. This molecular mass correlates well with the AcBBP1 protein mass determined by Welters et al. (1993) using biochemical methods (9-10 kDa). Two regions of the AcBBP1 protein were found to contain stretches of basic amino acid residues, which may indicate putative nuclear localization signals (NLSs; Garcia-Bustos et al., 1991; Raikhel, 1992; Figure 2-1A highlighted in red and green). DNA binding helix-turn-helix motifs were found in the central portion of AcBBP1 (residues 22-41; Figure 2-2B) as well as the C-terminus of the protein (residues 51-70; Figure 2-2C). Both of these regions contain conserved amino acid residues important for the formation of the DNA binding domain (Pabo and Sauer, 1984).

**Figure 2-2. Primary structure and deduced amino acid sequence of AcBBP1.**

**(A)** Deduced amino acid residues are shown in one-letter notation below the nucleotide sequence of the *Sty*I-*Sty*I fragment harboring *AcBBP1*. Underlined residues match the protein sequence obtained previously (Welters et al., 1993). Residues highlighted in red or in green may encode a nuclear localization signal (NLS).

**(B)** Alignment of a AcBBP1 helix-turn-helix DNA binding motif. AcBBP1 (this work), Lac Rep, λ Rep, λ Cro (lactose repressor, λ repressor, λ Cro protein; Pabo and Sauer, 1984). Residues highlighted in red represent residues thought to be important for maintaining structure (position 5 usually A, position 9 usually G, position 15 usually I or V). Green highlights identical residues while pale green denotes conservative substitutions in the alignment.

**(C)** Alignment of the second putative helix-turn-helix DNA binding motif in AcBBP1 with C proteins, immunity repressor proteins and a regulator of conjugal transfer and vegetative replication of the RK2 plasmid. AcBBP1 (this work), control elements: *Bgl*IIC, Anton et al., (1996); *Bam*HIC, Nathan and Brooks (1988); *Mun*IC, Siksnys et al., (1994); *Pvu*IIC, Tao et al. (1991); *Sma*IC, Heidemann et al. (1989); *Eco*RV, Bougueleret et al. (1984); Imrep ϕ105, Cully and Garro (1985); Dhaese et al. (1985); Imrep *E.coli*, Aiba et al. (1996), TrbA, Jagura-Burdzy et al. (1992). Red shading indicates those residues conserved in helix-turn-helix motifs while green shading denotes identical residues and pale green shading indicates conservative substitutions. Alignment was done using the method of Karlin and Brendel, (1994). Residues were grouped as follows: M, L, I, V, F, G, A, W; H, Q, N, P; C, S, T, Y; R, K, E, D.

**A**

*Sty*I

```
CCTTGGCCGCAAAGCGATCCTATACATACTATAGTTGATAATCTCGGA     48
GGGCGTAAATTGCGATCCGTCTGCGCATGGATATGCGCAAGCTGGTCG     96
                                  M  D  M  R  K  L  V

GCCGGAACTTCGCGCGCCTGCGTCAGGAGAAGGGCCTGACACAGGAGG    144
G  R  N  F  A  R  L  R  Q  E  K  G  L  T  Q  E

ACGTACAGACGCGATCCGGCTTCAGCCAGCAGTACATCAGCGGGCTCG    192
D  V  Q  T  R  S  G  F  S  Q  Q  Y  I  S  G  L

AACGCGGCCGGCGCAATCCCACTGTCATCACGCTCTATGAACTGGCAC    240
E  R  G  R  R  N  P  T  V  I  T  L  Y  E  L  A

AGGCGCTGGGCGTTAGCCACGAAGAGCTTGTTCGCGCTGACGGCAAGG    288
Q  A  L  G  V  S  H  E  E  L  V  R  A  D  G  K

ACTGAACCCCGGAGGGGAAGCACCGGCCGAAACCGCCCGATGCGTTGC    336
D  *

CGGCTCGCCCGCCGCGCCGGCCGCGAAGGGGGCGACCAAATCAGCGAA    384
AAGGTATTGCCGTCCTCGTCGTCGATCAGGTTGGGGGCGTGAAAGGTC    432
GGACCGTCGTCTTCGAATGCCCACCGCAATCTCCTTGG               470
                                    StyI
```

**B**

```
                1         5         10        15        20

AcBBP1      22  Q E D V Q T R S G F S Q Q Y I S G L E R  41
BglIIC      22  Q E K L A E I S K L D R T Y I G G V E R  41
BamHIC      25  Q E K L A F E C D L E R T Y I S D I E R  44
MunIC       25  Q E S F A A Q I D L D R T Y Y S S I E N  43
PvuIIC      27  Q E S L A D L V G I E R T Y I G S I E R  48
SmaI        30  Q E Q L A E I S G L E R T Y I G S V E R  49
EcoRV       42  Q G E V A K A L G K P Q S Y I S K I E Q  61
Phi105      18  Q V Q L A E K A N L S R S Y L A D I E R  37
ImRepEc     23  Q R R A A E L S G L T E S A I S T I E Q  42
TrbA        21  K E E L S E R A G V S I S F L S D L T N  40

          H e l i x ---turn--- H e l i x
```

**C**

```
                1         5         10        15        20

AcBBP1      51  L Y E L A Q A L G V S E E E L V R A D G  70
Lac Rep      6  L Y D V A E Y A G V S Y Q T V S R V A N  25
λ Rep       33  Q E S V A D K N G M G E S G V G A L F N  52
λ Cro       16  Q T K T A K D L G V Y Q S A I N K A I H  35

          H e l i x ---turn--- H e l i x
```

Secondary structure analysis of AcBBP1 using the PHD-sec program (Rost and Sander, 1993; 1994) revealed the presence of two regions containing two adjacent helical structures separated by a loop or turn with a high probability score (7-9 on a scale from 0-9; Figure 2-2 B, C).

A sequence similarity search was carried out using the BLAST algorithm (Altschul et al., 1990). This analysis revealed significant similarity of AcBBP1 to regulatory proteins. The most significant matches were found to be control elements in several type II restriction-modification (RM) systems (Bougueleret et al., 1984; Nathan and Brooks, 1988; Heidemann et al., 1989; Tao et al., 1991; Siksnys et al., 1994; Anton et al., 1996) , to an immunity repressor protein from the *Bacillus subtilis* phage ϕ105 (Cully and Garro, 1985; Dhaese et al., 1985), a randomly sequenced Kohara plasmid of *E. coli* (Aiba et al., 1996) and a regulator of vegetative replication and conjugal transfer of the RK2 plasmid (Jagura-Burdzy et al., 1992; Figure 2-3 and 2-4). AcBBP1 shares identical and similar amino acid residues with the C proteins throughout the amino acid sequence. AcBBP1 also shares similar residues in the N terminal regions of the repressor proteins and the replication/conjugal transfer regulator. Identity within helical domains thought to be important for the helix-turn-helix DNA binding motif was observed among the amino acid sequences in all classes of proteins (Tao et al., 1991; Dhaese et al., 1985; Van Kaer et al., 1987; Jagura-Burdzy et al., 1992).

The binding site for C.*Bam*HI, AcBBP1 (J. Brooks, personal communication; Welters et al., 1993) and the operator sequence to which the ϕ105 immunity repressor

**Legend for Figure 2-3. Alignment of AcBBP1 amino acid sequence to control elements in type II restriction-modification systems and to immunity repressors of ϕ-105 and *E. coli*.** AcBBP1 (this work), control elements: *Bgl*IIC, Anton et al. (1996); *Bam*HIC, Nathan and Brooks (1988); *Mun*IC, Siksnys et al. (1994); *Pvu*IIC, Tao et al. (1991); *Sma*IC, Heidemann et al. (1989); *Eco*RV, Bougueleret et al. (1984); Imrep ϕ105, Cully and Garro (1985); Dhaese et al. (1985); Imrep *E.coli*, Aiba et al. (1996). Boxed residues represent identity while shaded areas represent similarity to the AcBBP1 sequence. Identities were boxed if 50% of the aligned sequences contained the same residue. Sequences were aligned using the algorithm of Karlin and Brendel (1994). Residues were grouped as follows: M, L, I, V, F, G, A, W; H, Q, N, P; C, S, T, Y; R, K, E, D.

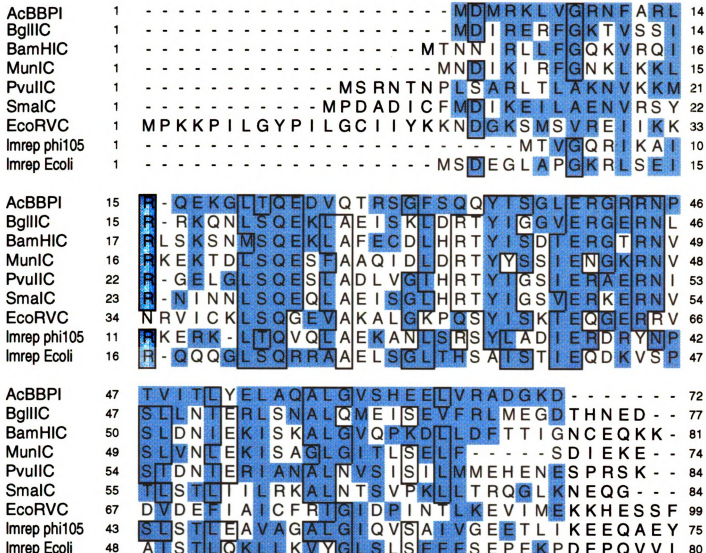



**Figure 2-3**. Alignment of AcBBP1 with control proteins found in type II restriction-modification systems and immunity repressor proteins from Phi 105 and *E. coli*.

```
AcBBP1  1   M D M R K L V G R N F A R L R Q E K G L T Q E D V   25
TrbA    1     M Y N Q I F F T N I L R L L D E R G M T K H E L   24

AcBBP1 26 Q T R S G F S Q Q Y I S G L E R G R R N P T V I T   31
TrbA   25 S E R A G V S I S F L S D L T N G K A N P S L K V   30

AcBBP1 32 L Y E L A Q L G V S H E E L V R A D G K D           72
TrbA   31 M E A I A D A L E T P L P L L L E S T D L D R E A   75
```

**Figure 2-4. Alignment of AcBBP1 with TrbA, a regulator of vegetative replication and conjugative transfer of the RK2 plasmid** (Jagura-Burdzy et al., 1992). Pink shading indicates identical residues, light green shading denotes conservative substitutions. Sequences were aligned using the algorithm of Karlin and Brendel (1994). Residues were grouped as follows: M, L, I V, F, G, A W; H, Q, N, P; C, S, T, Y; R, K, E, D.

binds were compared (Van Kaer et al., 1987; Van Kaer et al., 1988). Although these three types of binding sites do not share a high degree of similarity with each other, short stretches of identical nucleotide residues were observed in these binding sites (Figure 2-5).

**Creation and Characterization of An *A. caulinodans* AcBBP1 Deficient Strain**

In an attempt to define the function of AcBBP1, an ORS 571 strain lacking the ability to synthesize AcBBP1 was constructed (ORS 571-425). A kanamycin resistance ($Km^r$) cassette derived from pUC4K was used to replace a 470 bp *Sty*I fragment harboring the *AcBBP1* gene in addition to 74 bp (upstream) and 176 bp (downstream) of flanking region carried on plasmid pSF354. The resulting deletion/substitution mutation was used to replace the wild type gene via gene replacement (de Bruijn, 1987). The structure of the mutated *AcBBP1* locus in strain ORS 571-425 was confirmed by Southern blot analysis (see Figure 2-6). Whole cell extracts were prepared from the wild-type ORS 571 strain and the ORS 571-425 mutant and used in gel retardation assays using the fragment of the *S. rostrata glb*3 5' upstream region, harboring the binding site for AcBBP1 (BBS1; fragment 5'203; Welters et al., 1993; see Figure 2-7) as target DNA. No binding activity could be observed with extracts prepared from strain ORS 571-425 (Figure 2-7), confirming that the insertion/deletion mutation abolishes AcBBP1 production.

To further examine the phenotype of the AcBBP1 deficient mutant strain, the growth rate in the free living state was monitored. Cultures of the *A. caulinodans* wild type or the AcBBP1 deficient strain were grown in the presence of Cb 250 μg/ml until

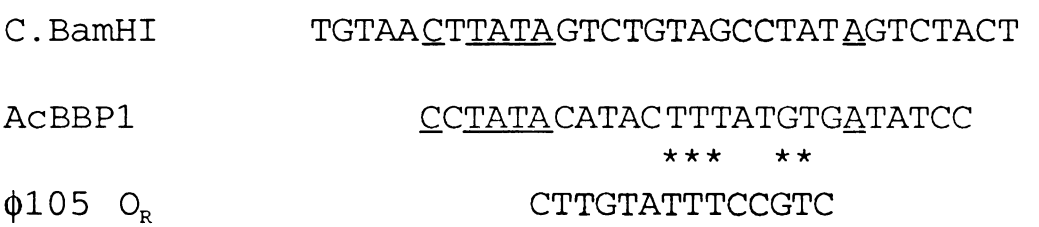



**Figure 2-5. Comparison of the binding sites for C.*Bam*HI, AcBBP1 and the ϕ105 immunity repressor**. Underlined nucleotides show identity between AcBBP1 and C.BamHI while * indicates identity between AcBBP1 and the immunity repressor from ϕ105. The red shading denotes identity between C.*Bam*HI and the binding site of the immunity repressor.

late log phase. The cultures were inoculated into fresh TY medium (Beringer, 1974) supplemented with Cb 250 µg/ml at a 1:1000 dilution. One milliliter samples were taken and measured every four hours until the cultures reached stationary phase. No differences in growth rate were observed (data not shown).

The AcBBP1 deficient mutant strain was also examined for dinitorgen dependent growth characteristics (Nif phenotype) under reduced oxygen conditions with or without the presence of ammonium as described by Pawlowski et al. (1987). No differences in growth or colony morphology were observed (data not shown).

The effect of the AcBBP1 mutation on nodulation and symbiotic nitrogen fixation (Nod and Fix phenotype) was also determined. For this purpose, sterile seedlings of *S. rostrata* were inoculated on the stems or on the roots with ORS 571 or ORS 571-425. The AcBBP1 deficient mutant strain was found to be capable of inducing both stem and root nodules on its host plant (Nod+). To determine the relative nitrogen fixation levels in wild type versus AcBBP1 deficient nodules, acetylene reduction assays were performed. The results of three independent root nodule experiments are shown in Figure 2-8.

