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I am submitting herewith a dissertation written by Crystal Bickley McAlvin entitled "Transgenic approaches to study nodulation in the model legume, *Lotus japonicus*". I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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Transgenic approaches to study nodulation in the model legume, *Lotus japonicus.*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Crystal Bickley McAlvin

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- This dissertation is dedicated to my parents, John and Debra Bickley, for giving me the gift of higher education, without which none of this would have been possible. It was a huge sacrifice for them to support me throughout my college career. In addition, their love and encouragement gave me the strength I needed to complete the enormous task of getting a Ph.D. in microbiology.

- In addition, I would like to dedicate this dissertation to my loving husband Jesse McAlvin. He patiently stood by my side during the completion of this work. His love and support gave me strength when I needed it the most.

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ABSTRACT

The soybean apyrase, GS52, characterized as an early nodulin, was further investigated for its possible role in nodulation. GS52 is expressed in roots and localized to the plasma membrane. In addition, it is rapidly induced upon rhizobial inoculation. Treatment of soybean roots with anti-GS52 antibodies blocked nodulation by *Bradyrhizobium japonicum*. Transgenic *Lotus japonicus* plants were generated expressing *gs52* and showed enhanced nodulation and infection thread formation upon inoculation with *Mesorhizobium loti* that correlated with expression of the transgene. Surprisingly, expression of GS52 allowed *L. japonicus* plants to be infected but not nodulated by *B. japonicum*, the natural symbiont of soybean. The data presented supports a critical role for the GS52 apyrase in nodulation and control of infection host specificity.

Similarly, the role of the plant defense response in nodulation was investigated via generation of transgenic plants. Salicylic acid (SA) is a central molecule in the plant defense response and plants that express salicylate hydroxylase, an enzyme that degrades SA to catechol, are deficient in SA cannot mount the defense response. *NahG L. japonicus* plants, expressing salicylate hydroxylase, were generated and characterized for their nodulation phenotype. NahG plants demonstrated enhanced nodulation and infection thread formation when inoculated with *M. loti.* However, other phenotypes were observed, such as increased root growth, that complicated interpretation of the results.

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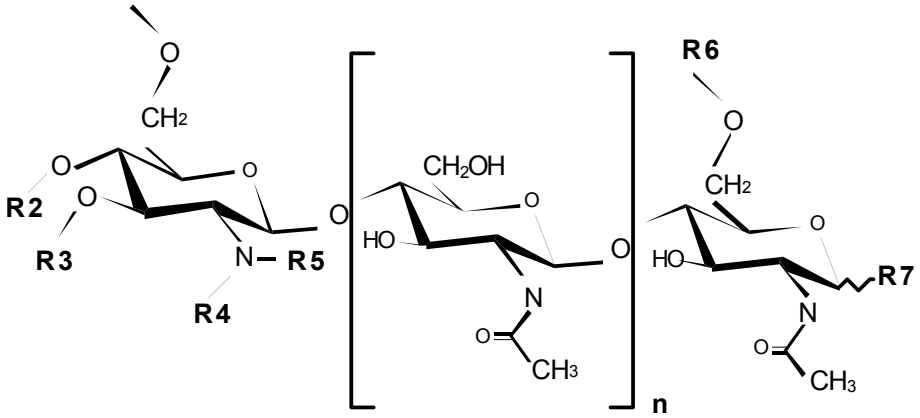
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CHAPTER 1: GENERAL INTRODUCTION

Overview of nodulation process

Bacteria in the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium,* and *Sinorhizobium* are symbiotic soil bacteria that have the unique ability to infect the roots of leguminous plants. This infection does not result in harm to the legume, but rather is a beneficial infection that results in nodule formation and atmospheric nitrogen fixation. Both partners benefit from this relationship as the legume plant is provided with a ready source of fixed nitrogen and the bacteria is, in turn, provided with a protected environment and usable carbon sources. The infection process is very specific in that it requires signal exchange from both the bacteria and legume. Legume roots secrete compounds called flavonoids that are perceived by symbiotic bacteria (Fellay et al., 1998). Recognition of the flavonoid signal leads to induction of the bacterial *nod* genes that are responsible for construction of the second component of the signal exchange, the lipo-chitin Nod signal. Bacterial *nod* genes are regulated by NodD, which is a LysR-type transcriptional regulator. NodD binds to the *nod* box, which is a specific DNA sequence that precedes each nodulation operon, and in the presence of plant flavonoids, induces expression of the *nod* genes (Fellay et al., 1998). The array of Nod signals produced is specific to the bacterial species, but generically they are composed of a chitin backbone of varying length (see figure 1-1). These molecules can have specific decorations attached to the reducing and non-reducing ends, such as fatty acid tails and sulfate groups (Fisher et al., 1992). Indeed, it is these modifications that are largely responsible for the species specificity of the Nod signal.



species	n	R1	R2	R3	R4	R5	R6	R7
B. japonicum (USDA110)	3	Ac	H	H	C18:1(9) C18:1(9,Me) C16:1(9) C16:0	H	2-*O*-MeFuc	H
B. elkanii	2,3	Ac	Cb	H	C18:1(11)	Me , H	2-*O*-MeFuc or Fuc	glycer -yl
R. sp. NGR234	3	Cb or H	Cb or H	Cb or H	C18:1(11) C16:0 C16:1	Me	3-*O*-SO_3H, 2-*O*-MeFuc or 4-*O*-Ac, 2-*O*-MeFuc	H
S. fredii (USDA 257)	1,2,3	H	H	H	C18:1(11)	H	2-*O*-MeFuc or Fuc	H
A. caulinodans	2,3	Cb	H	H	C18:1(11) C18:0(11)	Me	Ara or Fuc	H
R. loti	3	H	Cb	H	C18:1(11) C18:0	Me	4-*O*-Ac Fuc	H
R. etli	3	H	Cb or H	Cb or H	C18:1(11)	Me	4-*O*-Ac Fuc	H
R. tropici	3	H	H	H	C18:1(11)	Me	SO_3H	manno -syl
S. meliloti	1,2,3	Ac	H	H	C16:2(2,9) C16:3(2,4,9)	H	SO_3H	H
R. leguminosarum bv *viciae*	2,3	Ac	H	H	C18:1(11) C18:4(2,4,6, 11)	H	H or Ac [(a)]	H

Figure 1-1. Some examples of Nod signals produced by Rhizobia. Top, structure of the chitin backbone of the Nod signal. Bottom, various modifications to the Nod signal. Cb, carbamaoyl; Me, Methyl; Ac, acetyl.

The Nod signals made by symbiotic bacteria control many of the plant's early responses to rhizobia and are powerful morphogens that induce many morphological changes in the legume root, such as root hair deformation and curling and cortical cell division (Relić et al., 1994). Symbiotic bacteria attach to legume root hairs and root hair curling traps the invading rhizobia and initiates infection thread formation. Infection thread formation occurs as a result of actin rearrangements and invagination of the root hair cell wall (Kijne et al., 1992). This step seems to require rhizobial production of exopolysaccharides. These molecules may function as signaling factors, triggering developmental responses in the plant or modulating host defense responses. They may also play a structural role, enabling attachment to plant surfaces or protecting the symbiont from host defenses (Dickstein et al., 1991; van Rhijn et al., 1998; Gonzales et al., 1996). The rhizobia proceed to infect the root via the infection thread until they reach the foci of the cortical cell division, called the nodule primordia. Here, the rhizobia are released into the cortical cell, where they are enclosed in a membrane bound compartment called the symbiosome (Roth et al., 1991). In the symbiosome, the rhizobia cease to grow and differentiate into nitrogen fixing bacteroids. It is here, that the *nif* and *fix* genes are induced, which encode the ability to fix nitrogen (Fischer, 1994). It is interesting to note that there are two types of nodule morphologies that can form. Determinant nodules are round in shape because they have a non-persistent meristem that ceases to grow outward from the legume root. In contrast, indeterminant nodules are oblong in shape as they have a persistent meristem that continues to grow outward from the legume root.

Signal exchange in the Rhizobium-legume symbiosis

The lipo-chitin Nod signals

Most of the rhizobial nodulation genes code for enzymes responsible for synthesis of the Nod signal, which is a lipo-chitooligosaccharide (LCO) molecule (reviewed in Long, 1996, Mergaert et al., 1997, Spaink, 1992). The Nod signal consists of a chitin backbone of three to five β-1,4-linked N-acetylglucosamine residues that are modified at both the reducing and non-reducing ends in a species-specific manner. Figure 1-1 illustrates the chemical structure of the Nod signal. These signal molecules are active at sub-nanomolar concentrations and act as powerful morphogens that can trigger the onset of nodule organogenesis when applied to the developing legume root.

Host range in the legume-rhizobia symbiosis is determined, in part, by the specific modifications to the chitin oligosaccharide backbone of the Nod signal. Modifications to the Nod signal occur in a species specific manner by proteins encoded by host-specific nodulation genes. For example, in the *S. meliloti*-alfalfa symbiosis, the NodHPQ proteins function to add sulfate to the reducing end of the nod signal (Denarie et al., 1992). Mutations in the genes that encode these proteins, *nodHPQ*, abolish the ability of *S. meliloti* to nodulate *Medicago sativa* (alfalfa). Interestingly, these mutants retain their ability to nodulate an alternate host, *Vicia sativa* (vetch).

Plant-microbe interactions: pathogens and symbionts

Plant responses to pathogens

Plant-interacting microbes differ with respect to the nature of the responses that they elicit in their respective hosts. For example, some plant pathogens elicit a response that is detrimental to one of the two partners. The response is detrimental to the host in a compatible interaction and detrimental to the invading pathogen in an incompatible interaction. In the incompatible interaction, the host plant induces a defense response, either the hypersensitive response (HR), systemic acquired resistance (SAR), or both, that limits pathogen invasion and spread. The HR is an early plant defense response characterized by local tissue necrosis around infection sites serving to limit pathogen spread. This response is local in that only plant cells in the immediate vicinity of the invading pathogen undergo the HR and accumulate phenolic compounds, hydrogen peroxide (H_2O_2), and pathogenesis-related (PR) proteins. In addition, other general defenses many be employed during the HR, such as cell wall fortification (lignification) and programmed cell death, both serving to limit pathogen infection and spread. In contrast to the HR, SAR is a defense response that is characterized by a global or systemic plant resistance that is not limited only to the site of pathogen invasion. This type of resistance often develops over a period of several days to a week after the initial pathogen invasion. As mentioned, some pathogens have to the ability to elicit the plant defense response. The plant can recognize some pathogen-derived molecules as foreign and the foreign molecules are thus termed an elicitor because they induce or elicit the plant defense upon recognition by the host plant. On the other hand, some pathogen-

derived molecules have the ability to suppress the plant defense response upon recognition by the host plant and they are thus called suppressors.

Salicylic acid

The healing benefits of plants containing high levels of salicylic acid (SA) has long been known. Aspirin, acetylsalicylic acid, has been used for medicinal purposes since antiquity. In the plant kindom, SA is reported to be involved in many physiological processes, including plant growth (Gutiérrez-Coronado et al., 1998), senescence (Morris et al., 2000), abscission (Ferrarese et al., 1996), plant defense response (Gaffney et al., 1993), and nodulation (Martìnez-Abarca et al., 1998). Salicylic acid (SA) is a phenolic compound made throughout the plant kingdom via the phenylpropanoid pathway. Research efforts over the past decade have studied this molecule to elucidate its many roles in plant physiology. Interestingly, many reports have demonstrated that SA is a key molecule in the plant defense response. Although the actual mechanism of SA's action is not understood, it is clear that SA is intimately involved in the induction of both the hypersensitive response (HR) and systemic acquired resistance (SAR) (Durner, 1997; Feys et al., 2000). Studies adding exogenous SA to plants suggest that this compound can enhance defense gene induction, PR protein accumulation, production of H_2O_2, and programmed cell death (Draper, 1997). The initial evidence that SA was involved in the defense response came from the finding that SA treatment of tobacco plants could induce HR and hence provide enhanced resistance to tobacco mosaic virus (TMV). It was subsequently found that SA treatment could also induce SAR in a number of plant species, including cucumber and potato. In tobacco, SA induces the same set of nine

genes that are activated systemically by TMV infection (Ward et al., 1991). SA levels increase 40-fold in TMV inoculated leaves and 10-fold in uninoculated leaves. The marked increase in SA levels in both inoculated and uninoculated leaves parallels the induction of PR gene expression, HR, and SAR. Therefore, endogenous SA appears to play a key role in the signal transduction pathway that leads to activation of PR genes, HR, and SAR. Moreover, certain *Arabidopsis thaliana* mutants produce elevated levels of SA and show constitutive expression of PR genes and in some cases HR lesion formation even in the absence of pathogen challenge (Shah et al, 2001). In addition, it was reported that SA can reach 150 μM, a concentration sufficient to cause substancial inhibition of catalase and ascorbate peroxidase (Gaffney et al., 1993; Chen et al., 1993; Conrath et al., 1995; Enyedi et al., 1992; Durner et al., 1995). Conversely, plants that express the bacterial (*Pseudomonas putida*) *nahG* gene, encoding salicylate hydroxylase, are unable to accumulate SA, due to hydrolysis by NahG, and are deficient in the plant defense response (Gaffney et al, 1993). Figure 1-2 shows the reaction carried out by NahG in *Pseudomonas putida.* The NahG tobacco plants fail to establish SAR, and develop viral lesions that are larger than those produced on wild type plants when challenged with TMV.

In addition, salicylic acid and related derivatives have also been reported to have a pronounced antibacterial effect against *Bacillus cereus, Streptococcus pyogenes*, and *Mycobacterium fortuitum* (Corthout et al., 1994). Other reports also show that SA is antibacterial against other bacterial species (Kupferwasser et al., 1999; Muroi et al., 1993; Awad et al., 1991; Hartmann et al., 1988; Angelillo et al., 1977). In addition to the

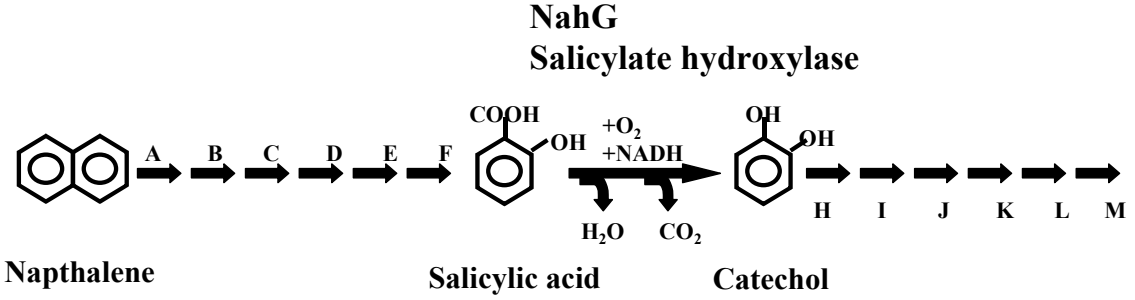


Fig. 1-2. Reaction carried out by the *Pseudomonas putida* NahG protein. The *Pseudomonas putida* napthalene degradation pathway involves many proteins, including: A, NahA; B, NahB; C, NahC; D, NahD; E, NahE; F, NahF; H, NahH; I, NahI; J, NahJ; K, NahK; L, NahL; M, NahM.

inherent antibacterial effect, phenolic acids have also been reported to have molluscicidal (Corthout et al., 1994) and antifungal activity (Bénigne-Ernest et al., 2002).

Interestingly, there are many reports that support the idea that SA could be the long-distance signal that is transported from the sight of infection throughout the plant to trigger SAR in distant parts of the plant. Shulaev et al., (1995) demonstrated that labeled salicylic acid was translocated to distal parts of tobacco plants infected with tobacco mosaic virus (TMV). Lower inoculated leaves of the plants were incubated in an $^{18}O_2$-rich environment allowing labeling of SA at the time of inoculation (the last step in SA biosynthesis is the O_2 dependent hydroxylation of benzoic acid). Subsequent analysis of the upper uninoculated leaves indicated that almost 70% of the salicylic acid was labeled. Despite this convincing evidence, grafting experiments refute this idea. Vernooj et al., (1994) used transgenic tobacco plants expressing *nahG* gene in grafting experiments with wild type plants. This approach worked because expression of NahG results in a product that converts SA to inactive catechol, and therefore NahG plants are unable to accumulate SA and unable to mount the HR or develop SAR. Experiments showed that inoculation of the NahG expressing graft with a pathogen induced SAR in distant parts of the grafted wild-type plant that was uninoculated. These results led to the conclusion that local infection results in the production of a yet unidentified mobile signal that is transported to distal tissues, where it initiates the accumulation of SA necessary for SAR induction.

It is also hypothesized that SA could function in the plant defense response by modulating the oxidative burst that occurs upon pathogen challenge. Durner et al. (1996) reports evidence that SA can both inhibit and protect catalase activity, depending on the concentration of H_2O_2. When H_2O_2 levels are low, SA inhibits catalase activity, which

allows reactive oxygen intermediates to rise in concentration and activate the plant defense response. In contrast, when H_2O_2 levels rise significantly in infected cells, SA can protect catalase from becoming inactivated. Catalase enzymes are inactivated by high levels of H_2O_2. However, in the presence of SA, this H_2O_2 induced inactivation is prevented and catalase activity increases significantly compared to controls where SA is absent. Therefore, it is hypothesized that SA can function in the plant to both potentiate and contain the oxidative damage associated with the HR.