In the first experiment, 13 plants were assayed for nitrogen fixation. The acetylene reduction (nitrogen fixation) activity of 8 independent root nodule samples harboring the AcBBP1 deficient strain were compared to 5 root nodule samples from control plants. A ~16% reduction in activity was observed (Figure 2-8 A). In the second trial, 18 plants were assayed. The nitrogen fixation activity of 13 independent nodule samples induced by the AcBBP1 deficient strain were compared to 5 samples from control plants. A

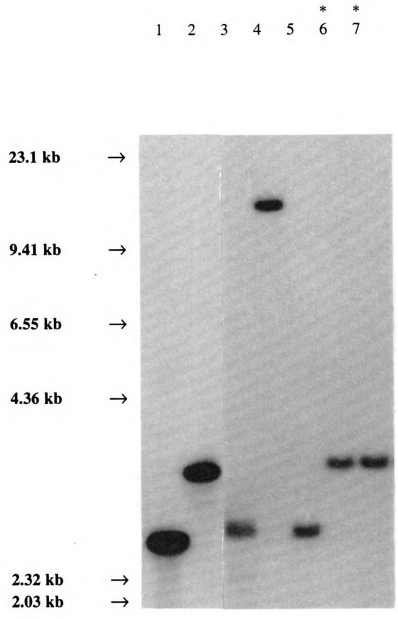
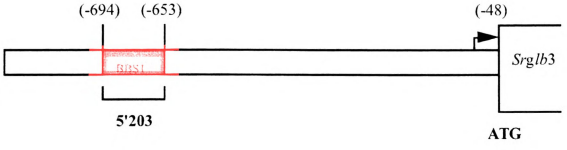



**Figure 2-6. Structure of the AcBBP1 ORS-4-25 deletion/insertion mutant**. **Lane 1**, *Sac*II fragment containing *AcBBP1*; **Lane 2**, *Sac*II without *Sty*I-*Sty*I fragment+kan cassette, **Lane 3**, plasmid pSF354 digested with *Sac*II, **Lane 4,** ORS 571 genomic DNA digested with *Pst*I, **Lane 5**, ORS 571 genomic DNA digested with *Sac*II, **Lane 6-7** genomic DNA digested with *Sac*II from transconjugants 346, 425. Plasmid DNA or 10 µg of total genomic DNA isolated from the above strains were digested as noted, separated on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with the entire wild type S*ac*II fragment as probe. *=gene replacement.

**A**




**B**

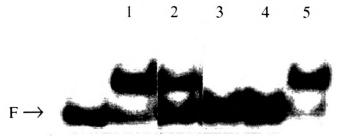
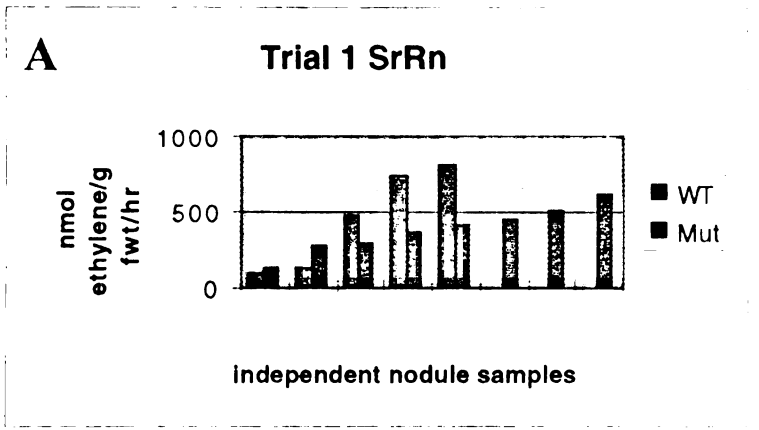


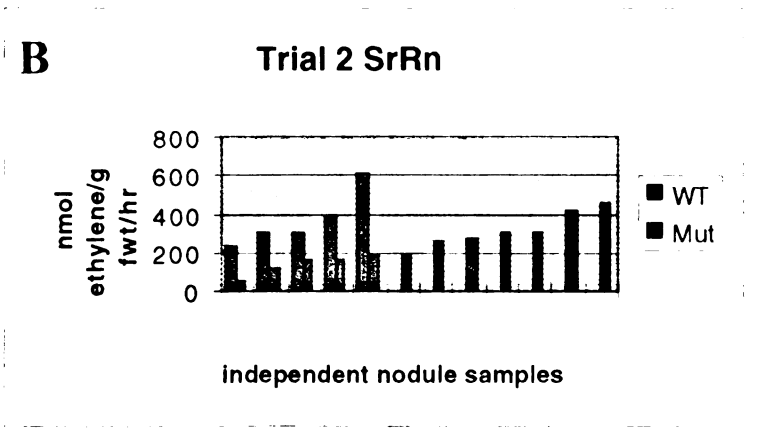

**Figure 2-7. Whole cell extracts prepared from the AcBBP1 deficient mutant lack binding activity**. Extracts prepared from ORS 571 and ORS 571-425 were tested for DNA binding activity using the BBS1 as target DNA. **(A)** Schematic of the *Srglb3* 5' upstream region. The arrow indicates the start point of transcription. The fragment 5'203 (shaded in red) containing the BBS1, was used as the target fragment in gel mobility shift assays. **(B) Lane 1**, extracts prepared from wild-type ORS 571 (high salt extraction), **Lane 2,** ORS 571 (low salt extract), **Lane 3**, extracts prepared from ORS 571-425 (high salt extraction) **Lane 4**, ORS 571-425 (low salt extraction), **Lane 5** control (wild-type extract isolated previously). The free fragment (F, 5'203) is indicated with an arrowhead.

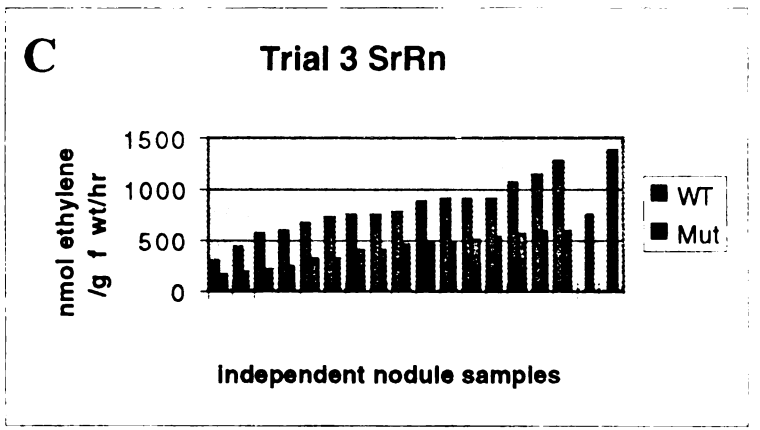
~36% reduction in activity was apparent (Figure 2-8 B). In the final trial, 34 plants were tested. The acetylene reduction activity of 18 root nodule samples induced by ORS 571-425 were compared to 16 samples from control plants. A 40% reduction in activity was detected (Figure 2-8 C). The overall nitrogen fixation activity is presented for each trial (Figure 2-8D)

*S. rostrata* stem nodules were also tested for nitrogen fixation activity. The results of three independent stem nodule experiments are presented in Figure 2-9. In the first experiment, 24 plants were tested. The acetylene reduction activities of 13 independent nodule samples harboring the mutant strain were compared to 11 nodule samples harvested from control plants. A 45% reduction in activity was observed. In the second experiment, 32 independent plants were assayed. Equal numbers of nodules induced by the AcBBP1 deficient strain were compared to nodules harboring the wild type strain. A ~16% reduction was detected in this trial. Finally, 13 plants were assayed for nitrogen fixation activity. Five independent nodule samples induced by ORS 571-425 were compared to 8 control samples. A 45% difference in acetylene reduction activity was observed. Thus it appears that the *AcBBP1* mutant strain induces nodules on *S. rostrata* plants that display an approximately ~16% reduction in nitrogen fixation activity, suggesting that AcBBP1 does play a role in symbiotic nitrogen fixation, but is clearly not essential.

**Figure 2-8. Nitrogen fixation activity is reduced in *S. rostrata* root nodules induced by ORS 571-425.** *S. rostrata* root nodules were tested for nitrogen fixation activity by the acetylene reduction assay. **(A)** Trial 1; **(B)** Trial 2; **(C)** Trial 3; **(D)** Average nitrogen fixation activity. Light bars = ORS 571; dark bars = ORS 571-425. All results are expressed as nmol ethylene produced/g fresh weight/hr.


A
Trial 1 SrRn
1000
500
0
nmol ethylene/g fwt/hr
WT
Mut
independent nodule samples


B
Trial 2 SrRn
800
600
400
200
0
nmol ethylene/g fwt/hr
WT
Mut
independent nodule samples


C
Trial 3 SrRn
1500
1000
500
0
nmol ethylene /g f wt/hr
WT
Mut
independent nodule samples

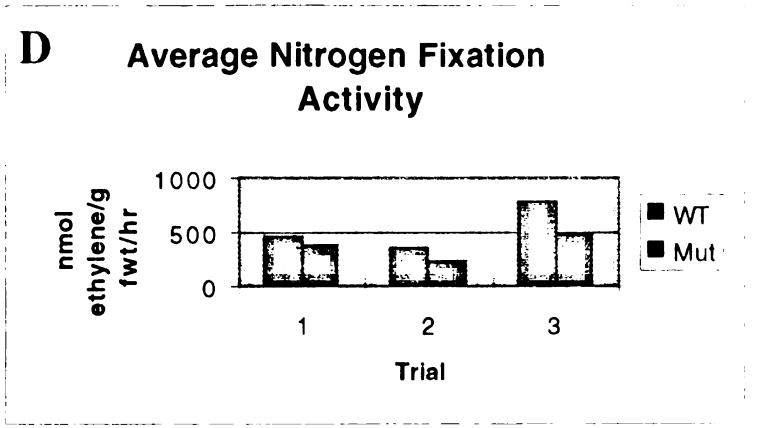
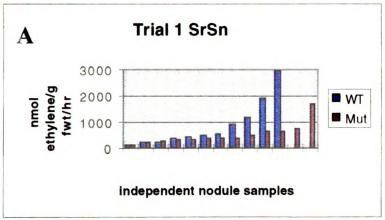
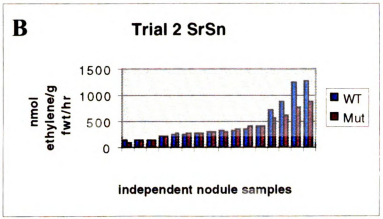
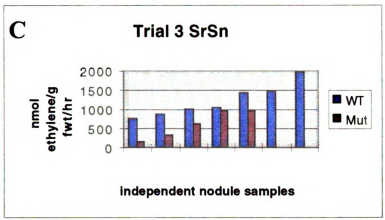
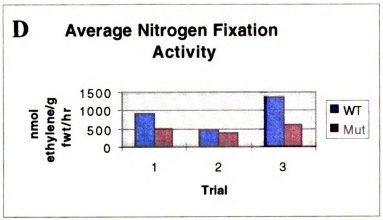

D
Average Nitrogen Fixation Activity
1000
500
0
nmol ethylene/g fwt/hr
WT
Mut
1
2
3
Trial

**Figure 2-9. Nitrogen fixation activity is reduced in *S. rostrata* stem nodules induced by ORS 571-425.** *S. rostrata* stem nodules were tested for nitrogen fixation activity by the acetylene reduction assay. (A) Trial 1; (B) Trial 2; (C) Trial 3; (D) Average nitrogen fixation activity. Light bars = ORS 571; dark bars = ORS 571-425. All results are expressed as nmol ethylene produced/g fresh weight/hr.


A
Trial 1 SrSn
nmol ethylene/g fwt/hr
3000
2000
1000
0
WT
Mut
independent nodule samples


B
Trial 2 SrSn
nmol ethylene/g fwt/hr
1500
1000
500
0
WT
Mut
independent nodule samples

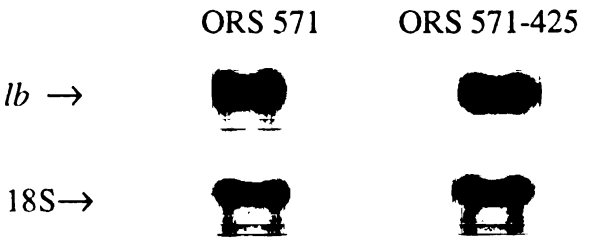

C
Trial 3 SrSn
nmol ethylene/g fwt/hr
2000
1500
1000
500
0
WT
Mut
independent nodule samples


D
Average Nitrogen Fixation Activity
nmol ethylene/g fwt/hr
1500
1000
500
0
1
2
3
Trial
WT
Mut

**Leghemoglobin (*lb*) Steady State mRNA Levels in *S. rostrata* Nodules Harboring Wild Type or AcBBP1 Deficient Bacteria**

The observed effect of the AcBBP1 mutation on symbiotic nitrogen fixation can be caused at various stages of the nodulation/nitrogen fixation process (see also Chapter 3 of this thesis). Since the target of the AcBBP1 protein is located in the *Srglb*3 5' upstream region and a role for AcBBP1 in infected cell specific expression of *lb* genes had been postulated (Welters et al., 1993), the relative levels of steady-state *lb* mRNAs in nodules elicited by ORS 571 and ORS 571-425 were examined. Total RNA from 5-week old nodules induced by wild-type *A. caulinodans* or the AcBBP1 deficient strain was hybridized with the *S. rostrata* leghemoglobin probe *Srclb*1 (Metz et al., 1988). High steady state levels of *lb* mRNA were detected both in nodules induced by the wild-type and in those induced by the mutant strain (Figure 2-10). Quantification of the *lb* gene hybridization signal versus the 18S rRNA gene control failed to reveal significant differences in *lb* mRNA levels between the two types of nodules, suggesting that the ~20% reduction of nitrogen fixation activity in the nodules harboring the mutant strain does not directly correlate with a reduction in total *lb* mRNA levels.