Ethylene

Ethylene is a plant hormone that is long known to be involved in many plant processes including plant growth and development, seed germination, root and shoot growth, flower development, senescence and abscission of flowers and leaves, and ripening of fruit, all of which discovered prior to 1940 (reviewed in Bleeker and Kende, 2000). Subsequently, ethylene was also shown to participate in plant responses to a variety of biotic and abiotic stresses. Ethylene is synthesized from its immediate precursor 1-Aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC oxidase. ACC is produced from S-Adenosyl-ι-methionine (SAM) by the enzyme ACC synthase (Adams and Yang 1979). Both reactions are part of the methionine (Yang) cycle, which is ubiquitous in the plant kindom as it plays important roles in the biosynthesis of polyamines and participates in a wide variety of methylation reactions. ACC synthase catalyzes the rate-limiting step in ethylene biosynthesis and therefore plays a pivotal role in regulating ethylene production (Boller *et al*., 1979; Yu *et al*., 1979). Interestingly, it was demonstrated in some cases that ethylene and SA work in concert to regulate plant

responses to biotic or abiotic stress. For example, Rao et al. (2002) reported that ozone-induced ethylene production is dependent on salicylic acid in *Arabidopsis thaliana*. Further, they demonstrated that both salicylic acid and ethylene are required to regulate ozone-induced cell death.

Ethylene is well known for its role in regulation of plant defense responses of plants when challenged or infected by pathogens (Reviewed in Ohashi and Ohshima, 1992). Moreover, ethylene was found to inhibit nodulation of both *Pisum sativum* and *Vicia sativa* ssp. Nigra (Lee and LaRue, 1992a; Lee and LaRue, 1992b; Zaat et al., 1989). An interesting report from van Workum *et al*, 1995, showed that exopolysaccharide-deficient mutants of *Rhizobium leguminosarum* bv. *viciae* could not nodulate *Vicia sativa* ssp. *nigra* unless ethylene production was inhibited. Penmetsa and Cook (1997) reported that the ethylene-insensitive *Medicago truncatula* mutant '*sickle'* was hyperinfected by its rhizobial symbiont. Ligero et al. (1991) showed that (1) a positive correlation between the nitrate concentrations in the growth medium and ethylene production in alfalfa roots, and (2) a negative correlation between ethylene evolution and nodulation. More recently, Ligero et al. (1999) demonstrated that nitrate inhibition of nodulation in soybean was mediated through ethylene. Thus it appears that ethylene inhibits nodulation in many legumes.

Pathogenesis related proteins

Associated with both the HR and SAR is the synthesis of five or more families of pathogenesis-related (PR) proteins and SA is implicated in several of these classes (Mur et al., 1996). Two of these families encode hydrolytic β-1,3-glucanase and chitinase

enzymes. These enzymes are intimately involved fighting against fungal pathogens by degradation of invading fungal cell walls. The precise function of the other families is not well understood, however, their expression is associated with resistance to a large number of viral, fungal, and bacterial pathogens.

SA-mediated signal transduction cascades also regulate the transcriptional activation of many PR genes (Zhu et al., 1996). Moreover, SA and ethylene were shown to act synergistically to further enhance the expression of PR genes. Some PR proteins, such as lipoxygenase, may contribute to the defense response by generating secondary plant defense signals such as jasmonic acid (JA) and lipid peroxides. Many PR proteins are also thought to act by producing a variety of toxic volatile and nonvolatile secondary metabolites with significant antimicrobial activity. For example, *trans*-2-hexanal, a C_6 volatile compound can inhibit growth of *Pseudomonas* bacteria and induce a subset of defense-related genes.

Oxidative burst

The production of reactive oxygen intermediates within minutes to hours of pathogen inoculation is termed the oxidative burst. These molecules are generated by many processes in plants, including: photorespiration, photosynthesis, oxidative phosphorylation, and the hypersensitive-response associated oxidative burst. In both compatible and incompatible interactions there is an oxidative burst that occurs very rapidly upon pathogen infection. However, in incompatible interactions there is a second, stronger, and more prolonged oxidative burst that occurs. This type of reaction is

usually followed by programmed cell death (PCD) to limit pathogen spread and the onset of HR and SAR.

Typical reactive oxygen intermediates that are detected during the oxidative burst are superoxide (O_2•–), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•–_. The mechanism that plants probably use to generate these molecules is thought to involve a plasma membrane-associated NADPH oxidase, which is similar but not identical to that used in mammalian neutrophils in the immune response (Khan et al., 1995). Superoxide anions produced outside plant cells are usually converted rapidly to H_2O_2, which can easily cross plant cell membranes and enter neighboring cells. Inside the cell it can then be converted into even more toxic reactive oxygen intermediates.

It is possible that reactive oxygen intermediates do not act directly to kill the pathogen and or host cells, but rather serve as a signal molecule to trigger the cell death pathway or transcription of defense related genes. For example, H_2O_2 induces the benzoic acid 2-hydroxylase enzyme, which is required for SA biosynthesis (Durner et al., 1996). H_2O_2 is also known to induce genes encoding proteins involved in cell protection mechanisms, such as glutathione S-transferase, glutathione peroxidase, and polyubiquiting, as well as peroxidases, catalases, and other enzymes involved in scavenging reactive oxygen species and pathogen resistance (Wobbe et al., 1996). The oxidative burst may also significantly alter the redox balance within plant cells. Many mammalian transcription factors are known to be redox-regulated, including NF-κB and AP-1, and it seems reasonable that certain plant transcription factors may be regulated in a similar way.

Plant responses to rhizobial symbionts

In contrast to plant pathogens, symbiotic bacteria in the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium,* and *Sinorhizobium*, do not usually elicit an obvious defense response. However, the invading symbiont is clearly recognized by the plant host since, during the progression of the rhizobium-legume symbiosis, there are numerous plant genes that are systematically activated. Associated with the change in gene expression are many physiological changes (e.g. root hair curling and calcium spiking). Table 1-1. Lists a number of events characterized thus far.

A role of the plant defense response in nodulation?

It has long been suggested that a defense response could be elicited during some rhizobial-plant interactions and this could play an important role in determining host range (Mellor and Collinge, 1995). For example, Vasse et al. (1993) reported that a plant defense response could be involved in the formation of aborted infection threads during normal infection of alfalfa by *Sinorhizobium meliloti.* This hypothesis is supported by the microscopic observation of localized root cell necrosis and accumulation of phenolic compounds at the site of infection thread arrest. It is well established that only a small percentage of the infection sites that are initiated are successful and it is possible that induction of a defense response could be responsible for limiting successful infection (reviewed in Mellor and Collinge, 1995). In addition, Santos et al. (2001) reported that alfalfa responds to wild-type *S. meliloti* by the transient production of reactive oxygen species, termed the oxidative burst. Alfalfa roots inoculated with rhizobial

Table 1-1: Plant responses to rhizobial inoculation.

Time after inoculation	Response	Reference
1 minute	Bacterial attachment	Turgeon and Bauer, 1982
1 minute	Ca^{2+} influx, Cl^- efflux in root hair cells	Felle et al, 1996
2 minutes	Membrane depolarization	Ehrhardt et al, 1996
2-10 minutes	Calcium spiking in root hairs	Erhardt et al, 1996
5 minutes	Intracellular alkalinization of root hairs	Felle et al, 1995
5-10 minutes	Actin de-polymerization	Allen and Bennett, 1996
10 minutes	Root hair deformation	Heidstra et al, 1994
1 hour	Leghemoglobin induction	Heidstra et al, 1997
1-2 hours	Root hair tip swelling and growth	Gehring et al, 1997
2 hours	Protein phosphorylation in root hairs	Boukli et al, 1999
3 hours	Enod12 induction	Journet et al, 1994; Pichon et al, 1992
3 hours	Rip1 induction	Peng et al, 1996
6 hours	GS52 apyrase induction	Day et al, 1999

Table 1-1. Continued

Time after inoculation	Response	Reference
8 hours	Enod5 induction	Vijn et al, 1995
5-17 hours	CHS induction	Mathesius et al, 1998
12 hours	Root hair curling	Turgeon et al, 1982
17 hours	GH3 induction	Mathesius et al, 1998
24 hours	PAL induction	Krause et al, 1997
12-24 hours	Enod40 mRNA induction (non-specific)	Minami et al, 1996
24 hours	Extensin mRNA	Arsenijecvic et al, 1997
24 hours	Visible infection thread	Turgeon and Bauer, 1982
24 hours	MtAnn1 (Annexin) induction	Niebel et al, 1998
24-96 hours	Cortical cell division and bacteroid formation	Newcomb et al, 1979; Turgeon and Bauer, 1982
4 days	Development of the nodule meristem	Turgeon and Bauer, 1982
4-5 days	Enod40 induction (specific)	Minami et al, 1996
5-6 days	Enod2 induction	Minami et al, 1996
4-10 days	Visible nodules	

These data are collective results from numerous legume species and does not imply that all responses are observed in all legumes (adapted from citations listed in table).

exopolysaccharide mutants, that are unable to fix nitrogen, appear to exhibit a defense response (Niehaus, 1993).

In this case, microscopy revealed a thickening of the cell walls in contact with the EPS mutant rhizobia. Moreover, pea roots inoculated with a lipopolysaccharide (LPS)-defective mutant showed a phenotype reminiscent of the HR, including reduced nodule colonization by the mutant, callose deposition leading to thickened host cell walls (known to limit pathogen spread), and sporadic host cell death (Perotto, et al. 1994). More recently, Martínez-Abarca et al. (1998) showed that SA accumulated in alfalfa roots inoculated with a *Sinorhizobium meliloti* Nod C mutant unable to synthesize the lipo-chitin Nod signal required for infection. This same report also showed that exogenous addition of SA resulted in both reduced and delayed nodule formation on alfalfa roots inoculated with wild-type *S. meliloti*. Subsequently, Bueno et al., (2001) showed that increased lipoxygenase activity and H_2O_2 accumulation in alfalfa roots following inoculation with wild-type *S. meliloti* and a Nod C mutant. Most recently, van Spronsen et al. (2003) reported that exogenous SA addition inhibited indeterminate nodulation (e.g., in vetch, with a persistent meristem) but not determinate nodulation (e.g., in *Lotus japonicus* with no persistent meristem). They correlated this response with the fact that *R. leguminosarum* bv. *viciae* that nodulates vetch produces a lipo-chitin nodulation signal with a 18:4 fatty acid. They postulate that this fatty acid may be active in oxylipin signaling, which is inhibited by SA. Rhizobia that form determinate nodules produce Nod signals lacking polyunsaturated fatty acids and, thus, these signals may act in a different way.

Interesting to the role of the plant defense response in nodulation, Cook et al. (1995) presented data on the identification of a Rhizobium-induced-peroxidase (*rip1*) that is rapidly induced (within 3 hours) upon rhizobial inoculation. Moerover, inoculation of plants with an incompatible rhizobium strain, which is not a natural symbiont of the legume, does not result in *rip1* induction, suggesting that *rip1* induction might be necessary for successful nodulation to occur. More specifically, Ramu et al. (1999) showed that induction of rip1 and the subsequent development of an oxidative burst are dependent on the structural decorations present on the bacterial nod signal.

Recent work demonstrated that *Bradyrhizobium japonicum USDA110*, the soybean symbiont, synthesizes cyclic 1,3-1,6-β-glucans during symbiosis (Rolin et al., 1992; Gore and Miller, 1993). These molecules are interesting in that they are produced during many pathogenic fungal interactions through the action of plant chitinases, which degrade the invading fungal cell wall. In general, the generation of these elicitor molecules sets off signal cascades that induce the plant defense response, including generation of phyotalexin (including SA) compounds. In addition to elicitor generation, some fungal pathogens have the ability to actively generate suppressor molecules that can suppress the defense response. Interestingly, β-glucans synthesized by *B. japonicum* did not induce the accumulation of phytoalexins at physiological concentration (1 μM). In contrast, β-glucans produced by non-symbiont rhizobia, that do not normally infection soybean, did induce phytoalexin accumulation (Mithöfer et al., 1996). These results further confirm the hypothesis that the successful establishment of rhizobium-legume symbiosis may occur via a novel mechanism whereby rhizobium suppresses the legume plant's defense response.

Other reports demonstrate yet another mechanism by which rhizobia might be challenged with the plant defense response. Chitinases are common pathogenesis related (PR) proteins whose expression is signature of the plant defense response. Interestingly, numerous reports indicate that rhizobial inoculation or Nod signal treatment of roots can cause differential expression of plant root chininases (Kim et al., 2002; Salzer et al., 2000; Xie et al., 1999). In fact, Staehelin et al. (1994) proposed a model for the determination of host-specificity in the rhizbium-legume symbiosis based on the hypothesis that structural modifications to the Nod signal influence their stability against hydrolysis by plant root chitinases. Incubation of different concentrations of *R. meliloti* Nod signals in the presence of root chitinases from *Medicago* (host plant) and *Vicia* (non-host plant) showed that the Nod signals were degraded at different rates. Chitinases from *Vicia* had a greater capacity to degrade the Nod signals produced by *R. meliloti*. The finding of differential degradation of Nod signals by plant chitinases raises the question of whether Nod signal stability could act as a host-range determinant. Further, it is possible that plant chitinases may be involved in controlling the biological activity of Nod signals by degrading them.

Symbiotic bacteria have been found to contain genes encoding type III secretion systems (Viprey et al., 1998). Type III secretion systems (TTSS) are not just secretion systems, but rather complex weapons for close combat used by many gram(-) pathogenic bacteria. Found in both plant and animal pathogens, these loci are generally clustered on the genome and encode products required for both secretion and translocation of effector proteins into the respective host cell (He, 1998; Cornelis, 2000). TTSSs encode proteins that make up the secretion apparatus, which is generally an appendage-like structure that

is believed to serve as a hollow conduit through which the effector proteins can travel. TTSSs also encode proteins necessary for the translocation process. These proteins contain hydrophobic domains and are thought to insert themselves into the host membrane and form a pore. Pore formation is very important, as it allows the translocation of effector proteins into the host cell. It is not known how the effector proteins travel through the secretion and translocation apparatus. However, once in the intracellular space or the host cell cytoplasm, these effectors serve to mimic, suppress, or modulate the host cytoskeleton and /or defense pathways to enhance the survival and fitness of the invading pathogen (He, 1998 and Cornelis, 2000).

Therefore, the finding that numerous rhizobia strains have genes encoding TTSSs suggests similarities among symbionts and their pathogenic ancestors. One of the best studied TTSSs in symbiotic bacteria is the system in *Rhizobium* NGR234. *Rhizobium* NGR234 is a broad host strain that can nodulate at least 112 legume genera including the non-legume, *Parasponia andersonii* (Pueppke and Broughton, 1999). Most of the known nodulation genes in this strain are found on a 536 kb replicon (pNGR234a) (Perret and Broughton, 1997). Studies in this system provide evidence for the secretion of at least eight proteins in a TTSS dependent manner during the infection of the legume host. However only two of these proteins have been identified, namely NolX and Y4XL. The NolX protein is predicted to be involved in the translocation process because it has homology to HrpF, which is thought to act as a translocator in *Xanthomonas campestris*. In contrast, the Y4XL protein, however, has no known homologues in the database and is predicted to be a cytoplasmic protein (Viprey et al., 1998).

Plant responses to rhizobial infection

In addition to the many physiological responses to rhizobia (e.g., root hair curling and calcium spiking), it is well characterized that numerous plant genes are transcriptionally activated upon rhizobial inoculation and infection of the legume root. These genes are classified as early or late nodulins based on the time course of their activation. Nodulins were originally thought to function exclusively in the nodulation process. However, it is now known that some of the nodulins are expressed in other plant tissues where the may play additional roles in plant development. Moreover, homologues to nodulins have been found in non-legume plants (Crespi et al., 1994; Cook et al., 1995; Bauer et al., 1996). Despite the possibility of dual roles, nodulins are now broadly classified as plant genes whose expression is markedly enhanced during the stages of nodule development. Examples of early nodulins include *enod2*, *enod12*, and *enod40* (Scheres et al., 1990; Pichon et al., 1992; Kouchi and Hata, 1993) and these are discussed in more detail in later sections that describe Nod signal recognition. Late nodulins include, but are not limited to, leghemoglobin and uricase (Papadopoulou et al., 1995; Heidstra et al., 1997).

Nod signal perception

Morphogenic activity of Nod signals

Interestingly, many of the plant developmental responses to nodulation can be induced by purified Nod signals even in the absence of bacteria. Nod signals are capable of eliciting root hair deformation (HAD) and curling (HAC) (Lerouge et al., 1990; Roche et al., 1991; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; van Brussel et al., 1992; Carlson et al., 1993; Relic et al., 1993; Ardourel et al., 1994; Heidstra et al., 1994; Kurkdjian, 1995; Orgambide et al., 1996) and cortical cell division (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1994; Ardourel et al., 1994; Yang et al., 1994; Bloemberg et al., 1995; Orgambide et al., 1996). Reports also show that external application of Nod signals to some legume roots leads to nodule formation (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans and Peters, 1994; Cardenas et al., 1995; Demont-Caulet et al., 1999). As mentioned previously, Nod signal activity is in some cases dependent on specific chemical decorations to the reducing and non-reducing ends and the structure of the acyl residue as well as the length of the chitooligosaccharide backbone. In some cases relatively minor changes in Nod signal structure led to loss or gain of function. In addition, the stringency for Nod signal structural requirements varies greatly among legume plants.

Furthermore, some plant responses to Nod signals can be uncoupled by their requirements for chemical decorations. *R. leguminosarum* bv. *viciae* Nod signals NodRlv-V (Ac, C18:4) and NodRlv-IV(Ac, C18:4) elicited HAD and nodule meristem formation, while their non-O-acetylated derivative (NodRlv-V(Ac, C corresponding

18:1)) showed only HAD activity (Spaink et al., 1991). Double nodF/nodL mutant of S. meliloti produced Nod signals modified in the acyl moiety and missing the O-acetyl group. These mutants were unable to initiate infection thread formation but were still able to induce root hair branching (Ardourel et al., 1994). The observed differences in Nod signal stringency were crucial in that they support the hypothesis that two types of receptors may be involved in signal perception and transduction (Ardourel et al., 1994). It seemed reasonable that a low stringency signaling receptor exists that activates cellular mechanisms involved in root hair deformation and infection thread initiation. On the other hand, a second receptor with stringent Nod signal structural requirments would be responsible for specific recognition of bacteria. This receptor is hypothesized to work at the surface of root hairs and discriminate for rhizobia producing Nod signals with the correct decorations.