**A**




**B**

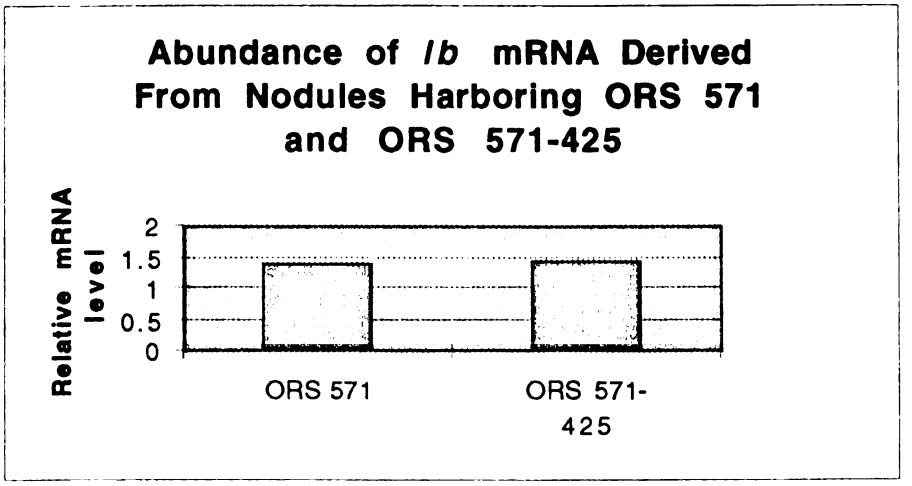



**Figure 2-10. Northern blot analysis of leghemoglobin gene expression.** Ten micrograms of total RNA from 5 week old *S. rostrata* stem nodules harboring wild-type or AcBBP1 deficient bacteria was isolated and hybridized with radiolabeled *Srclb*1, a cDNA encoding a leghemoglobin from *S. rostrata*. as a probe (Metz et al., 1988). The blot was stripped and re-hybridized with the 18S rRNA gene from rice as a loading control.

# DISCUSSION

In this chapter, the gene encoding the *A. caulinodans* ORS 571 AcBBP1 DNA binding protein that was previously shown to interact with a portion of the 5' upstream region of the *Srglb3* gene was isolated and characterized. This protein (AcBBP1) was identified during a screen for DNA-binding proteins (*trans*-acting factors) interacting with *cis*-acting elements in the 5' upstream region of a leghemoglobin gene from the legume host of *A. caulinodans*, namely *S. rostrata* (*Srglb3*; Welters et al., 1993). The binding site for AcBBP1 in the promoter region of the *S. rostrata glb3* gene (BBS1), was found to play a role in *Srglb3* promoter activity since a BBS1 insertion mutation displayed a substantially reduced level of *Srglb3-uidA* reporter gene expression in transgenic legume plants (Welters et al., 1993).

It was surprising that the AcBBP1 protein was of bacterial origin and that it could interact with a plant gene promoter, however this is not an entirely new idea. Leghemoglobin gene expression appears to be infected cell specific. In the case of indeterminate nodules, strong evidence has been presented in support of this idea as *lb* transcripts in alfalfa nodules are only found in infected cells (de Billy et al., 1991). In determinate nodules, although the precise localization of *lb* transcripts is not clear, transgenic *L. corniculatus* plants carrying chimeric *lb* promoter-reporter gene constructs appear to only be expressed in the infected cells (Szabados et al., 1990; Lauridsen et al., 1993; Ramlov et al., 1993; Szczyglowski et al., 1994).

*lb* genes are not expressed in alfalfa nodules that do not contain intracellular rhizobia such as those which spontaneously form on the roots of specific alfalfa lines (Truchet et al., 1989), or induced by the treatment of auxin transport inhibitors (Hirsch et al. 1989) or by particular mutant strains of rhizobia (Dickstein et al., 1988; de Bruijn et al., 1989). These data have suggested that a rhizobial factor produced by the intracellular bacteria or related to the release of bacteria from the infection thread may be essential for activation of *lb* genes (Nap and Bisseling, 1990; de Billy et al., 1991; Dickstein et al., 1991; de Bruijn and Schell, 1992). Furthermore, Wullstein and Bruening (1972) showed that rhizobial metabolites could be transferred to the nuclei of clover nodule cells and hypothesized that rhizobia may induce clover cells to begin leghemoglobin synthesis by the transfer of a bacterial produced inducer. Truchet et al. (1980) proposed the existence of a non-diffusable *Rhizobium*-related signal involved in the differentiation of the central infected tissue of the nodules and de Billy et al. (1991) postulated that such a factor may play a role in infected cell specific expression.

The observation that the *A. caulinodans* AcBBP1 protein could bind to a leghemoglobin promoter suggest that a bacterial protein is transported out of the cell and is directed to the plant nucleus. The concept of bacterial-plant transkingdom signaling is not a completely novel one. In the *Agrobacterium*-plant interaction, the transfer of the T-DNA from the bacterium to the plant cell is mediated by two discrete DNA binding proteins, VirD2 and VirE2. These two proteins direct and protect the T-DNA strand on its journey from *Agrobacterium* to the plant cell. Both VirD2 and VirE2 contain bipartite NLSs (Howard et al., 1992; Citovsky et al., 1992) and the presence of an intact NLS

correlates with the ability of this bacterium to form tumors. Recently, avirulence proteins have been shown to be recognized directly in the plant cell. Yang and Gabriel (1995) first indicated that *Xanthomonas avr/pth* gene products contained NLSs and could be directed to the plant nucleus, suggesting that these proteins are deposited in the plant cell. Furthermore, Van den Ackerveken et al. (1996) showed that the AvrBs3 protein contains a functional NLS and that a functional targeting signal correlates positively with HR. In symbiosis, the *fixF* gene product could direct chimeric reporter gene constructs to the plant nucleus. So far this is the only example of a rhizobial protein in which a functional NLS has been tested using chimeric NLS-reporter gene constructs. This observation indicates that bacterial proteins may be important in establishing or maintaining symbiosis (Jabbouri et al., 1996).

To further investigate what role the *A. caulinodans* AcBBP1 protein may play in *Srglb3* gene expression, the gene encoding this factor was cloned via a reverse genetics approach using oligonucleotide primers designed to the amino acid sequence previously determined by Welters et al. (1993). Upon inspection of the amino acid sequence encoding this protein, putative NLSs and two regions with similarities to helix-turn-helix DNA binding motifs were identified (Figure 2-2). Both helix-turn-helix regions contain conserved amino acid residues that are important for the formation of the DNA binding domain. Secondary structure analysis using the PHD-Sec computer program (Rost and Sander, 1993; 1994) predicted two adjacent helical structures separated by a loop or turn with fairly high probabilities (7 to 9 on a scale of 0-9).

Small patches of basic residues that may serve to direct this protein to the plant nucleus were detected. Although, no consensus sequences for NLSs are known in any system (Garcia-Bustos et al., 1991; Raikhel, 1992), a general feature consistent among these targeting sequences is the presence of clusters of basic residues. The basic R-K residues at position 4 and 5 followed by a patch of residues in which 3 out of 6 are basic R-L-R-Q-E-K could act as a bipartite NLS. The second patch of basic residues G-L-E-R-G-R-R-N-P-T has some similarities to a previously identified NLS of the regulatory protein R from maize (NLS-M; Figure 2-2). NLS-M in combination with one other NLS found in the R protein was necessary for efficient transport of chimeric NLS-reporter gene constructs to the nucleus (Shieh et al., 1993). NLS-M and the second putative NLS in AcBBP1 (highlighted in green) are similar in sequence and both are localized to the N-terminal portion of the helix-turn-helix DNA binding motif. The MyoD1 regulatory protein was found to contain an NLS in the helix-turn-helix motif although the precise location within the domain was not delimited (Tapscott et al., 1988). The observation that the transcription factors MyoD1 and R have NLSs within the DNA binding motif presents an interesting correlation. The NLS may have evolved to co-localize with the essential DNA binding domain.

A sequence similarity search using the BLAST algorithm (Altschul et al., 1990) revealed that AcBBP1 was similar to a class of control elements found in type II restriction-modification systems: *Eco*RV (Bougueleret et al., 1984), *Bam*HI (Nathan and Brooks, 1988), *Sma*I (Heidemann et al., 1989), *Pvu*II (Tao et al., 1991), *Bgl*II (Anton et al., 1996), *Mun*I (Siksnys et al., 1994), *Eco*721 (Rimseliene et al., 1995); an immmunity

region in the *B. subtilis* phage ϕ105 (Cully and Garro, 1985; Dhaese et al., 1985), a randomly sequenced ORF from *E.coli* (Aiba et al., 1996), and a regulator of vegetative replication and conjugal transfer of the plasmid RK2 (Jagura-Burdzy et al., 1992). All of these proteins contain putative helix-turn-helix motifs which are fairly conserved between these proteins (Pabo and Sauer, 1984; Tao et al., 1991; Figure 2-2 B, C).

The most significant match to AcBBP1 was to the *Bacillus subtilis Bgl*II control gene product (Anton et al., 1996). *C* genes have been identified in some type II restriction-modification (RM) systems but not all. *C* genes encode proteins that have been implicated to activate and/or repress expression of the essential restriction endonuclease (R) and modification methylase (M) genes (Nathan and Brooks, 1989; Tao et al., 1991; Tao and Blumenthal, 1992; Ives et al., 1992; Ives et al., 1995). These control genes were found as small ORFs upstream of or in close proximity to the *R* gene (Tao et al., 1991). The best studied *C* genes are *ba*mHIC and *pvuII*C from the *Bam*HI and *Pvu*II RM systems respectively. ORFs similar to these have been identified in a number of bacterial RM systems but currently, no functions for the rest of these genes have been assigned. All *C* genes or putative homologs share a few common characteristics: 1) the deduced amino acid sequence of all members identified thus far encode small proteins of about 9-10 kDa and 2) a helix-turn-helix DNA binding motif is located in the central region of the protein and fairly conserved between all members. The AcBBP1 protein exhibits both characteristics.

The AcBBP1 protein shares amino acid sequence similarity with an immunity repressor from the phage ϕ105 (Cully and Garro, 1985; Dhaese et al., 1985) and a randomly sequenced Kohara plasmid from *E. coli* (Aiba et al., 1986) proposed to have a similar function. Both of these amino acid sequences contain helix-turn-helix DNA binding motifs at the N-terminus. The AcBBP1 sequence contains similar amino acid residues to the immunity repressor proteins throughout the sequence. The immunity repressor from ϕ105 was found to be involved in the regulation of lysogeny and superinfection (Cully and Garro, 1985, Dhaese et al., 1985). It is known that phage encoded repressors are required for lysogeny in response to infection, so that lytic genes are not expressed. These repressors are also responsible for the immunity of secondary infection by the same or closely related phage termed "superinfection".

The binding site for C.*BamHI*, AcBBP1 and the ϕ105 immunity repressor have been determined (Figure 2-4; Van Kaer et al, 1987; Van Kaer et al, 1988; Welters et al., 1993; J. Brooks, personal communication). All three sites share some common nucleotides that may be important for protein recognition.

The AcBBP1 protein shares amino acid sequence similarity to a vegetative replication/conjugal transfer *trans*-acting factor of the RK2 plasmid. Conjugal transfer has been implicated as a means to transfer bacterial factors to the plant cell. It was hypothesized by Stachel and Zambryski in 1986 that conjugal transfer may be a mechanism which *Agrobacterium* uses to mobilize the T-complex from the bacterium to the plant. The identification of surface appendages or pili on *Agrobacterium tumefaciens*

(*vir*B-dependent pilus; Fullner et al., 1996) involved in T-DNA transfer and more recently the Hrp pilus (Roine et al., 1997) produced by *Pseudomonas syringae* pv. *tomato* suggest that this mechanism may be involved in delivering bacterial factors to the plant cell.

The phenotype of the AcBBP1 deficient strain under free-living and nitrogen fixing conditions was monitored. No difference in growth rate was observed under either of these conditions. The ORS 571-425 mutant strain was tested for nodule formation on *S. rostrata*. Nodules were induced on both the stems and roots of *S. rostrata* indicating a ($Nod^+$) phenotype. To determine the relative nitrogen fixation levels in nodules harboring wild-type versus the AcBBP1 deficient mutant, acetylene reduction assays were performed. In three separate trials, a total of 65 root nodule samples were tested. In all cases a ~20% reduction in total nitrogen fixation was detected by the acetylene reduction assay. In three separate *S. rostrata* stem nodule trials, a total of 69 samples were tested for nitrogen fixation levels and a 15-20% reduction was observed in nodules harboring the mutant strain. Since a difference in total nitrogen fixation ability was detected in nodules induced by the AcBBP1 deficient strain, the leghemoglobin (*lb*) steady state mRNA levels in nodules harboring the wild-type or mutant strain were compared. Total RNA isolated from 5-week old S. rostrata stem nodules harboring the wild-type or mutant strain was hybridized with the *S. rostrata* leghemoglobin probe *Srclb*1. Quantification of the *lb* gene hybridization signal versus a loading control (18S) failed to reveal any significant differences in these two types of nodules indicating that the reduction of nitrogen fixation

in nodules harboring mutant bacteria does not directly correlate with a reduction in *lb* mRNA levels.

Since *lb* genes comprise a small gene family of highly homologous members (~95-97% identical) in *S. rostrata*, it is not too surprising that transcripts remained unchanged in the two types of nodules tested. The g*lb*3 gene product represents a minor form of leghemoglobin in *S. rostrata* (Bogusz et al., 1987). Additionally, northern blot analysis can only assess the global *lb* mRNA levels in the plant rather than the expression pattern of a particular member because gene specific probes are almost impossible to obtain. Therefore, small changes in Srg*lb*3 gene expression could be masked in northern blots. Experiments to directly investigate the role of AcBBP1 in *Srglb*3 gene expression will be addressed in Chapter 4 of this thesis.

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# CHAPTER 3

## ROLE OF AcBBP1 IN INFECTION AND IMMUNOLOCALIZATION OF THE PROTEIN IN *Sesbania rostrata* NODULES

Portions of this chapter will be submitted as an article to the journal Molecular Plant-Microbe Interactions.

## ABSTRACT

As discussed in Chapter 2, a gene encoding a bacterial DNA binding protein that can bind to a specific region in the *Srglb3* promoter was isolated and characterized. A microscopical study was initiated to determine if any structural differences between nodules harboring the wild type or the AcBBP1 deficient mutant existed. Using transmission electron microscopy, it appeared that nodules harboring bacteria deficient in the production of AcBBP1 were delayed in the infection process. Fewer infected plant cells were found in the central infected zone of the nodule when compared to the wild type. In addition, antibodies raised against AcBBP1 were generated. These antibodies compete with *A. caulinodans* extracts for complex formation in gel mobility shift assays and therefore probably recognizes an epitope that interferes with DNA binding. Western blot analysis detected the presence of the AcBBP1 protein only in whole cell extracts or in *Sesbania rostrata* stem nodule extracts harboring wild type *Azorhizobium caulinodans.* Extracts prepared from the AcBBP1 deficient mutant or *S. rostrata* stem nodules harboring these mutant bacteria do not express the AcBBP1 protein. Localization of AcBBP1 in nodules was attempted using immunogold labeling techniques. The colloidal gold marker was located within the bacteroids and in the peribacteroid membrane and was not detected in the plant cytoplasm or nucleus.

# INTRODUCTION

Leghemoglobin (Lb) is a late nodulin which functions to facilitate oxygen diffusion to the actively respiring bacteroids within the infected cells of the nodule (Appleby, 1984). In the tropical legume *Sesbania rostrata,* a small family of leghemoglobin genes exists. Seven distinct leghemoglobin components have been identified in *S. rostrata* and can be distinguished by their biochemical properties and primary protein sequence (Bogusz et al., 1987).

The expression of *lb* genes appears to be confined to the infected cells of the nodule as chimeric leghemoglobin promoter-reporter gene fusions seem to only be expressed in these cell types (Stougaard et al., 1986, Stougaard et al., 1987; de Bruijn et al., 1989; Stougaard et al., 1990; Szabados et al., 1990; de Bruijn and Schell, 1992; Ramlov et al., 1993; Szczyglowski et al., 1994). *Cis*-acting elements that are essential for tissue specific expression of leghemoglobin genes have been identified (Stougaard et al, 1986; 1987; 1990; Szabados et al., 1990; Ramlov et al., 1993; Szczyglowski et al., 1994). In particular, the tissue-specific NICE (Nodule Infected Cell Expression) element found in the *Srglb*3 5' upstream region was delimited and could confer a nodule-specific expression pattern on a normally root-enhanced promoter (Szczyglowski et al., 1994). To complement the *cis* analysis of leghemoglobin promoters, *trans*-acting factors interacting with these 5' upstream regions were identified using gel mobility shift assays (Jensen et al., 1988; Metz et al., 1988; Jacobsen et al., 1990; Welters et al., 1993).

In a search to find factors that could bind to the promoter region of the *Srglb3* gene, Welters et al. (1993) observed DNA binding activity in *S. rostrata* nodule extracts which could specifically interact with a portion of the *Srglb3* 5' upstream region. (BBS1). Interestingly, the factor responsible for the binding activity was derived from the symbiont of *S. rostrata*, *Azorhizobium caulinodans* (AcBBP1). The AcBBP1 protein in addition to the BBS1 within the *Srglb3* promoter was characterized Welters et al., 1993). The analysis of the BBS1 in transgenic *Lotus corniculatus* plants using chimeric promoter-reporter constructs suggested that the BBS1 was important for modulating levels of *Srglb3* promoter activity but was clearly not essential.

The identification of a bacterial protein which has the ability to bind *in vitro* to a plant gene promoter is very intriguing and if proven to be biologically significant, would support the concept of transkingdom signaling that was described in plant-pathogenic interactions (Howard et al., 1992; Citovsky et al., 1992; Yang and Gabriel, 1995; Van den Ackerveken et al., 1996). Targeting motifs have been identified in a few bacterial proteins which have the ability to be delivered to the plant nucleus. These reports, which involve *Agrobacterium tumefaciens* and some members of the *Xanthomonas* genus and plants, not only showed that bacterial proteins were able to direct chimeric reporter gene constructs to the plant nucleus but also is supported by a correlation with the presence of a nuclear localization signal (NLS) and a pathogenic phenotype (Howard et al., 1992; Citovsky et al., 1992; Van den Ackerveken, 1996).

Proteins can be directed to the nucleus of a cell by several mechanisms. In some cases, a nuclear localization signal (NLS) found within the amino acid sequence can target

a protein to this compartment. Although there are no consensus sequences for NLSs in any system, the majority are characterized by short stretches of basic amino acids and involve cellular components which actively transport the protein through the nuclear pore (Garcia-Bustos et al, 1991; Dingwall and Laskey, 1991; Raikhel, 1992). However, it is also believed that proteins smaller than 40-60 kDa may passively enter the nucleus by simple diffusion (Dingwall and Laskey, 1986). Furthermore, proteins could be shuttled or ride "piggyback" into the nucleus by interacting with another NLS containing protein in the cytoplasm (Kang et al., 1994). As mentioned previously, the AcBBP1 amino acid sequence contains patches of basic residues which could act as NLSs. However, this protein is also very small (9 kDa) and could easily diffuse through the nuclear pore complex and not be actively transported.

The gene encoding AcBBP1 was cloned and characterized. To assess the role of AcBBP1 in symbiosis, an *A. caulinodans* strain unable to produce this protein was constructed. Growth in the free-living state and under a reduced oxygen environment was tested and no significant differences were observed. However, when nitrogen fixation activity was assayed, *S. rostrata* nodules induced by the AcBBP1 deficient strain displayed a ~20% % reduction in activity. The steady state level of *lb* mRNA in *S. rostrata* stem nodules was monitored. Quantification using a densitometer indicated no difference in *lb* expression between the wild-type or mutant nodules.

Determining the ultrastructure of a tissue in combination with the subcellular location of a protein can greatly aid in deducing this protein's biological role. Therefore *S. rostrata* stem nodules harboring wild-type or AcBBP1-deficient bacteria were analyzed

under the transmission electron microscope. In addition the subcellular localization of AcBBP1 in nodules was attempted.

One of the goals of this study was to investigate whether or not this bacterial protein plays a role in symbiosis. In an attempt to address this question, a study at the cellular level was initiated. Data obtained by microscopy suggest that the infection of plant cells within nodules harboring the AcBBP1-deficient mutant is delayed. Polyclonal antibodies raised against AcBBP1 interfere with the DNA-binding domain, and appear to be specific. Localization studies using the colloidal gold marker indicate that AcBBP1 is localized in the bacteroids and the region surrounding the bacteroids of *S. rostrata* nodules and does not appear to be present in any other compartment.

# MATERIALS AND METHODS

## Transmission electron microscopy

Five week old *S. rostrata* stem nodule slices were prepared for microscopy as described by Subba-Rao et al. (1995). In brief, the nodule slices were fixed in a solution containing 4% glutaraldehyde and 1% formaldehyde in 50 mM Na Cacodylate buffer for 2 hours under vacuum. After brief washing in 50 mM Na Cacodylate buffer, the samples were post fixed in 1% osmium tetroxide for 2 hours at room temperature. After dehydration through a graded acetone series, the samples were infiltrated with Spurr's resin (Electron Microscopy Sciences; Fort Washington, PA). Infiltrated samples were positioned in molds and polymerized in a 60°C oven for 24 hours. Thin sections (1-2 μm) were fixed to glass slides by briefly passing them over a flame. Subsequently, the samples were stained with toluidine blue to visualize the tissue integrity under the light microscope. Ultra thin (90 nm) sections were mounted on flamed copper grids and stained with 1% uranyl acetate and 16.6% lead citrate. The sections were carbon coated and examined under a transmission microscope (Model CM10, Phillips, New Jersey).

## Production of Anti-AcBBP1 Antibodies

In order to overexpress AcBBP1 in *E. coli*, it was necessary to place the AcBBP1 coding region in the proper reading frame of an *E. coli* expression vector (pET $22b^+$; Novagen, Madison, WI) so that the protein would be translated correctly. To facilitate

insertion of the target gene into pET 22b+, the AcBBP1 gene was modified using PCR to generate an *Nde*I site at the 5' end and an *Xho*I restriction site at the 3' end. Plasmid DNA from pSF354 was used in the modification/amplification reaction. Insertion of the target gene into the *Nde*I site of the vector allowed translation of the recombinant protein to begin with the ATG found in *AcBBP1* gene. Furthermore, the *Xho*I site not only made insertion of the *AcBBP1* gene into the expression vector easy, it also mutated the native stop codon in the *AcBBP1* gene so that a 6X histidine (His) tag was translationally fused to the C-terminus of the recombinant AcBBP1 protein. This 6X His tag serves as an affinity tag for one-step purification by metal chelation chromatography (His Bind Resin, Novagen) which can facilitate the purification of the recombinant protein. To confirm that the target gene was in the proper reading frame, the nucleotide sequence of the construct was determined. This plasmid was introduced into *E. coli* strain BL21(DE3) (Novagen) via electrotransformation (Sambrook et al., 1989) and the T7 RNA polymerase gene was induced with 1 mM IPTG to express the *AcBBP1* gene. Induced cells were collected by centrifugation, resuspended in 50 mM Tris pH 7.5, 2 mM EDTA, and sonicated at 5 second intervals every 20 seconds for about 2 minutes until slightly viscous. The extract was purified over a nickel column as the 6X tag binds to divalent cations ($Ni^{2+}$) immobilized on the His Bind resin and then eluted. The recombinant protein was further purified by separation on SDS-PAGE and visualized by equilibration in 0.19 M Tris-HCl followed by immersion in 0.3 M $CuCl_2$ (Lee et al. 1987). The band corresponding to the His-tagged AcBBP1 protein was electroeluted from the gel. The purity of the recombinant protein was monitored by SDS-PAGE and Coomassie blue staining. The

protein was concentrated using Centricon-10 units (Amicon, Beverly, MA) in phosphate-buffered saline (PBS, Sambrook et al. 1989). The adjuvant Titermax (CytRx Corporation, Norcross, GA) was used to immunize rabbits following the instructions in the manufacturer's manual. Anti-AcBBP1 serum was affinity purified using Affigel 10 (Bio Rad, Hercules, CA), according to the manufacturer's instructions.

**Protein Gels and Immunoblot Analysis**

Cultures of bacterial strains expressing the AcBBP1 protein were grown to saturation, pelleted, and resuspended in 50 mM Tris pH 7.6. Protein extracts from plant tissues were obtained by grinding them in liquid nitrogen with a mortar and pestle and boiling the mixture for 10 min in protein extraction buffer (100 mM Tris, pH 6.8, 5% SDS, 0.5% β-mercaptoethanol). For western blot analysis, 50 ug of total proteins from bacterial cells or plant tissues were separated by SDS-PAGE (Laemmli, 1970) using 18% acrylamide gels, and electroblotted overnight onto 0.45 μm nitrocellulose membranes (Protran BA 85, Schleicher and Schuell; Keene, NH) in Towbin buffer (Towbin et al., 1989). The blocking, binding, and washing steps were performed using a solution containing 1% BSA (w/v), 0.05% Tween 20, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (VandenBosch, 1991). AcBBP1 serum was used at a 1:1000 dilution. Antibody detection was achieved using goat anti-rabbit antibodies conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and developed using nitroblue tetrazolium (NBT, United States Biochemical; Cleveland, OH) and 5-choloro-4-bromo-3-

indoyl phosphate (BCIP, United States Biochemical), as described by Harlow and Lane (1988).

**Gel Mobility Shift Assays**

Extracts of ORS 571 bacteria were prepared as described by Welters et al. (1993). A fragment of the *Srglb3* promoter containing the BBS1 (fragment 5'203) was end labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Gibco BRL, Grand Island, NY), as described by Sambrook et al. (1989). Binding reactions were carried out as described previously (Welters et al., 1993). Antisera raised against AcBBP1, pre-immune serum, or purified anti-AcBBP1 antibodies were included in the binding reactions at the concentrations described in the legends of the appropriate figures.

**Immunocytochemistry**

Sixteen day old *S. rostrata* stem nodules were sliced and fixed in a solution containing 4% formaldehyde, 1% glutaraldehyde, in 50 mM potassium phosphate buffer pH 7.2 and vacuum infiltrated for two hours at room temperature. The samples were rinsed twice for 15 min in 50 mM potassium phosphate buffer (pH 7.2) and dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 3x 100%, 15 min per step, 3 hours to overnight in 100% ethanol). Dehydrated samples were infiltrated with London Resin White (Electron Microscopy Sciences; Fort Washington, PA) and polymerized in aluminum weighing dishes (Fisher, St. Louis, MO) at 58°C. Ultra-thin sections were

prepared using an Ultracut E Microtome (Reichert-Jung, Vienna, Austria) and mounted on Pioloform-coated 300 mesh nickel grids (Ted Pella, Inc., Redding, CA).

The grids with samples were incubated in a blocking solution (10 mM Tris-HCl pH 7.4, 150 mM NaCl, .05% Tween 20, 1% BSA) twice for 30 min and then incubated in a solution of purified AcBBP1 antibody and blocking solution (1:1) for 1 hr at room temperature. After incubation, the grids were washed in 1 X TBS and put into blocking buffer again for 30 min. The grids were then incubated in blocking solution with the secondary antibody (protein A conjugated to 15 nm colloidal gold, Ted Pella, Inc., Redding, CA) diluted 1:50. The grids were rinsed with 1X TBS followed by a brief rinse in water. The samples were stained with a solution of uranyl acetate and potassium permanganate (4:1 saturated uranyl acetate:1% potassium permanganate solution) for 5 min, thoroughly rinsed in water and dried. Sections were viewed under a transmission electron microscope (Model CM10, Phillips, New Jersey).

# RESULTS

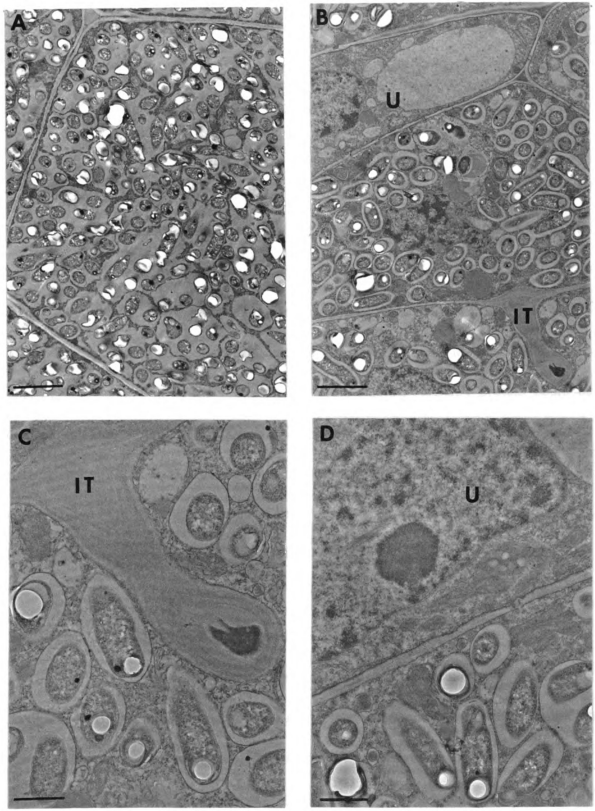
## Infection of *S. rostrata* by an AcBBP1 Deficient Mutant Strain

Nodules harboring the wild-type *A. caulinodans* strain ORS 571 or the AcBBP1 deficient mutant ORS 571-425 were examined by transmission electron microscopy. *S. rostrata* cells harboring wild-type ORS 571 bacteria were found to be densely packed with bacteroids. Up to ten bacteroids were enclosed within the peribacteroid membrane (symbiosome) in infected plant cells (Figure 3-1). In nodules induced by the AcBBP1 deficient strain, the infected cells were found to be less densely packed with bacteroids (Figure 3-1 B, C, D). Uninfected cells and recently infected cells could easily be detected (Figure 3-1 B, C). The cytoplasm of individual infected cells was found to be more prominent in the cells harboring the AcBBP1 deficient strain than in cells harboring the wild-type strain. Moreover, the majority of symbiosomes contained a single bacteroid encased by a peribacteroid membrane. No other ultrastructural differences could be detected in nodules harboring the AcBBP1 deficient mutant versus the wild-type strain.