Moreover, many of the morphological responses of the host plant to Nod signals can also be induced by cytokinins and auxin transport inhibitors. Auxin inhibitors (N-(1-naphthyl)-phthalamic acid (NPA) and 2,3,5-triiodobezoic acid (TIBA)) induced nodule-like structures on alfalfa roots (Hirsch et al., 1989). In addition, Nod signals acted similarly to NPA on the expression of the auxin responsive promoter (GH3) isolated from soybean (Hagen et al., 1984). Application of cytokinins (isopenthenyladenine, 6-bezylaminopurine, kinetin), or inhibitors of auxin transport (NPA, TIBA) also induced the formation of pseudonodules on *Macroptilium atropurpureum* roots similar to those induced by Nod signals (Relic et al., 1994). Interestingly, cytokinins are also found to induce the expression of some of the *enod* genes, but unable to have a significant effect on root hair deformation or curling (Relic et al., 1994). These observations support the

hypothesis that Nod signals could modulate the cytokinin/auxin ratio in the plant cells leading to the activation of phytohormone signal transduction pathways through yet unidentified components.

Nod signal binding proteins

A bona fide Nod signal receptor should bind Nod signals and specifically trigger biological responses specific to Nod signals. In addition, since Nod signals can induce biological responses at extremely low concentrations, the corresponding receptor should possess a very high affinity to the Nod signal ligand. Interestingly, none of the Nod signal binding proteins described to date can meet these requirements.

Binding proteins for Nod signals have been demonstrated to be present in microsomal membrane fractions of alfalfa (Bono et al., 1995; Niebel et al., 1997; Gressent et al., 1999) and in the root hairs of the legume Dolichos biflorus (Etzler et al., 1999). In alfalfa, two binding sites, termed Nod Factor Binding Site 1 and 2 (NFBS1 and NFBS2) were identified in root preparations from *Medicago truncatula* and *Medicago varia* cell suspension cultures using tritium labeled Nod signal from *Rhizobium meliloti* (the major Nod signal from *S. meliloti*, NodRm-1(Ac,S, C16:2)) (Bono et al., 1995). NFBS2 was found to have a high affinity (K_d=4nM) to the Nod signal compared to NFBS1 (K_d= 86 nM). Studies demonstrated that binding to NFBS2 was dependent on substitutions to the nod signal backbone, i.e. O-acetylation, N-acylation, and C-4 hydroxylation, but not dependent on sulfation. This finding was significant because sulfation was previously thought to be critical in Nod signal activity in alfalfa.

As previously discussed, there is evidence that several receptors with different stringencies for Nod signal recognition may be involved in signal transduction leading to the nodulation process. Therefore, NFBS1 and NFBS2 seem to be likely candidates for Nod signal receptors. NFBS2 meets several requirement of a specific receptor molecule as the binding site was saturable, reversible, and has a high affinity for the Nod signal ligand (Gressent et al., 1999). However, the indifference of NFBS2 to the sulphate group Nod signal decoration has to be considered, because it was reported to be essential in *Medicago* symbiosis (Roche et al., 1991). NFBS1 also seems not to be the key Nod signal receptor involved in signal transduction. Plant responses induced by very low Nod signal concentrations would certainly require receptor(s) with a much higher affinity to the ligand than that reported for NFBS1 (Bono et al., 1995). Nevertheless, it cannot be excluded that NFBS1 or NFBS2 may be a receptor that works in concert with other Nod signal receptors.

Interesting work from both Stracke et al., 2002 and Endre et al., 2002 implicate a role for a plant receptor-like protein kinase in signal transduction during nodule initiation and establishment of vesicular-arbuscular mycorrhizal infection of *L. japonicus* and *Medicago sativa*, respectively. Both researchers used a map-based cloning approach to identify the affected gene in symbiotic mutants blocked in both processes (Nod-, Myc-). Endre et al. (2002) identified an alfalfa mutant blocked in nodulation. Map-based cloning allowed the isolation of the affected gene led to its identification as a receptor kinase. This gene was termed 'nodulation receptor kinase', NORK. Similarly, Stracke *et al*., 2002 analyzed Lotus SYMRK mutants (Symbiotic Receptor Kinase) and found that interuption of the receptor kinase resulted in a nodulation phenotype blocked in the early

stages of infection. Following inoculation with *M. loti*, SYMRK mutants showed branched or deformed root hairs but never curled. Bacteria were not entrapped in infection pockets and infection threads were never observed, indicating that SYMRK is required for infection thread initiation.

Further, Stracke et al., 2002 showed evidence that SYMRK is required for Nod signal-mediated induction of leghaemoglobin (LB) expression. In wild-type L. japonicus plants, LB expression is detected within hours of Nod signal treatment or inoculation with *M. loti*, but not after inoculation with a NodC- mutant of *M. loti* that is unable to synthesize Nod signals. In contrast, LB induction by Nod signal treatment or inoculation is absent in SYMRK mutants.

The discussed data strongly suggests that SYMRK is a likely candidate as a Nod signal receptor. However, the results are not definitive because to date no one has shown that Nod signals bind to SYMRK. As mentioned, a true Nod signal receptor must have a very high affinity for the Nod signal ligand. In addition, Stracke et al., 2002 showed evidence that Nod signal-dependent signaling leading to HAD is independent of *Lotus* SYMRK. Neither the root hairs on wild-type nor SYMRK plants responded morphologically (ie. HAD) to inoculation with a Nod C - ::Tn5 strain of *M. loti*. Despite the similarity in this case, wild-type and SYMRK plants differ dramatically when inoculated with *M. loti*. On wild-type Lotus, it was observed that only a subset of root hairs within the infection zone show morphological responses to inoculation with *M. loti*. In contrast, virtually all the root hairs on the SYMRK mutants responded to inoculation via root hair deformation (HAD), suggesting that SYMRK is instead involved in negative regulation for root hair cell competence.

Lectins

Lectins, found in both prokaryotes and eukaryotes, share a common feature: they possess at least one non-catalytic domain that can bind reversibly to a specific saccharide (Peumans and Van Damme, 1995). Throughout the plant kingdom, lectins have been investigated for their role in plant development (Hirsch et al., 1995), defense (Sequeira and Graham, 1977; Shibuya et al., 1987; Knibbs et al., 1991; Peumans and Van Damme, 1995), cytokinin interactions (Gregg et al., 1992), and more recently, for their hypothesized role as Nod signal binding proteins (Etzler et al., 1999).

The postulated role for lectins in nodulation has long been controversial. Hamblin and Kent (1973) first provided evidence for a role for a lectin as a determinant for host-specificty in the rhizobium-bean (*Phaseolus vulgaris*) symbiosis. Subsequently, Dazzo and Hubbell (1975) suggested a role for a lectin as a host-specificity determinant in the rhizobium-clover symbiosis. Further work by Schmidt and Bohlool (1981) suggested a role of lectins in nodulation, and thus, provided the foundation for the 'legume-lectin recognition hypothesis'. This hypothesis postulated that a lectin served as a connector for the attachment of rhizobia to the plant root surface. Halverson and Stacey (1985) proposed a different role in the *Bradyrhizobium*-soybean symbiosis. Their work showed that pretreatment of a *B. japonicum* nodulation-deficient mutant with soybean root extracts, containing a galactose-specific lectin, or purified soybean seed lectin could restore the mutant's ability to nodulate soybean. These results suggested that the lectin acted as a signal to modify the nodulation characteristics of *B. japonicum* and may be involved in events prior to bacterial attachment to root hairs.

More recently, Diaz et al. (1989) demonstrated the involvement of a *Pisum sativum* (garden pea) seed lectin (PSL) in determining host-specificity. Transgenic clover plants expressing PSL were shown to be nodulated by *Rhizobium leguminosarun* b.v. *viciae*, the natural symbiont of garden pea, which normally does not nodulate clover. Subsequent site-directed mutagenesis revealed that the carbohydrate-binding domain of the lectin was responsible for the change in host specificity (Kijne et al., 1994; van Eijsden et al., 1995). This was the first report that provided evidence that the host range in rhizobium-legume symbiosis is at least in part determined by rhizobium-plant root lectin interactions. Criticism of this work is that *R. leguminosarum* bv. *viciae* could nodulate wild-type clover roots (i.e. not expressing PSL) at very low levels. Therefore, this work was not unequivocal with regard to the role of lectins in host range determination. A more recent study, van Rhijn et al. (2001), on the same subject showed that expression of PSL in alfalfa plants led to enhanced infection thread formation by *S. meliloti.* In addition, this report showed that the transgenic alfalfa plants could be infected by *R. leguminosarum* bv. *viciae*, a natural symbiont of pea and not alfalfa, and that the sugar-binding activity is critical for this phenomenon. Further, this report demonstrated that *B. japonicum*, a natural symbiont of soybean, could not infect the transgenic alfalfa plants showing that the host extension was specific to the symbiont of pea.