## Detection of AcBBP1 in Cultures and in Nodules

The full length AcBBP1 protein was synthesized in *E. coli*, using a pET-based vector system which adds a 6X Histidine tag to facilitate purification of the recombinant protein. A recombinant protein of expected molecular weight was found to accumulate in the soluble fraction of *E. coli* cells harboring the plasmid pET $22b^{+}$/Ac4 following induction (Figure 3-2).

**Figure 3-1. *S. rostrata* nodules harboring AcBBP1 deficient bacteria are delayed in the infection process.** **(A)** Overview of a plant cell infected with wild-type *A. caulinodans* ORS 571 **(B)** Overview of a plant cell infected with mutant *A. caulinodans* ORS 571-425 **(C)** A magnified view of an infection thread and an **(D)** uninfected cell near other infected cells within a nodule harboring mutant bacteria. For A, B bar = 2μm. For C, D bar = 1 μm.


A
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This recombinant protein was purified over a $Ni^{2+}$ column and further purified by SDS-PAGE. The AcBBP1 protein was electroeluted from the gel and used to raise polyclonal antibodies. Western blot analysis of total protein extracts from bacterial cultures or *S. rostrata* nodules induced by the wild-type or AcBBP1 deficient strain of *A. caulinodans* was performed (Figure 3-3). A single band of about 9 kDa was consistently detected in extracts of wild-type ORS 571 bacteria and in extracts from nodules induced by the wild-type strain (Figure 3-3). The protein band detected in nodule extracts was usually very faint and diffuse. This may be due to the low abundance of the protein or to difficulties in liberating the protein from the bacteroid/symbiosomes. The diffuse nature of the band may be due to large amounts of Lb protein found in the nodule which obscures the separation of the proteins. No cross-reacting AcBBP1 protein could be detected in extracts prepared from AcBBP1 deficient bacteria or nodules induced by this mutant strain (Figure 3-3). These results support and extend our previous observations (see Chapter 2) to the absence of the AcBBP1 protein in free living cultures of mutant bacteria or in nodules induced by strain ORS 571-425.

**Specificity of the Anti-AcBBP1 Antibodies**

To provide further evidence that the anti-AcBBP1 antibodies specifically recognize AcBBP1, antisera and purified anti-AcBBP1 antibodies were incorporated in gel mobility shift assays using the AcBBP1 binding site as the target, as described by Boulanger et al. (1987) and L'Etoile et al. (1994). A "supershift" effect can be observed if the antibody recognizes its corresponding protein in the extract. The protein-DNA

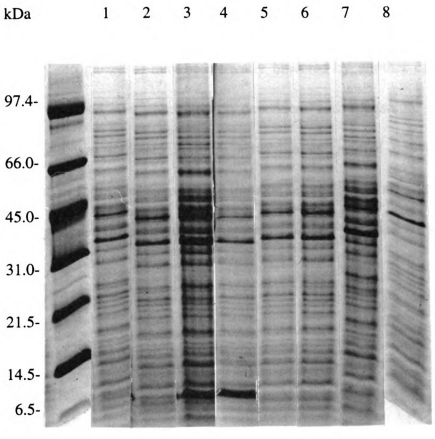



**Figure 3-2. Overexpression of AcBBP1 in *E. coli* using the pET system**. Production of AcBBP1 protein after **Lane 1**, 0 hours; **Lane 2**, 1 hour; **Lane 3**, 2 hours; **Lane 4**, 3 hours after inducing cultures with 1 mM IPTG. A control strain harboring the vector alone **Lane 5**, 0 hr, **Lane 6**, 1 hr; **Lane 7**, 2 hr; **Lane 8**, 3 hr after inducing cultures under the same conditions yield no accumulation of protein of expected molecular weight.

complex will be shifted to a higher molecular weight and migrate slower in the gel due to the formation of a complex with the antibody. Competition rather than a super shift was observed when antiserum or purified antibodies generated to AcBBP1 were incorporated into the DNA binding assays (Figure 3-4). This observation can be explained by the fact that the antibodies generated specifically recognize an epitope on the AcBBP1 protein which interferes with the DNA binding domain. The DNA binding domain may be directly recognized or may be sterically hindered by another epitope. The competition event observed was concentration dependent and appears to be specific as complex formation was inhibited as more antiserum or purified antibodies were added. The addition of four times as much pre-immune serum did not affect complex formation. This result provides further evidence that the antibodies generated are specific to the AcBBP1 protein.

**Immunolocalization of AcBBP1**

Before the *S. rostrata* nodule samples were fixed and embedded in resin, the fixative was tested to determine if the formaldehyde or glutaraldehyde destroyed or modified the antigen located on the AcBBP1 protein. Therefore, western blots were briefly incubated in the fixative which contained 4% formaldehyde, 1% glutaraldehyde in 50 mM potassium phosphate buffer, or in solutions of formaldehyde or glutaraldehyde alone and developed to determine if this treatment would affect antigenicity (VandenBosch, 1991). No masking of AcBBP1 detection was observed (data not shown).

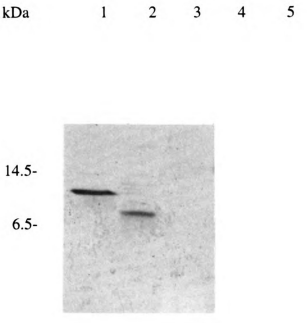
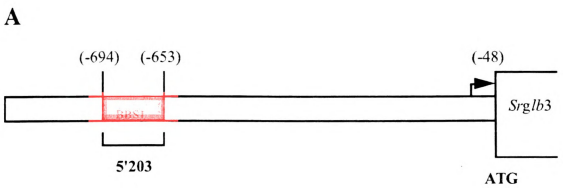



**Figure 3-3. Detection of AcBBP1 protein in free living cultures of *A. caulinodans* ORS 571 and in *S. rostrata* nodules harboring *A. caulinodans ORS 571*. Lane 1**, recombinant AcBBP1; **Lane 2** and **3**, extract prepared from *A. caulinodans* ORS 571 and *A. caulinodans* ORS 571-425 respectively; **Lanes 4** and **5**, total protein extracted from *S. rostrata* nodules harboring wild-type or mutant bacteria respectively. Western blots were probed with anti-AcBBP1 serum.



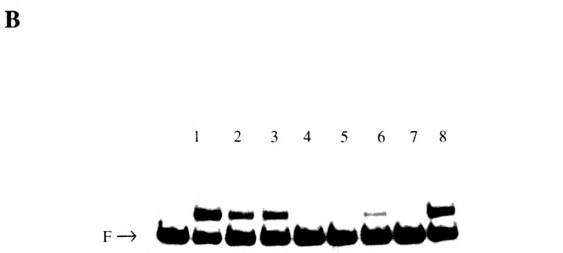




**Figure 3-4. Characterization of AcBBP1 antibodies using DNA binding assays.** AcBBP1 antibodies were incubated with extracts prepared from ORS 571 and were tested for DNA binding activity using the BBS1 as target DNA. **(A)** Schematic of the *Srglb3* 5' upstream region. The arrow indicates the start point of transcription. The fragment 5'203 (shaded in red) containing the BBS1, was used as the target fragment in gel mobility shift assays. **(B)** ORS 571 extracts were incubated with the following: **Lane 1**, no additions; **Lanes 2-4**, 0.005 µg, 0.025 µg, 0.05 µg affinity purified AcBBP1 antibody respectively; **Lanes 5-7**, 0.05 µg, 0.01, 0.1 µg AcBBP1 antisera respectively; **Lane 8**, 0.4 µg pre-immune serum. The free fragment(F, 5'203) is indicated with an arrowhead.

The anti-AcBBP1 antibodies characterized earlier were used to immunolocalize AcBBP1 at the subcellular level. Slices of *S. rostrata* stem nodules induced by the wild-type strain ORS 571, were fixed, infiltrated with resin and labeled with the colloidal gold marker as described in the Materials and Methods. Labeling of AcBBP1 with colloidal gold was observed predominantly in the bacteroids and the region surrounding them (Figure 3-5). This result is not too surprising since AcBBP1 is of bacterial origin. Background levels or no labeling was detected in the plant cell cytoplasm within the infected cell or in an uninfected cell bordering the infected zone. Although the concentration of particles was not very high, the labeling appears to be specific.

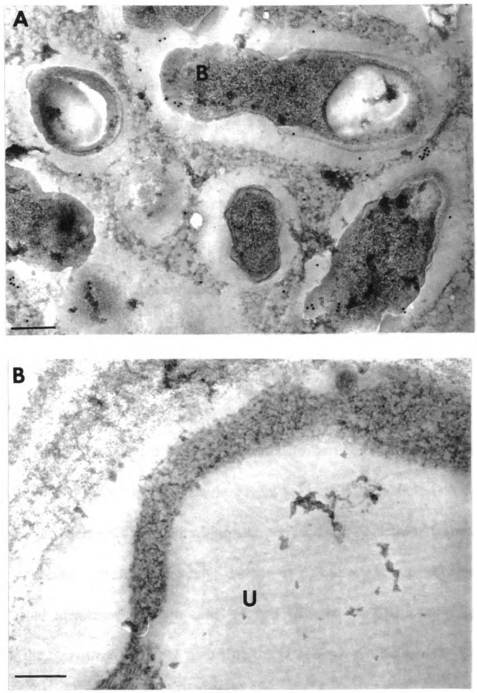



**Figure 3-5. The colloidal gold marker locates the AcBBP1 protein.** **(A)** Bacteroids within an infected cell of a *S. rostrata* nodule harboring *A. caulinodans* ORS 571. **(B)** A peripheral uninfected cell in the same sample. Bars = 300 nm.

# DISCUSSION

In this chapter, *S. rostrata* nodules induced by wild-type *A. caulinodans* and the AcBBP1 deficient mutant were compared at the ultrastructural level. It was shown in Chapter 2 that nitrogen fixation activity was about 20% lower in nodules harboring the AcBBP1 deficient strain. To investigate this reduction in nitrogen fixation a microscopical study was carried out to determine if any differences in the ultrastructure of nodules induced by the *A. caulinodans* mutant existed. Using transmission electron microscopy sections of *S. rostrata* nodules induced by the wild-type or AcBBP1 deficient strain were observed. The plant cells of nodules induced by mutant bacteria appear to be slightly delayed in the infection process. Structures associated with the infection process such as infection threads were visible. Uninfected plant cells were also observed. The number of bacteroids within the infected cell also appeared to be reduced when compared to the nodules colonized by wild-type bacteria. Figure 3-1 suggest that there is a difference in the number of bacteroids encased within the peribacteroid membrane (symbiosome). It can be observed that approximately one or two bacteroids are surrounded by a peribacteroid membrane in plant cells infected with the AcBBP1 deficient strain. In contrast, the *S. rostrata* nodules harboring the wild-type *A. caulinodans* were densely packed with bacteria with virtually no plant cytoplasm visible.

It was also of interest to localize the AcBBP1 protein subcellularly in the infected plant cell. The deduced amino acid sequence of AcBBP1 has stretches of basic residues which may be involved in targeting this protein to the plant nucleus. On the other hand,

AcBBP1 is a small protein of about 9 kDa and could passively diffuse through the nuclear pore complex and gain access to the plant nucleus. To localize this bacterial derived protein, antibodies specific to AcBBP1 were produced. The AcBBP1 protein was overexpressed in *E. coli* (pET system, Novagen, Madison, WI) and purified over a nickel column using the 6X His tag that was fused to the recombinant protein. The AcBBP1 recombinant protein was further purified by electroelution and injected to rabbits.

A few lines of evidence indicated that antibodies generated were specific to AcBBP1. First, these antibodies recognized the recombinant AcBBP1 protein. In addition, a unique band of about 9 kDa was detected in extracts prepared from free living cultures of *A. caulinodans* or extracts obtained from nodules harboring wild-type bacteria. The band observed in nodule extracts is usually diffuse and is not very abundant. This may indicate that the protein is not synthesized in large quantities in the bacteroid or that the protein is difficult to extract from the nodule tissue. The bacteroid is very membranous and may entrap the protein making detection a little difficult. The band may also be obscured by all the soluble proteins found in nodule extracts such as leghemoglobin which may affect the way the protein sample separates on the gel. The difference in the molecular weight of the recombinant protein and the native protein is most likely due to the 6X histidine tag that was fused to AcBBP1 for purification purposes. Second, gel mobility shift assays also support the idea that these antibodies are specific to AcBBP1 since incorporating the immune serum or purified antibodies to the binding reactions lead to competition of complex formation. Pre-immune serum however, was unable to compete for complex formation. These two pieces of evidence suggest that

the antibodies that were generated do in fact specifically recognize the AcBBP1 protein. The binding studies also indicate that the epitope to which the antibodies are generated affect DNA binding since competition rather than a super shift was observed.

Because no established protocols were available for immunolocalization of proteins in this tissue, a great deal of experimentation with fixation and infiltration was necessary. The dehydration step was extremely important for good fixation of *S. rostrata* nodule tissues. A graded ethanol series with smaller steps in addition to long incubation periods in 100% ethanol was important (see Materials and Methods) for retaining structure. These small modifications made a difference.

Gold particles were detected in the bacteroids of the infected cells, most often within the bacteroid or within the symbiosome. The observed labeling pattern in the region surrounding the bacteroid may reflect the secretion of the protein into this region or may be a fixation or sectioning artifact. Labeling in the bacteroids was not very intense and may be due to the presence of a large population of bacteroids within a section which could titrate out the antibody. Alternatively, it may reflect the *in vivo* concentration of the protein within the bacteroid. Nothing is known about the abundance of this protein in this differentiated state. The colloidal gold marker was not detected in the plant nucleus or other plant organelles or compartments.

NLS-reporter protein fusions are classic methods to determine if a protein can be targeted to the nucleus. Recently, Avrb6, PthA, AvrBs3 and FixF proteins were shown to have functional NLSs which could direct fusion proteins into the plant cell nucleus (Yang and Gabriel, 1995; Van den Ackerveken et al., 1996, Jabbouri et al., 1996). Similar

experiments using the *uid*A reporter gene fused to entire *AcBBP1* gene were tested in onion cells. In this assay, the chimeric construct is precipitated onto gold particles and bombarded into onion epidermal layers. Localization of the chimeric protein can be observed using the histochemical Gus activity assay (Jefferson, 1987). AcBBP1 did not localize to the nucleus of onion cells (data not shown). This result may indicate that AcBBP1 does not have an NLS. Alternatively, this result could be due to the addition of the β-glucuronidase protein to AcBBP1. The reporter protein could mask or sterically hinder the putative NLSs previously identified in the deduced amino acid sequence (Shieh et al., 1993). On the other hand, this protein may not need an NLS to enter the plant nucleus since it is so small (Dingwall and Laskey, 1986). The addition of the reporter gene may prevent the protein from freely diffusing through the nuclear pore or interacting with other cellular components (Kang et al., 1994) which could possibly direct it to the nucleus.

In conclusion, differences were observed in nodules induced by the *A. caulinodans* wild-type and AcBBP1 deficient strain. The plant cells inhabited by the mutant strain appear to be delayed in the infection process as infection threads and uninfected cells were clearly visible in the central portion of the nodule. Immunogold localization showed that AcBBP1 is localized to the bacteroid and within the symbiosome. Unfortunately, this location does not provide much evidence as to the role this protein may play in *Srglb*3 gene regulation. AcBBP1 was not localized to the nucleus using immunochemical methods or by the chimeric NLS-reporter gene system. Since the AcBBP1 protein is

small, it may simply diffuse into the nucleus or may be shuttled in by another nuclear localized protein. On the other hand, AcBBP1 could be present in the plant nucleus but is undetectable by the antibodies that were generated. To test the hypothesis that AcBBP1 plays a role in *lb* gene expression, transgenic plants harboring the *Srglb*3 promoter-reporter construct will be used. Nodules induced on these transgenic plants by a BBP1 deficient *R. loti* strain will be monitored for reporter protein activity and compared to the nodules induced by the wild-type strain. These experiments will be discussed in Chapter 4 of this thesis.

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# CHAPTER 4

# ANALYZING THE ROLE OF BBP1 IN TRANSGENIC *LOTUS CORNICULATUS*

Portions of this chapter will be submitted as an article to the journal Molecular Plant-Microbe Interactions.

## ABSTRACT

The gene from *Rhizobium loti* which is similar to the *AcBBP1* (Bacterial Binding Protein) gene from *Azorhizobium caulinodans* was isolated. The sequence of the *R. loti* gene (RlBBP1) is 63% identical to the *AcBBP1* gene at the nucleotide level. At the amino acid level, the two BBP1 proteins are 78% identical and 85% similar. Putative nuclear localization signals and helix-turn-helix DNA binding domains are conserved. Factors present in extracts prepared from *R. loti* interacted with the same region of the 5' upstream region of the *Sesbania rostrata* leghemoglobin *glb*3 gene to which AcBBP1 binds (BBS1; Welters et al., 1993). An *R. loti* strain deficient in the production of the RlBBP1 protein was constructed by inserting a kanamycin resistance cassette into the RlBBP1 gene. Extracts prepared from this mutant strain did not form any complexes with the BBS1 in gel mobility shift assays. The interaction between RlBBP1 and the BBS1 region within the *Srglb*3 promoter was further examined by monitoring the activity of *Srglb3* promoter-*uid*A reporter gene fusions in transgenic *Lotus corniculatus* plants. Transgenic *L. corniculatus* plants were inoculated with the wild-type or the RlBBP1 deficient strain. Gus enzymatic activity within nodules was quantified using a fluorometric assay. No statistically significant differences in Gus expression between nodules induced by the wild-type and the RlBBP1 deficient strains could be observed, raising doubt that BBP1 is involved in *Srglb*3 gene expression.

# INTRODUCTION

Symbiotic nitrogen fixation is a unique example of a complex and highly regulated biological process which occurs in a novel plant structure, the nodule. A complex and fine-tuned exchange of signals between bacteria belonging to the genus *Azorhizobium*, *Bradyrhizobium, Rhizobium* or *Sinorhizobium* and legume plants leads to the formation of a plant organ in which atmospheric nitrogen is converted into ammonium (Nap and Bisseling, 1990; de Bruijn and Schell, 1992; Mylona et al., 1995; Long, 1996). A collection of genes encoding nodule-specific proteins, collectively known as nodulin genes (van Kammen, 1984) have been isolated and characterized from many species of legumes. These genes have been classified as early or late depending on their developmental appearance (Nap and Bisseling, 1990). Early nodulin genes correlate with the infection process and nodule ontogeny while late nodulin genes are involved in nodule functioning and maintenance.

Nodulin gene expression depends on direct or indirect signals originating from both the plant and symbiotic bacteria in addition to the tissue or cell specific receptiveness in the plant. One line of research to elucidate the signal transduction pathway involved in nodulin gene induction has focused on the identification of *cis*-acting elements within the promoters of these genes and *trans*-acting factors which interact with these regions (de Bruijn and Schell, 1992).

Leghemoglobin (*lb*) genes belong to a class of highly studied late nodulin genes and encode proteins which function to facilitate oxygen diffusion to the actively respiring

bacteroids of the nodule. Functional analysis of two leghemoglobin genes have defined *cis*-acting elements important for specific expression in the infected cells of nodules (Stougaard et al., 1986, 1987; Szabados et al., 1990; Ramlov et al., 1933 and Szczyglowski et al., 1994). The apparent expression of the *lb* promoter in the infected cells of transgenic *Lotus corniculatus* nodules suggest that the physical presence of bacteria may be essential for expression of this gene (de Bruijn and Schell, 1992).

To complement this work, several DNA binding proteins which interact with the promoters of these genes have been identified (Jensen et al., 1988; Metz et al., 1988; Welters et al., 1993), although none of these factors have been directly implicated in modulating gene expression. Welters and co-workers (1993) showed that a protein of bacterial origin (AcBBP1) was able to bind to the leghemoglobin *Srglb*3 5' upstream region and that disruption or deletion of its binding site, the Bacterial Binding Site 1 (BBS1), decreased expression of a chimeric promoter-reporter gene construct in *Lotus corniculatus*. This finding provided a possible explanation for the infected cell specific expression pattern of the *Srglb*3 gene and warranted more investigation.

The gene encoding AcBBP1 from *Azorhizobium caulinodans* AcBBP1 was cloned and characterized, and an AcBBP1 deficient strain was constructed. No obvious differences in growth of the mutant strain under free living or reduced oxygen conditions were observed. The AcBBP1 deficient strain was able to induce nodules on *S. rostrata* plants that were able to fix nitrogen (Nod+, Fix+). However, the nitrogen fixation activity was ~20% lower in the mutant strain as assayed by acetylene reduction. The ultrastructure of nodules harboring this mutant revealed that plant cells appeared to be at

a different stage of the infection process. Less bacteroids appeared to be encased within the peribacteroid membrane and provides additional evidence for delay in the infection process.

Welters et al. (1993) showed that a factor similar to AcBBP1 exists in nodules of *L. corniculatus* and in free living extracts of its symbiont, *Rhizobium loti* strain NZP 2037 (Pankhurst et al., 1986). This factor binds to the identical site (BBS1) in the *S. rostrata* promoter as the AcBBP1 protein. It was therefore postulated, that these two factors play similar roles in their respective interactions. To address the biological significance of these proteins in leghemoglobin gene expression, a heterologous transgenic plant system was used which had previously been employed to study *lb* promoter activation (Stougaard et al., 1986; 1987; 1990; Szabados et al., 1990; Ramlov et al., 1993; Welters et al., 1993; Szczyglowski et al., 1994).

To directly test the effect of BBP1 on *glb3* promoter activity, the homolog of AcBBP1 in *R. loti* (RlBBP1) was identified. The corresponding gene is 63% identical at the nucleotide level, and its protein product is 78% identical and 85% similar at the deduced amino acid level. RlBBP1 binds *in vitro* to the same target in the 5' upstream region of the *Srglb3* promoter as AcBBP1. A RlBBP1 deficient mutant was constructed by insertional mutagenesis and gel mobility shift assays confirmed the loss of complex formation in extracts of this mutant. The wild type and RlBBP1 deficient strain were inoculated on the roots of transgenic *L. corniculatus* plants harboring the *Srglb3* 5' upstream region fused to the *uid*A reporter gene and nodules of the resulting transgenic

plants were monitored for GUS reporter gene activity. No differences in GUS activity levels were detected in nodules harboring the wild-type or BBP1 deficient mutant strain suggesting that the BBP1 protein is not involved in *Srglb3* gene expression.

# MATERIALS AND METHODS

## Bacterial Strains and Plasmids used

The bacterial strains and plasmids used in this study are described in Table 2.

## Southern Blot Analysis

Total bacterial genomic DNA was isolated as described by Meade et al. (1989). Ten ug of total genomic DNA was completely digested with restriction enzymes, separated on 0.8% agarose gels, and transferred to nylon filters (Hybond-N; Amersham, Arlington Heights, IL) according to standard procedures (Sambrook, 1989). Membranes were prehybridized and hybridized in a solution containing 4 X SSC, 5 X Denhardt's solution (Sambrook, 1989), 0.5% SDS (w/v) and 100 μg/ml denatured sheared salmon sperm DNA at 65°C. The membranes were washed once for 15 min in 4 X SSC, 0.1% SDS (w/v), twice for 15 min in 1 X SSC, 0.1% SDS (w/v), and twice for 15 min in 0.5 X SSC, 0.1% SDS (w/v) at 65°C. The full length AcBBP1 gene probe was labeled with [α-$^{32}$P]dATP using a random primer kit (Boehringer Mannheim) following the manufacturer's instructions.

**Construction of an *R. loti* Partial Library**

Total genomic DNA from *R. loti* NZP 2037 (Pankhurst et al., 1986) was digested with *Pst*I and 10 µg of DNA was separated on a 0.8% preparative agarose gel. A one centimeter area in the size range of the fragments found to hybridize with the *AcBBP1* gene probe were excised and the DNA isolated using a phenol freeze-thaw method. The purified fragments were cloned into pBluescript KS$^-$ (Stratagene, La Jolla, CA) digested with *Pst*I. Positive colonies were identified using a pooled plasmid mini preparation method. Ten individual colonies were grown in LB media with the appropriate antibiotics, pooled together and the plasmid DNA isolated (Sambrook et l., 1989). Southern blot analysis was carried out as described in the previous section using the *AcBBP1* gene as the probe. Plasmid DNA was isolated from individual cultures of positive hybridizing pools and were screened again using Southern analysis. Out of ten pools of ten colonies each (100 colonies screened), two positive hybridizing strains were found.

**DNA Sequencing and Computer Analyses**

A plasmid carrying a 6-kb fragment hybridizing with the AcBBP1 probe was mapped using single or multiple enzyme digests. The smallest fragment containing the RlBBP1 gene was cloned and the nucleotide sequence determined (pCla3). DNA sequencing was performed using a Sequenase 7-Deaza-dGTP DNA sequencing kit (United States Biochemical, Cleveland, OH), according to the manufacturer's instructions. Computer analysis of DNA sequences was carried out using Sequencher 2.1 (Gene Codes

Corp., Ann Arbor, MI) and SeqEd (Applied Biosystems) software. Multiple sequence alignment using Pileup in the GCG (Genetics Computer Group, Madison, WI) program and SeqVu 1.01 (Garvan Institute of Medical Research; Sydney, Australia). Analysis of predicted protein sequences was performed using PHDsec (Rost and Sander, 1993; 1994). Homology searches were performed using the BLAST algorithm (Altschul et al., 1990).

**Construction of the RlBBP1 Deficient Strain**

A plasmid carrying a 6-kb *Pst*I fragment hybridizing with the AcBBP1 gene probe was mapped using single or multiple enzyme digest. The location of the RlBBP1 gene was delimited using Southern blot analysis. The 6-kb *Pst*I fragment harboring the RlBBP1 locus was cloned into the pBluescript $KS^-$ vector (pSF99C) and used for gene replacement. A *Bam*HI digested fragment carrying the kanamycin cassette from pUC4K (Pharmacia Biotech; Uppsala, Sweden) was introduced into the unique *Bgl*II site 80-bp from the beginning of the *RlBBP1* ORF (pSF99CKm). The fragment containing the disrupted RlBBP1 gene was liberated from plasmid pSF99CKm and cloned into the *Pst*I site of the suicide vector pSup202 (Simon et al., 1983). The vector was mobilized from *E. coli* to NZP 2037 using the helper plasmid pRK2013 (Ditta et al., 1980). Transconjugants were selected as described by Simon et al. (1983) on S-20 medium (Chua et al., 1985) supplemented with tetracycline (Tc) 10 μg/ml and kanamycin (Km) 200 μg/ml initially and were later scored for the lack of growth on media containing Tc which confirmed gene replacement into the genome.

**Gel Mobility Shift Assay**

Extracts of *R. loti* NZP 2037 and the mutant NZP 2037-11 strains were prepared as described by Welters et al., (1993). The fragment of the *Srglb*3 promoter containing the BBS1 (fragment 5'203) was used in all reactions under conditions previously described (Welters et al., 1993).

**Plant Transformation and Nodulation**

Transgenic *L. corniculatus* plants cv *Rodeo* were generated according to Szczyglowski et al. (1994). Binary vectors carrying the 5' upstream region of *Srglb*3 fused to the reporter gene *uid*A (LP14, Szczyglowski et al., 1994)) were transferred to *Agrobacterium rhizogenes* strain A4 (Tempe and Casse-Delbart, 1989) using the freeze thaw method (Hofgen and Willmitzer, 1988). After plants were regenerated, multiple cuttings from a plant with average expression levels were propagated and allowed to root in sterile "sandy "soil (3:1 sand:metromix) or "mixed" soil (3:3:1:1 sand:medium vermiculite:metromix:Arabidopsis mix) for two weeks in growth chambers with an 16-hr light (24°C) and 8-hr dark (18°C) cycle. Plants were inoculated with 1ml of a two-day old culture of NZP 2037 or NZP 2037-11 grown in the presence of antibiotics but washed with sterile water to remove antibiotics and residual medium. The pelleted cells were resuspended in sterile water and 1 ml of this suspension was inoculated onto the roots of transgenic *L. corniculatus* plants. Twenty-eight days after infection, all nodules from each plant with the exception of four were harvested and frozen in liquid nitrogen until

analyzed. The remaining four nodules were used to resolute bacteria from the nodule (Pawlowski et al., 1987) to ensure that no cross contamination took place.

**Quantification of GUS enzymatic activity**

GUS enzymatic activity was quantified using the fluorescence assay (Jefferson, 1987; Jefferson et al., 1987) with a fluorescence spectrophotometer (Model F-2000, Hitachi). Enzymatic activity was expressed as picomoles of 4-methylumbelliferone produced per minute per milligram of protein. Protein concentration of the extract was determined using the Bradford assay using BSA as a standard (Bradford, 1976).