Considerable interest was shown again when van Rhijn et al. (1998) demonstrated that expression of soybean lectin gene, *Le1*, in *Lotus corniculatus* resulted in the ability of *B. japonicum* to nodulate *L. corniculatus*. *B. japonicum* showed no nodulation ability on *L. corniculatus* in the absensce of Le1 expression. The findings of this study are very

significant and differ from that of Diaz et al. (1989), described above, because the phylogenetic difference between *B. japonicum* and *S. loti* is greater than that of the of the two biovars that nodulate pea and clover (*R. leguminosarum* bv *viciae* and bv *trifolii*, respectively). Moreover, the results of van Rhijn et al. (1998) demonstrate that the soybean lectin can change specificity in the plant's response to nod signal. Further, this report demonstrated that (1) Le1 was properly targeted to *L. corniculatus* root hairs, (2) that infection threads were formed within the root hair cells, but rarely penetrated into the next cell layer, (c) that mutating the sugar-binding site of Le1 eliminated infection thread formation.

Significant new evidence has recently emerged through the work of Etzler *et al*. (1999), who described a novel lectin purified from the roots of the legume *Dolichos biflorus*. This lectin, unlike classical seed lectins, is not an agglutin. The *D. biflorus* lectin, termed DB46, was found to bind Nod signals from a variety of rhizobia. The highest binding affinity was observed to Nod signals from *B. japonicum* and *Rhizobium* sp. NGR234, both of which can nodulate the roots of *D. biflorus*. Sequence analysis and comparison suggested that the DB46 was an apyrase (i.e. NTPase) and subsequently Etzler *et al*.(1999) showed that DB46 does have apyrase activity, which is enhanced upon Nod signal binding. Therefore, DB46 was characterized as a lectin-nucleotide phosphohydrolase (LNP). Pre-treatment of roots with anti-DB46 antibodies was sufficient to block nodulation of *D. biflorus*. The results of these experiments provided strong evidence for a role for the DB46 apyrase in nodulation. More specificially, Etzler *et al*. hypothesized that DB46 was involved in Nod signal recognition and subsequent cellular transduction of the signal via enhanced apyrase activity. Finally, Kasi et al.,

2000, localized DB46 to root hair cells through immunocytochemistry and showed that the protein re-localizes to the tips of root hair cells upon Nod signal treatment or rhizobial inoculation. Interestingly, DB46 expression was found to be strongest in young emerging root hairs and found to diminish in mature root hairs, correlating with the ability of root hairs to be infected by rhizobia and thus strongly supporting a role for this lectin in nodulation. In addition, expression of DB46 in root hairs was inhibited by treatment with NO_3- or NH_4, both of which inhibit nodulation.

Apyrases

Apyrases (ATP diphosphohydrolase, EC 3.6.1.5) are enzymes that function to break down nucleotide triphosphates, e.g. ATP, GTP, etc., and can be divided into two distinct classes based on the localization of their catalytic domain. Ecto-apyrases have their catalytic domain localized outside of the cell, while endo-apyrases have their catalytic domain localized inside of the cell (Komoszynski and Wojtczak, 1996). It is hypothesized that apyrases function in diverse activities such as Nod signal binding proteins (Etzler et al., 1999), neurotransmission (Edwards and Gibbs, 1993), blood platelet aggregation (Marcus and Safier, 1993), and protein glycosylation in the lumen of the golgi (Abeijon et al., 1993; Gao et al., 1999). The functional role of endo-apyrases can be best understood by the work of Abeijon et al. (1993) and Gao et al. (1999). Their work used a yeast model to study the role of endo-apyrases in protein glycosylation in the golgi. (Abeijon et al., 1993) demonstrated that mannosylation of *N*- and *O*-linked oligosaccharides in *Saccharomyces cerevisiae* is predominantly regulated by a GDPase (endoapyrase) that converts GDP to GMP. This was demonstrated through the use of a *S.*

cerevisiae Δ*gda1* null mutant. More specifically, this work showed that the mutation in *gda1* caused four very significant phenotypes. First, the mutation caused a block in the elongation of *O*-linked carbohydrate chains in mannoproteins and secreted chitinases. Second, the mutation caused a disruption in the elongation of *N*-linked carbohydrates and carboxypeptidase Y. Third, they observed reduced glycosylation of invertase, and finally, the impaired biosynthesis of mannosylinositophorylceramides. Hence, the results suggest that the yeast apyrase GDA1 is required for GDP dependent mannosylation of a number of membrane proteins needed for cell wall biosynthesis.

To further characterize the role of endo-apyrases in cell wall biosynthesis, Gao et al. (1999) identified and characterized a homologue to GDA1, called YND1. Complementation of the *S. cerevisiae* Δ*gda1* null mutant with YND1 showed that the glycosylation defect of invertase could be restored, suggesting that YND1 function is at least partially redundant to GDA1. Interestingly, when a double mutant was constructed (Δ*ynd1*Δ*gda1*), the phenotype observed was impaired cell growth and germination. Moreover, microscopic analysis showed a severe defect in cell wall integrity further supporting the role for yeast endo-apyrases in cell wall organogenesis.

In animal cells, extracellular ATP is a regulatory molecule. It can bind to purinergic receptors and trigger signaling cascades that lead to diverse responses (Ralevic and Burnstock, 1998). In turn, ectoapyrases, with their active site in the extracellular matrix, play an important role of quenching the signal effects of ATP (Komoszynski and Wojtczak, 1996), which can reach as high as millimolar concentrations in the extracellular matrix of stressed or injured cells (Di Virgilio, 1995). Interestingly, the recent results of Lew and Dearnaley (2000) show that extracellular ATP can induce

changes in the membrane potential of Arabidopsis root hairs, supporting the idea that ATP may serve as a regulatory molecule in plants as well.

In addition to the signaling role, another role for ectoapyrases was suggested by Thomas et al., 2000. This report showed that yeast and plants might use an ATP gradient across the plasma membrane to help drive a symport process to drive the efflux of various compounds across a multidrug resistance transporter called P-glycoprotein, or PGP1. PGP1 is a membrane protein in the family of ATP-binding cassette (ABC) proteins, many of which are involved in export of diverse chemicals from cells (Gros and Hanna, 1996). Thomas et al., 2000 demonstrated that transgenic yeast and arabidopsis that over express PGP1 are more resistant to the toxic drug cyclohexamide. In addition, they found that transgenic over expression of a pea (Pisum sativum) ectoapyrase in yeast and Arabidopsis similarly enhanced xenobiotic resistance. A correlation was found with the ability of the organism to degrade extracellular ATP with its ability to be resistant to toxins. These results were interpreted to mean that ATP hydrolysis by ectoapyrases may play a critical role in PGP1-mediated xenobiotic resistance.

In addition to DB46, mentioned previously, a pea (*Pisum sativum*) apyrase, psNTP9, shows interesting characteristics that suggest it may also play a role in nodulation. It has been studied for a number of years and experiments show that it is calmodulin-stimulated, nuclear-localized, and involved in mediating the phytochrome response (Chen and Roux, 1986; Chen et al., 1987). Although the ability of the pea apyrase to bind Nod signals was not demonstrated, significant to nodulation is the mRNA expression pattern of this apyrase because it is largely governed by factors that can influence nodulation. For example, Hsieh et al. (1996) demonstrated that the pea apyrase

is expressed in root tissues of light-grown plants and in stems and roots of dark-grown plants. In contrast, expression was not found in leaves and stems of light-grown plants. The results of these experiments suggest that the pea apyrase is inhibited by light, which is also known to regulate symbiotic nitrogen fixation.

Further, Hsieh et al. (2000) characterized the pea apyrase and showed that it could be regulated by calmodulin and casein kinase II. Here they demonstrated that the apyrase has a preference for ATP over other nucleoside triphosphates. In addition, they found that calmodulin binds the apyrase indicating regulation by calcium. Moreover, casein kinase II can phosphorylate the apyrase and this phosphorylation is inhibited by calmodulin binding. Simultaneously, Steinbrunner et al.(2000) reported on an *Arabidopsis* apyrase (Atapy1) that is also able to bind calmodulin. To date, these are the only two apyrases shown to contain a functional calmodulin-binding domain.

More recently, Thomas et al. (1999) characterized a the nuclear-localized pea apyrase psNTP9, and showed that it is involved in phosphate transport when phosphate was supplied as inorganic phosphate or as ATP. Transgenic *Arabidopsis thaliana* plants expressing the apyrase showed enhanced growth and phosphate transport under limiting phosphate conditions. This evidence does not support or negate the role of the pea apyrase in nodulation. Instead, they provide evidence of a role for the pea ecto-apyrase in phosphate nutrition.

Another interesting role for ecto-apyrases is presented in work that studies the pea NTPase/apyrase and its role in the plant defense response. It has long been known that plants have the ability to sense invading pathogens, and perhaps the best characterized systems is that of plant-fungal interactions. During these interactions, fungal cells have

the ability to release metabolites such as elicitors and suppressors. These molecules can influence the plant defense response to elicit a plant response (i.e., elicitors) or to suppress a plant response (i.e., suppressors)(Knogge et al., 1996; Hahn et al., 1996). Some of these molecules are generated via the action of plant chitinases as they degrade the invading fungal cell wall. In contrast, others are actively generated and secreted into the germination fluid of conidiospores that carry and spread the fungal infection. The elicitors and suppressors of the pea pathogen *Mycosphaerella pinodes* are very well characterized in their ability to elicit or suppress the pea plant defense response, respectively. However, the first definitive evidences for the mechanism by which the elicitor and suppressor mediate the plant defense response comes from Kiba *et al*. (2003). This work identified a NTPase protein that is present in the pea cell wall and regulated by the fungal suppressor and elicitor. Interestingly, the NTPase co-purified as a peroxidase complex and superoxide generation was regulated by the elicitor and suppressor. This data supports the hypothesis that apyrase activity, modulated by the elicitor and suppressor, could affect superoxide generation. Superoxide generation is a very important aspect of the plant defense response and this subject is discussed in more detail below. Hence, this data provides evidence for the ability of a plant ecto-apyrase to modulate the plant defense response.

Nod signal transduction

G protein coupled receptor

A pharmacological approach in conjunction with transgenic plants harbouring a *Mtenod12*-GUS construct strongly suggested that Nod signal transduction maybe modulated by a heterotrimeric G protein (Pingret *et al.,* 1998). Like Nod signals, G protien agonists mastoporan and Mas7 (Higahijima *et al*., 1988) induced expression of the *Mtenod12-GUS* in transgenic plants. In turn, bacterial pertussis toxin, a G protien antagonist, or the addition of nitrogen to the plant blocked the induction of *Mtenod12-GUS* caused by mastoporan and Nod signal, respectively. Involvement of G proteins would suggest a seven-transmembrane-spanning receptor for the perception of the Nod signal. Until now only a few putative G protien coupled receptors are known in plants (Hooley, 1999).

The role of Ca^{2+} in Nod signal transduction

One of the first changes in plant cells in response to externally applied Nod signals is an increase in intracellular Ca^{2+} concentrations followed by delayed but periodic calcium spikes. The increase in intracellular Ca^{2+} concentrations in *Vigna unguiculata* root hairs by application of Nod signals preceded or occurred in parallel to the depolarization of *Medicago sativa* root hairs (Gehring et al., 1997). The increase in calcium correlated with the ability of the Nod signal to cause root hair deformation (had) in *V. unguiculata.* None of these factors, however, were able to affect Ca^{2+} concentrations in root hairs of non-legumes *Arabidopsis thaliana* or *Zea mays.* Further,

addition of calcium to growing root hairs of *Phaseolus vulgaris* caused calcium influxes and increases in intracellular Ca^{2+} concentrations in root hairs (Cardenas et al., 1999). Therefore, it circumstancial evidence suggests that Ca^{2+} concentrations play a role in Nod signal perception.

CHAPTER 2: THE ROLE OF SALICYLIC ACID IN NODULATION AND ROOT GROWTH IN THE MODEL LEGUME, *LOTUS JAPONICUS.*

Abstract

Salicylic acid (SA) is well established as a signal molecule involved in plant defense. Previous research showed that addition of exogenous SA to plant roots could inhibit indeterminate nodulation, suggesting a possible role of this signal in nodulation. We initially attempted to address this hypothesis by adding exogenous SA to roots of the model legume, *Lotus japonicus*. However, the levels of SA, which affected nodulation, also strongly inhibited growth of the symbiont, *Mesorhizobium loti.* Since endogenous SA is likely the mediator of any physiologically relevant effect on nodulation, we sought to modulate these levels by expression of salicylate hydroxylase, encoded by the bacterial *nahG* gene, in transgenic *Lotus japonicus* plants. Several independent transgenic lines, expressing NahG, were analyzed for their nodulation and root growth phenotype after inoculation with *Mesorhizobium loti*. Expression of the transgene correlated with a significant increase in mean nodule number per plant when compared to wild-type controls. Examination of infection thread formation indicated that the *nahG* plants had enhanced infection as a consequence of an increase in the length of the root infection zone. Interestingly, the *nahG* plants also showed a significant increase in root growth that correlated to the nodulation phenotype. Analysis of plant extracts indicated that SA levels in the *nahG* transgenic plants were significantly reduced, compared to wild-type controls. These data suggest that SA may play a role in suppression of nodulation. However, interpretation of these results is complicated by the concomitant effects on root

growth. The *nahG* transgenic plants should be a valuable resource for further investigating the mechanism of SA action in legumes.

Introduction

Salicylic acid (SA) is a phenolic compound made throughout the plant kingdom via the phenylpropanoid pathway. Research efforts over the past decade have studied this molecule to elucidate its many roles in plant physiology. Interestingly, many reports have demonstrated that SA is a key molecule in the plant defense response. Although the actual mechanism of SA's action is not understood, it is clear that SA is intimately involved in the induction of both the hypersensitive response (HR) and systemic acquired resistance (SAR) (Durner, 1997; Feys et al., 2000). The HR is an early plant defense response characterized by local tissue necrosis around infection sites serving to limit pathogen spread. This response is local in that only plant cells in the immediate vicinity of the invading pathogen undergo the HR and accumulate phenolic compounds, hydrogen peroxide (H_2O_2), and pathogenesis-related (PR) proteins. In addition, other general defenses may be employed during the HR, such as cell wall fortification (lignification) serving to limit pathogen infection and spread. In contrast, SAR is a defense response that is characterized by a global or systemic plant resistance that is not limited only to the site of pathogen invasion. Studies adding exogenous SA to plants suggest that this compound can enhance defense gene induction, the production of H_2O_2, and cell death (Draper, 1997). Moreover, certain *Arabidopsis thaliana* mutants produce elevated levels of SA and show constitutive expression of PR genes and in some cases HR lesion formation even in the absence of pathogen challenge (Shah *et al*, 2001). Conversely,

plants that express the bacterial *nahG* gene, encoding salicylate hydroxylase, are unable to accumulate SA, due to hydrolysis by NahG, and are deficient in the plant defense response (Gaffney *et al*, 1993).

Interestingly, salicylic acid and related derivatives have also been reported to have a pronounced antibacterial effect against *Bacillus cereus, Streptococcus pyogenes*, and *Mycobacterium fortuitum* (Corthout *et al*., 1994). Other reports also show that SA is antibacterial against other bacterial species (Kupferwasser *et al*., 1999; Muroi *et al*., 1993; Awad *et al*., 1991; Hartmann *et al*., 1988; Angelillo *et al*., 1977). In addition to the inherent antibacterial effect, phenolic acids have also been reported to have molluscicidal (Corthout *et al*., 1994) and antifungal activity (Bénigne-Ernest *et al*., 2002).

Plant-interacting microbes differ with respect to the nature of the responses that they elicit in their respective hosts. For example, some plant pathogens elicit a response that is detrimental to one of the two partners. The response is detrimental to the host in a compatible interaction and detrimental to the invading pathogen in an incompatible interaction. In the incompatible interaction, the host plant induces a defense response, either the HR or SAR or both, that limits pathogen invasion and spread. However, in the case of symbiotic bacteria in the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium,* and *Sinorhizobium*, an obvious defense response is usually not elicited. Instead, a beneficial relationship is established that results in nodule formation and atmospheric nitrogen fixation. Both partners benefit from this relationship as the legume plant is provided with a ready source of fixed nitrogen and the bacteria is, in turn, provided with a protected environment and usable carbon sources (Stacey et al., 1995).

Sufficient data suggests that a defense response could be elicited during some rhizobial-plant interactions and this could play an important role in determining host range or regulating nodule formation (Mellor and Collinge, 1995). For example, Vasse et al. (1993) reported that a plant defense response could be involved in the formation of aborted infection threads during normal infection of alfalfa by *Sinorhizobium meliloti.* This was supported by the microscopic observation of localized root cell necrosis and accumulation of phenolic compounds at the site of infection thread arrest. It is well established that only a small percentage of the infection sites that are initiated are successful and it is possible that induction of a defense response could be responsible for limiting successful infection (reviewed in Mellor and Collinge, 1995). In addition, Santos et al. (2001) reported that alfalfa responds to wild-type *S. meliloti* by the transient production of reactive oxygen species, termed the oxidative burst. Alfalfa roots inoculated with rhizobial exopolysaccharide mutants, that are unable to fix nitrogen, appear to exhibit a defense response (Niehaus, 1993). In this case, microscopy revealed a thickening of the cell walls in contact with the EPS mutant rhizobia. Moreover, pea roots inoculated with a lipopolysaccharide (LPS)-defective mutant showed a phenotype reminiscent of the HR, including reduced nodule colonization by the mutant, callose deposition leading to thickened host cell walls, and sporadic host cell death (Perotto et al. 1994). More recently, Martínez-Abarca et al. (1998) showed that SA accumulated in alfalfa roots inoculated with a Nod C mutant of *Sinorhizobium meliloti* unable to synthesize the lipo-chitin Nod signal required for infection. This same report also showed that exogenous addition of SA resulted in both reduced and delayed nodule formation on alfalfa roots inoculated with wild-type *S. meliloti*. Subsequently, Bueno et

al., (2001) showed that increased lipoxygenase activity and H_2O_2 accumulation in alfalfa roots following inoculation with wild-type *S. meliloti* and a Nod C mutant.