**Statistical Analysis**

Each set of data was analyzed using the non-parametric Mann Whitney test. Friedman's test was used to determine if data from different trials could be pooled. The pooled data was subsequently analyzed using the Mann Whitney test (Gardener and de Bruijn, 1997).

# RESULTS

## Identification and Cloning of the *R. loti* RlBBP1 Gene

The gene encoding RlBBP1 from *R. loti* strain NZP 2037 was isolated to directly test the effect of BBP1 on *Srglb3* promoter activity. The *AcBBP1* gene from *A. caulinodans* was used as a probe to identify any sequences cross hybridizing in the *R. loti* genome. Southern blot analysis of *R. loti* NZP 2037 genomic DNA digested with *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I revealed single hybridizing bands, suggesting that the RlBBP1 gene is unique (Figure 4-1). This analysis revealed that the RlBBP1 gene was located on a ~6-kb *Pst*I fragment. Single hybridizing bands were observed in closely related strains of *R. loti* (NZP 2235, NZP 2234, and PN184) genomic DNA but not in *Rhizobium meliloti* 1021 or two *E. coli* strains (Figure 4-1B). To isolate the complete *RlBBP1* locus, a partial *Pst*I library of *R. loti* NZP 2037 was constructed, enriching for fragments of ~6 kb and screened via a pooled plasmid mini preparation method using the AcBBP1 gene as a probe. This screening procedure yielded two positive clones. The recombinant plasmids from one colony was isolated, mapped with several restriction enzymes and re-hybridized with the *AcBBP1* gene probe (pSF99C). The RlBBP1 gene was located on a ~500 bp *Cla*I-*Eco*RI fragment and its nucleotide sequence was determined.

## Analysis of the AcBBP1 Gene and Its Protein Product (RlBBP1)

The gene encoding RlBBP1 consists of 201 bp and is 63% similar to the *AcBBP1* gene at the nucleotide level. The BBP1 proteins are about 78% identical and about 85%

**Table 2.** Strains and plasmids used in this study

| | Relevant characteristics | Source or reference |
|---|---|---|
| **Strains** | | |
| *Escherichia coli* | | |
| HB101 | recA⁻ strain for cloning experiments | Boyer and Roulland (1969) |
| INVαF' | recA⁻ strain for cloning experiments | Invitrogen (Carlsbad, CA) |
| *Rhizobium loti* | | |
| NZP 2037 | wild type, Nod⁺, Fix+ | Pankhurst et al. (1986) |
| NZP 2037-11 | *RlBBP1*⁻,Nod⁺, Fix⁺ Nod (delayed) | This work |
| NZP 2234 | wild-type, Nod⁺, Fix⁺ | provided by Dr. DB Scott |
| NZP 2235 | wild-type, Nod⁺, Fix⁺ | provided by Dr. DB Scott |
| PN 184 | derivative of NZP 2037, Cm$^r$ | Chua et al., (1985) |
| **Plasmids** | | |
| pBluescript II KS/SK (+/-) | Cloning and sequencing vector, Ap$^r$ | Stratagene (La Jolla, CA) |
| pK18 | Cloning and sequencing vector, Km$^r$ | Pridmore (1987) |
| pRK2013 | Tra⁺, Mob⁺, IncP, Km$^r$, helper plasmid in triparental conjugations | Ditta et al. (1980) |
| pSup202 | Mob+, Tra-, Apr, Cmr, Tcr | Simon et al. (1984) |
| pUC4k | Km$^r$ cassette | Pharmacia Biotech (Uppsala, Sweden) |
| pSF99C | NZP 2037 genomic DNA containing *RlBBP1* in pBluescript II KS⁻ Ap$^r$ | This work |
| pSF99CKm | Insertion of Km$^r$ cassette into *Bgl*II site of pSF99C | This work |

similar at the amino acid level. The RlBBP1 protein retains the two regions found to contain stretches of basic amino acid residues which may act as nuclear localization signals (NLSs; Garcia-Bustos et al., 1991; Raikhel, 1992; Figure 4-2). The DNA binding helix-turn-helix motifs in the central portion of RlBBP1 (residues 22-41) as well as the C-terminus of the protein (residues 51-70) that were identified in AcBBP1 are also retained. Secondary structure analysis of RlBBP1 using the PHD-sec program (Rost and Sander, 1993; 1994) predict the presence of regions containing two adjacent helical structures separated by a loop or turn with a high probability (7-9 on a scale from 0-9).

The RlBBP1 amino acid sequence aligns well with the control elements in several type II restriction-modification (RM) systems (Bougueleret et al., 1984; Nathan and Brooks, 1988; Heidemann et al., 1989; Tao et al., 1991; Siksnys et al., 1994; Anton et al., 1996), immunity repressor proteins from phage $\phi$105 and *E. coli* (Cully and Garro, 1985; Dhaese et al., 1985; Aiba et al., 1996) and the regulator of vegetative replication and conjugal transfer of the plasmid RK2 (Jagura-Burdzy et al., 1992) previously identified to have significant similarity to AcBBP1. Identity within the helical domains thought to be important for the helix-turn-helix DNA binding motif in these proteins was also conserved in RlBBP1 (Tao et al., 1991; Dhaese et al., 1985; Van Kaer et al., 1987; Jagura-Burdzy et al., 1992).

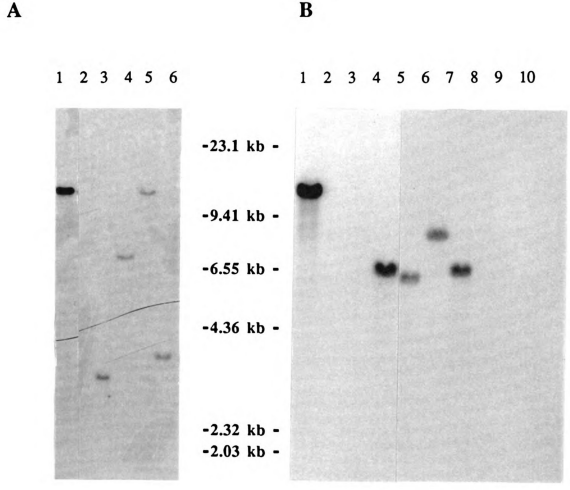



**Figure 4-1. Organization of the RlBBP1 gene in the genome of *R. loti* NZP 2037 and other bacterial strains. (A)** Ten micrograms of *R. loti* NZP 2037 genomic DNA was completely digested with restriction enzymes **Lane 1** positive control: ORS 571 digested with *Pst*I; **Lane 2**, blank; **Lane 3**, *Bam*HI; **Lane 4**, *Eco*RI; **Lane 5**, *Hind*III; **Lane 6**, *Pst*I separated on a .8% agarose gel and probed with [$\alpha$-$^{32}$P]dATP labeled AcBBP1 DNA. **(B)** Southern blot hybridization of genomic DNA. Lanes 1, 3 were digested with *Pst*I. Lanes 4-10 were digested with *Eco*RI. **Lane 1**, ORS 571; **Lane 2**, blank; **Lane 3**, ORS 571-425; **Lane 4**, *R. loti* NZP 2037; **Lane 5** NZP 2235; **Lane 6**, NZP 2234; **Lane 7**, PN184; **Lane 8**, *R. meliloti* 1021; **Lane 9**, *E. coli* strain INF$\alpha$'; **Lane 10** *E. coli* strain HB101.

**Figure 4-2. Alignment of RlBBP1 with AcBBP1.** **(A)** Nucleotide sequence; asterisks (*) indicates identity, **(B)** Amino acid sequence; asterisks (*) indicates identity, semi-colon (:) indicates conservative substitutions.

**A**

```
AcBBP1     ATGGATATGCGCAAGCTGGTCGGCCGGAACTTCGCGCGCCTGCGT    45
           *************** ******* ** **         *  *  *
RlBBP1     ATGGATATGCGCAAGTTGGTCGGACGAAATGCACGCAGGATCAGG    45

AcBBP1     CAGGAGAAGGGCCTGACACAGGAGGACGTACAGACGCGATCCGGC    90
            ** **   *** **** ****** *  *           ******
RlBBP1     GAGAAGGCCGGCTTGACGCAGGAGCAGCTTGCCGAGATCTCCGGC    90

AcBBP1     TTCAGCCAGCAGTACATCAGCGGGCTCGAACGCGGCCGGCGCAAT   135
           ************************** **    **     * **
RlBBP1     TTCAGCCAGCAGTACATCAGCGGGCTGGAGAAGGGTAAAAGGAAC   135

AcBBP1     CCCACTGTCATCACGCTCTATGAACTGGCACAGGCGCTGGGGTTA   180
           *****  ** ******  ************** *  **  *  *
RlBBP1     CCCACCATCGTCACGCTTTATGAACTGGCACAAGCCCTCCGTGTC   180

AcBBP1     CGCCACGAAGAGCTTGTTCGCGCTGACGGCAAGGACTGA        210
            * **    ** ** ** **  ***** *
RlBBP1     AGTCATATCGATCTGGTGCGACCTGACTGA                 201
```

**B**

```
AcBBP1     MDMRKLVGRNFARLRQEKGLTQEDVQTRSGFSQQYISGLE    40
           **********: *:* : ***** :   ************
RlBBP1     MDMRKLVGRNARRIREKAGLTQEQLAEISGFSQQYISGLE    40

AcBBP1     RGRRNPTVITLYELAQALGVSHEELVRADGKD            72
            * ****::********* *** :***:*
RlBBP1     KGKRNPTIVTLYELAQALRVSHIDLVRPD               69
```

**Creation and Characterization of An *R. loti* RlBBP1 Deficient Strain**

To directly determine if BBP1 proteins play a role to modulate *Srglb3* gene expression, an *R. loti* NZP 2037 strain lacking the ability to synthesize RlBBP1 was constructed (NZP 2037-11). A kanamycin resistance ($Km^r$) cassette derived from pUC4K was inserted into the unique *Bgl*II site 80 bp from the beginning of the ORF. The resulting insertion mutation was used to replace the wild-type gene via gene replacement (Simon et al., 1983). The structure of the mutated RlBBP1 locus in strain NZP 2037-11 was confirmed by Southern blot analysis (see Figure 4-3). Whole cell extracts were prepared from the wild-type NZP 2037 strain and the NZP 2037-11 mutant and used in gel retardation assays using the fragment of the *S. rostrata glb3* 5' upstream region, harboring the binding site (BBS1; fragment 5'203; Welters et al., 1993: see Figure 4-4) as target DNA. No binding activity could be observed with extracts of the RlBBP1 deficient strain (Figure 4-4) confirming that the mutation created abolishes RlBBP1 production.

**Directly Testing the Effect of BBP1 on *Srglb3* Promoter Activity**

To directly test the effect of BBP1 on *Srglb3* promoter activity, transgenic *L. corniculatus* plants harboring the *Srglb3* promoter fused to the *uidA* reporter gene were produced as described by Szczyglowski et al. (1994). The resulting plants were tested for tissue specific reporter gene (GUS) activity. All plants had detectable GUS activity in the infected cells of nodules (data not shown). One plant with average GUS activity of ~600 pmol methylumbelliferone (MU) per minute per mg protein was chosen for further study.

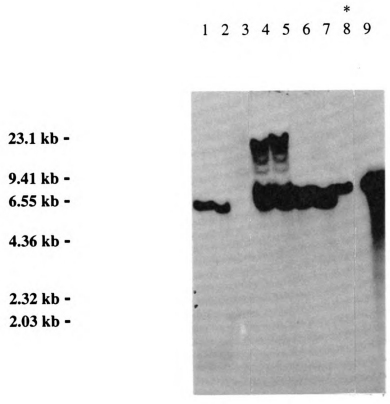
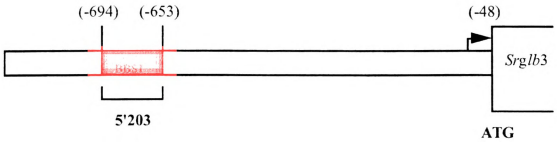



**Figure 4-3- Structure of the RlBBP1 insertion mutant.** All lanes were digested with *Pst*I. **Lane 1**, NZP 2037 genomic DNA; **Lane 2**, NZP 2235 genomic DNA; **Lane 3** blank; **Lane 4**, transconjugant 1; **Lane 5**; transconjugant 8; **Lane 6**, transconjugant 9; **Lane 7**, transconjugant 10; **Lane 8,** transconjugant 11; **Lane 9**, *Pst*I fragment containing the inserted kanamycin cassette. The entire *Pst*I-*Pst*I fragment containing the RlBBP1 gene (see Methods and Materials) was used as a probe. Asterisk (*) denotes the strain selected for use in future studies as the RlBBP1 deficient mutant.

**A**




**B**

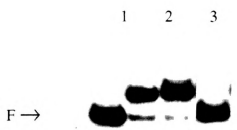



**Figure 4-4. Whole cell extracts prepared from the RIBBP1 deficient mutant lack binding activity**. Extracts prepared from NZP 2037 or NZP 2037-11 were tested for DNA binding activity using the BBS1 as target DNA. **(A)** Schematic of the *Srglh3* 5' upstream region. The arrow indicates the start point of transcription. The fragment 5'203 (shaded in red) containing the BBS1, was used as the target fragment in gel mobility shift assays. **(B) Lane 1**, extracts prepared from wild type ORS 571, **Lane 2,** NZP 2037 **Lane 3**, extracts prepared from NZP 2037-11.

The effect of the RlBBP1 mutation on nodulation and symbiotic nitrogen fixation was determined. Vegetatively propagated transgenic *L. corniculatus* plants harboring chimeric *Srglb3* promoter-*uidA* fusions were inoculated with either the wild-type or *R. loti* strain lacking the ability to produce RlBBP1 in a sandy soil or a mixed soil. The RlBBP1 deficient mutant strain was capable of inducing root nodules on its host plant ($Nod^+$). In order to determine the *Srglb3* promoter activity in nodules harboring wild-type or RlBBP1 deficient bacteria, GUS activity assays were performed as described by Szczyglowski et al. (1994) using the fluorescence assay (Jefferson, 1987). The results of three separate GUS activity assays in sandy soil were pooled and presented in Figure 4-5.

Thirty-six plants grown in the sandy soil were assayed for GUS activity. The GUS activity in nodules harvested from eighteen transgenic *L. corniculatus* plants inoculated with the RlBBP1 deficient strain was compared to the GUS activity in nodules induced by the wild-type strain. The GUS activity was measured and the non-parametric Mann-Whitney statistical test was applied. No statistically significant difference in GUS activity was detected between the two treatments suggesting that BBP1 proteins do not play a role in *Srglb3* gene expression (Figure 4-6).

Twenty-two plants grown in mixed soil were assayed for GUS activity. Nodules harvested from eleven plants inoculated with the *R. loti* mutant strain were compared to nodules harvested from eleven plants inoculated with the wild-type strain. The GUS activity was measured and the non-parametric Mann-Whitney statistical test was applied. No statistically significant difference in GUS activity was detected between the

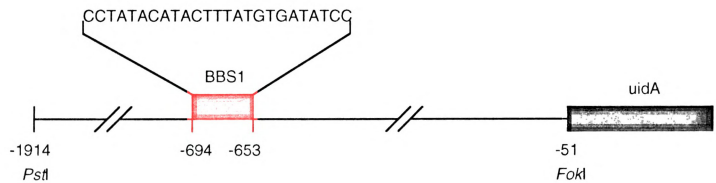



**Figure 4-5.** Schematic of the LP14 construct. The construct encompasses the 5' upstream region from -1914 to -51 where transcription begins. The *uid*A gene (shaded in blue) is fused to the entire promoter region. The BBS1 site is highlighted in red and its sequence designated above.

two treatments which suggest that BBP1 proteins do not play a role in *Srglb3* expression (Figure 4-6).

**Figure 4-6. β-glucuronidase activity in 26 day-old *L. corniculatus* nodules harvested from sandy soil.** In three independent experiments, transgenic *L. corniculatus* harboring the LP14 construct (*Srglb3* promoter fused to *uidA*) were nodulated with the wild-type or the RlBBP1 deficient *R. loti* mutant. Light bars = wild-type, dark bars = mutant. All results are expressed as pmol MU/min/mg protein. Non-parametric statistics indicate that there is no significant difference between treatments.

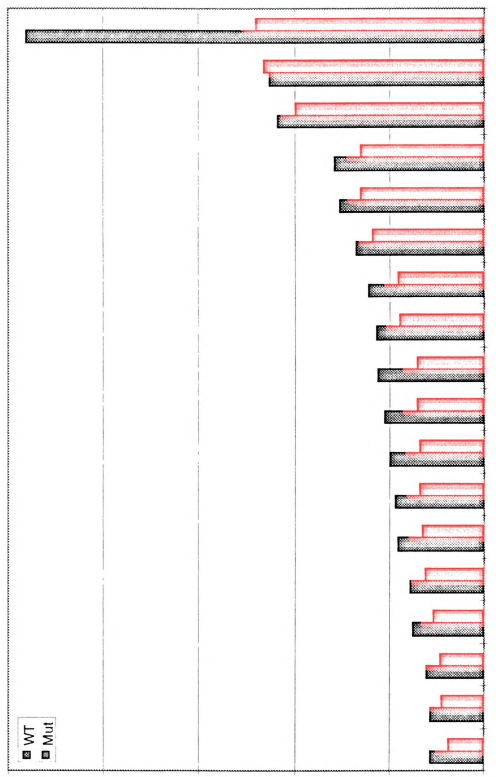
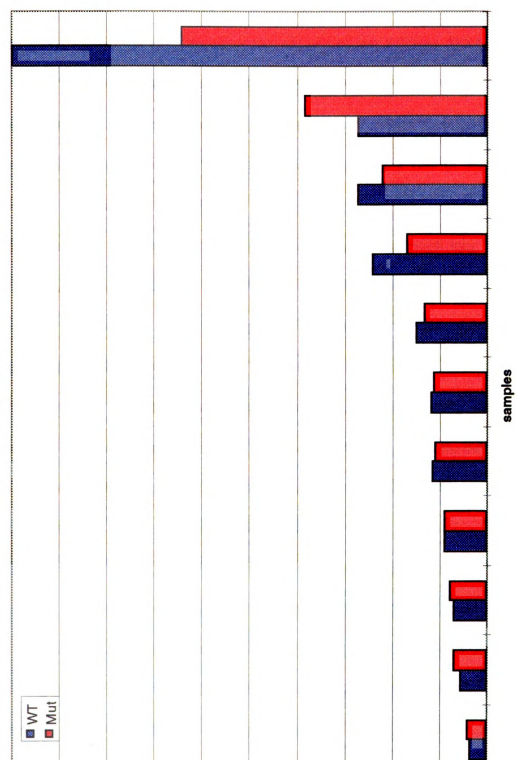

GUS Activity-Sandy Soil
WT
Mut
2500
2000
1500
1000
500
0
pmol MU/min/mg protein
samples

**Figure 4-7. β-glucuronidase activity in 26 day-old *L. corniculatus* nodules harvested from mixed soil.** In three independent experiments, transgenic *L. corniculatus* harboring the LP14 construct (*Srglb3* promoter fused to *uid*A) were nodulated with the wild-type or the RlBBP1 deficient *R. loti* mutant. Light bars = wild-type, dark bars = mutant. All results are expressed as pmol MU/min/mg protein. Non-parametric statistics indicate that there is no significant difference between treatments.


GUS Activity-Mixed
WT
Mut
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
pmol MU/min/mg protein
samples

# DISCUSSION

In this chapter, the gene encoding the *Rhizobium loti* RlBBP1 DNA binding protein which is homologous to the AcBBP1 protein from *Azorhizobium caulinodans* was isolated and characterized. This protein was previously shown to bind to the 5' upstream region of the *Srglb3* gene and was implicated to play a role in *Srglb3* gene expression (BBS1, Welters et al., 1993).

Since previous indirect evidence indicated that *lb* gene expression may require a bacterial signal, the observation that a protein originating from the symbiotic bacterium could bind to a region of an *lb* gene was intriguing. Efforts to define the role of AcBBP1 in *Srglb3* gene expression are described earlier in this thesis. Unfortunately, a clear answer was not obtained. Therefore, a system using transgenic plants carrying the *Srglb3* promoter fused to the *uidA* reporter gene was used to directly test what role BBP1 plays in *Srglb3* gene expression. In addition, the homologous gene from *R. loti* was isolated and characterized and an RlBBP1 deficient mutant constructed.

To investigate what role BBP1 proteins play in *Srglb3* gene expression, the gene encoding the *R. loti* RlBBP1 protein was cloned using the *A. caulinodans AcBBP1* gene as a probe. Upon inspection of the nucleotide and amino acid sequence of the two proteins, the *RlBBP1* gene is 63% identical to the *AcBBP1* gene at the nucleotide level while the two BBP1 proteins are 78% identical and 85% similar to each other at the amino acid level. Putative NLSs such as the NLS identified in the R protein is conserved in RlBBP1 (Shieh et al., 1993). In addition, the helix-turn-helix DNA binding domains are also conserved

spatially and in amino acid content. Secondary structure analysis using the PHD-Sec computer program (Rost and Sander, 1993; 1994) predicted two adjacent helical structures separated by a loop or turn with fairly high probabilities (7 to 9 on a scale of 0-9).

The RlBBP1 sequence also aligns well with the class of control elements found in type II restriction-modification systems (Bougueleret et al., 1984; Nathan and Brooks, 1988; Heidemann et al., 1989; Tao et al., 1991; Anton et al., 1996), to the two immunity repressor proteins previously mentioned (Cully and Garro, 195; Dhaese et al., 1985; Aiba et al., 1996) and to the TrbA protein which is a regulator of vegetative replication and conjugal transfer of the RK2 plasmid. (Jagura-Burdzy et al., 1992). The most significant sequence similarities were to the C proteins which are found in some type II restriction-modification systems. The overall sequence similarity at the nucleotide and amino acid level is striking when comparing the BBP1 proteins and the C proteins.

The phenotype of the RlBBP1 deficient strain under free living conditions was monitored. No difference in growth rate was observed. The NZP 2037-11 mutant strain was tested for nodule formation on *L. corniculatus*. Nodules were induced on the roots indicating a ($Nod^+$) phenotype. Gel mobility shift assays in which the BBS1 site in the *Srglb3* promoter region was incubated with extracts prepared from the wild-type or RlBBP1 deficient strain, confirmed that the binding activity was abolished in the mutant strain.

Transgenic *L. corniculatus* plants were inoculated with a two-day culture of NZP 2037 or NZP 2037-11. The bacterial cultures were pelleted and washed with sterile water

to remove residual medium and antibiotics. Three independent experiments were carried out in sandy soil. A total of 36 plants were assayed for GUS activity and the data analyzed using the non-parametric Mann-Whitney statistical test. In the mixed soil, nodules from two experiments involving a total of 22 plants were assayed for GUS activity and the data analyzed using the Mann-Whitney non-parametric test. No statistically significant differences were detected in the two treatments. However, in almost all cases, nodules harboring RlBBP1 deficient mutants appear to have less activity. From these GUS activity data, it appears that BBPs are not involved in *Sr*glb3 gene expression.

Since AcBBP1 does not seem to play a role in *Srglb*3 gene expression, does this bacterial protein have a function in the plant? It seems clear that the BBS1 in the 5' upstream region of the *Srglb*3 gene may play a role in modulating leghemoglobin gene expression. Welters et al. (1993) investigated the importance of the binding site (BBS1). Insertional mutagenesis and deletion of the site reduces promoter activity. This suggests that more than one factor can bind to this site *in vivo*. Disruption of this site could affect the binding of a *trans*-acting factor that has not yet been identified. Perhaps one of the other two binding activities obtained by Welters et al. (1993) could produce the results observed. The question that was addressed in this chapter investigated the interaction of a DNA binding protein and its *in vitro* target within the *Srglb*3 promoter. In this case, it appears that AcBBP1 fortuitously binds to this region *in vitro* and does not appear to affect leghemoglobin expression. Although the hypothesis that bacterial proteins are involved in *lb* gene expression has not been proven, bacterial proteins could still play a

role in symbiotic plant gene expression. The recent identification of the bacterial protein FixF, which contains functional NLSs is a candidate for interkingdom signaling in the *Rhizobium*-legume plant symbiosis (Jabbouri et al., 1996). Although no targets have been identified, mutations within the *fixF* gene lead to a Fix$^-$ phenotype.

What role does RlBBP1 and AcBBP1 play in their respective bacterium? This is a mystery. The BBP1 proteins have similarities to bacterial regulators, both activators and repressors. The most logical guess is that the BBP1 proteins may be related to the C proteins found in some type II restriction-modification (RM) systems. The features of the BBP1 proteins are strikingly similar to the class of C proteins already identified. One kb of DNA flanking the AcBBP1 gene has been sequenced, but no matches have been found in the databases. This suggests that the BBP1 proteins are novel and possess a function which is still unknown. Alternatively, these proteins may have a function homologous to the C proteins but were unable to be detected in the flanking sequences. The organization of the locus may be different from the type II RM systems identified thus far or similarities in the primary protein sequence are below the level to be scored as significant in a database search.

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# CHAPTER 5

# CONCLUSIONS

The observation that a bacterial protein, AcBBP1, from *Azorhizobium caulinodans* binds to a region in the *Sesbania rostrata glb3* leghemoglobin promoter (Welters et al., 1993) was an intriguing finding. If proven biologically significant, this observation would provide the first piece of direct evidence that bacterial proteins are involved in leghemoglobin (*lb*) gene expression. Since the early 1970's, many have speculated that a signal from the symbiotic bacterium was necessary for the induction of *lb* genes. The interaction between AcBBP1 and its target in the *Srglb3* promoter (BBS1) was a nice system to investigate this idea. The goal of this thesis project was to test the hypothesis that bacterial proteins are involved in *lb* gene expression. To begin the investigation, the gene encoding AcBBP1 was cloned. The deduced amino acid sequence revealed two putative nuclear localization signals and two helix-turn-helix DNA binding motifs in the central and C-terminal end. The AcBBP1 protein shares sequence similarity with a number of regulatory proteins. The best match was to a class of control proteins found in some type II restriction-modification systems. To address the biological significance of the AcBBP1 protein in *Srglb3* gene expression, a deletion/insertion mutant lacking the ability to produce AcBBP1 was constructed. Gel mobility shift assays and western blot analysis confirmed that the protein was not produced. The wild-type and mutant *A. caulinodans* strain was inoculated onto the stems and roots of *S. rostrata*. The nitrogen fixation ability of nodules induced by the wild-type and mutant bacteria was determined using the acetylene reduction assay. On average, the nodules induced by the AcBBP1 deficient strain fixed about 20% less nitrogen than its wild-type counterpart. Since a difference in nitrogen fixation was observed, total leghemoglobin mRNA steady

state levels were monitored. The expression levels of leghemoglobin in nodules harboring wild-type or mutant bacteria were identical suggesting that the AcBBP1 protein does not significantly alter the total leghemoglobin levels in the nodules induced by the mutant strain.

These observations led to the next phase of this thesis project, in which experiments designed to further elucidate potential roles of the AcBBP1 protein were initiated. Transmission electron microscopy revealed that plant cells harboring wild-type or mutant bacteria were at different stages of the infection process. The plant cells harboring mutants were delayed in the infection process as more uninfected cells were present in the nodule tissue. In addition, only one or a few bacteroids were encased by a peribacteroid membrane while up to ten wild-type bacteroids were surrounded by this membrane. Antibodies specific to AcBBP1 were produced and used to localize this protein to the bacteroid and peribacteroid membrane. This was not an unexpected result as this protein is of bacterial origin. The AcBBP1 protein was not found in the uninfected cells or in the nucleus of infected cells. This suggests that the protein may not be directed to the plant nucleus or at least can not be detected in this compartment.

It was previously shown that a homologous factor in *R. loti* extracts could bind to the BBS1 in the 5' upstream region of the *Srglb3* gene (Welters et al., 1993). To directly monitor what role the BBP1 proteins play in *Srglb3* gene expression, two tools were developed. First, transgenic *Lotus corniculatus* plants harboring the 5' upstream region of the *Srglb3* gene fused to the reporter gene *uidA* were made. Second, an *R. loti* mutant deficient in the production of BBP1 was created. The AcBBP1 homolog in *R. loti*

(RlBBP1) was cloned. The *RlBBP1* gene is 63% identical to *AcBBP1* at the nucleotide level and the two proteins are 78% identical and 85% similar at the amino acid level. An RlBBP1 deficient mutant was constructed by insertional mutagenesis and was used to nodulate the transgenic *L. corniculatus* plants harboring the chimeric promoter-reporter gene construct. Nodules harboring wild-type or RlBBP1 deficient bacteria were assayed for GUS activity and no statistically significant differences were detected. Therefore, our hypothesis that bacterial proteins are involved in *lb* gene expression can not be substantiated at this time.

The investigation of this interesting phenomenon was undertaken and the goals set forth were accomplished. The biological significance of the interaction between AcBBP1 and its target in the *Srglb3* promoter was explored and it appears that this bacterial DNA binding protein does not modulate *Srglb3* gene expression. However, examples in other plant-bacterial systems implicate that bacterial components (i.e. proteins or DNA) to be necessary to obtain specific plant responses. As more knowledge is gained in this area, the concept of transkingdom signaling may become more universal.