Most recently, van Spronsen et al. (2003) reported that exogenous SA addition inhibited indeterminate nodulation (e.g., in vetch, with a persistent meristem) but not determinate nodulation (e.g., in *Lotus japonicus* with no persistent meristem). They correlated this response with the fact that *R. leguminosarum* bv. *viciae* that nodulates vetch produces a lipo-chitin nodulation signal with a 18:4 fatty acid. They postulate that this fatty acid may be active in oxylipin signaling, which is known to be inhibited by SA. Rhizobia that form determinate nodules produce Nod signals lacking polyunsaturated fatty acids and, thus, these signals may act in a different way.

In our current work, we initially set out to repeat the exogenous-SA studies of Martinez-Abarca et al. (1998) and van Spronsen et al. (2003) using the symbiosis between the model legume *Lotus japonicus* and *Mesorhizobium loti*, which results in determinate nodules. However, we found that exogenous SA significantly affected the growth of the bacterial symbiont. Since endogenous SA levels would mediate any affect of SA on nodulation, we opted to modulate these levels by construction of transgenic plants expressing salicylate hydroxylase, encoded by the bacterial *nahG* gene. Decreased SA levels in the *nahG* plants correlated with a significant increase in mean nodule number when compared to wild-type controls. However, other pleotropic effects were also noted. For example, *nahG* plants also showed significantly greater root growth.

Materials and Methods

Chemicals and enzymes

Na-^{32}Phosphate was purchased from ICN (Costa Mesa, CA). Protease inhibitor phenylmethylsulfonyl flouride (PMSF) was obtained from Sigma Chemical Company (St. Louis, MO). Unless specified, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA). Most enzymes in this study were purchased from Promega (Madison, WI) or Fisher Scientific (Pittsburgh, PA).Bacterial strains and plasmids Bacterial strains, plasmids, and plant material are listed in Table 2-1.

Bacterial culture media and growth conditions

Escherichia coli strains were grown and maintained on LB medium (Sambrook *et al.,* 1989). The antibiotics used for plasmid maintenance and selection in *E. coli* is 200μg/ml ampicillin. *Bradyrhizobium japonicum* USDA 110 was grown and maintained on RDY medium (SO *et al.,* 1987) at 30°C. Minimal medium (MM) (Bergersen, 1961) was used for growth of *B. japonicum* USDA 110 in nodulation assays. Minimal medium consists of 0.3 g/L KH_2PO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 1 mL of 1000X trace elements solution, 0.5 g/L NH_4NO_3, 0.4% glycerol, 0.0002 g/L biotin, 0.0001 g/L thiamine, and0.0001 g/L calcium pantothenate, pH 7.0. *Mesorhizobium loti* NZP2235 was cultivated on yeast mannitol broth (YMB) medium (Handberg et al. 1992) at 30°C. The *Agrobacterium* strain was grown at 30°C on yeast extract peptone (YEP) medium (Vervliet et al., 1975). Antibiotic concentrations were 2 μg/ml tetracycline and 100 μg/ml carbenicillin for *M. loti* NZP2235 and *A. tumefaciens* LBA4404, respectively.

Table 2-1. Bacterial strains, plasmids and plant material used in this study.

Strain, plasmid, or plant material	Relevant Characteristics	Source or Reference
Escherichia coli JM109	*EndA1, recA1, gyrA96, thi, hsdR17,* (r_k^-, m_k^+)*, relA1, supE44, Δ(lac-proAB), F', traD36, proAB,* $lacI^qZ\Delta M15$	Messing, 1983
Mesorhizobium loti NZP2235	*HemA-lacZ* fusion, Tc^r	Schauser, 1998
Agrobacterium tumefaciens LBA4404	Cb^r	Stiller, 1997
PCR2.1	Ap^r, Km^r, T7 promotor	Invitrogen, San Diego, CA
Lotus japonicus ecotype "Gifu"		Stiller, 1997

Growth assays to determine SA's effect on *M. loti* was performed as follows: 4 ml *M. loti* cultures were grown in YMB (see above) for 2 days. Equal aliquots of this culture was then used to inoculate 200 ml of YMB (rich media) or MM (minimal media) (see above). A 1 mM stock of salicylic acid sodium salt (Sigma, CA) was used to add the various concentrations of SA to the medium at the time of inoculation. Immediately after subculture, the initial absorbance ($O.D._{600}$) of the culture was read and recorded as time zero. The absorbance was then read every 24 hours to monitor culture growth.

Construction of NahG transgenic Lotus japonicus

The *nahG* construct was kindly provided by Dr. Kay Lawton (Syngenta Biotechnology Inc., Research Triangle Park, NC). Construction of the plant binary vector pCIB200 expressing *nahG* from the cauliflower mosaic virus *35s* promoter was described previously (Gaffney *et al*., 1993). This construct was used to transform *Lotus japonicus* ecotype "Gifu" via *Agrobacterium*-mediated hypocotyl transformation as described in Stiller et al. (1997).

Seed sterilization and germination

Wild-type and *nahG Lotus* seeds were first scarified by rubbing briefly between two sheets of fine 150 grain sandpaper. This was done until the seed coat was visibly roughened. The scarified seeds were then soaked in 3% hydrogen peroxide (H_2O_2), 95% ethanol (EtOH) for fifteen minutes with shaking at room temperature. The seeds were germinated in square petri dishes on a 0.5 cm stack of sterile Whatman #1 filter paper soaked in sterile, distilled, de-ionized water. The petri dishes were sealed with parafilm

and placed in a Percival model CU-32L (Percival Scientific, Boone, IA) incubator for one week with a light cycle of eight hours, 22°C day and 16 hour, 20°C night. The relative humidity was 70-80%.

Segregation analysis of transgenic NahG Lotus japonicus

One-week-old germinated seedlings were transferred to ½ x B5 (Gamborg's B5 medium; Gamborg, 1970) agar plates containing 5 μg/ml G418 (Sigma, CA) for segregation analysis. The seedlings were allowed to grow on selection medium for 4 weeks before scoring for antibiotic resistance. Antibiotic resistance was based on growth phenotype compared to wild-type plants that are obviously sensitive to the antibiotic. Resistant plants will have enhanced root growth compared to wild-type sensitive plants and in most cases the roots of resistant plants grow in a curled and/or wavy pattern. In addition to an obvious root phenotype, resistant plants have healthy, green leaves, while sensitive plants appear chlorotic.

Infection thread assays

Lotus seeds were sterilized and germinated as described previously. After one week, the germinated seedlings were transferred to sterile Leonard jars containing sterile vermiculite for further growth and nodulation assays. To ensure sterility, all containers, vermiculite, and water and/or nutrient solutions used in these experiments were autoclaved. The plants were watered with B and D nutrient solution as described by Broughton and Dilworth, (1971) and grown for an additional two weeks as described above.

The three-week-old plants were inoculated with 1ml each of a three day *M. loti* (*hemA-lacZ*) culture washed with sterile water and diluted to an $O.D._{600}$ of 0.1. The culture inoculant was manually applied to each plant with a pipette. The plants were allowed to grow for an additional 2 weeks post-inoculation before the roots were harvested, fixed, and stained for visualization of infection threads.

At the time of harvest, the plants were removed from the Leonard jars by flooding gently with water. The roots were further washed gently in water and subsequently detached from the plant using a razor blade. The detached roots were immediately fixed in glutaraldehyde and stained for LacZ (β-galactosidase) expression as described by Boivin *et al*. (1990). Roots were stored in the dark in sterile, distilled, water at 4°C until use.

The roots were measured and photographed using a stereoscope (Olympus SZX12) equipped with a Nikon DXM1200 digital camera. The infection zone was defined as an area on the root showing the most abundant infections (c.f. Calvert *et al*., 1985). The edges of the infection zone were arbitrarily determined as the point where no additional infections were apparent within 3 mm.

Nodulation assays

Lotus seeds were sterilized and germinated as described above. One-week-old seedlings were planted in four-inch plastic pots containing sterile vermiculite and given B and D nutrient solution (Broughton and Dilworth, 1971). After planting, the seedlings were allowed to grow for two additional weeks (as described above) and then inoculated with a *M. loti* culture as described above. The plants were placed back into the growth

chamber and allowed to grow for an additional four weeks (as described above). At four weeks post-inoculation, the plants were harvested and scored for nodule number.

HPLC analysis of plant material (Conducted by Dr. Maria Jose Soto Misfut)

Lotus seeds were scarified by treatment with sulfuric acid for 3 min. After washing with distilled water, the seeds were surface-sterilized by shaking in 0.5% bleach containing 0.02% Tween 20 for 20 minutes. This was followed by several washes with sterile distilled water. Finally, the seeds were spread in petri dishes containing sterile water-soaked filter paper and incubated in the light at 22ºC for 7 days. *Lotus* seedlings were grown in hydroponic culture as described by Olivares et al. (1980).

Leaf and root tissues were collected from 4-week-old wild-type and transgenic *nahG* plants, weighed and frozen in liquid nitrogen. For each sample, 0.1-0.3 g of the frozen tissue was extracted and quantitated for total SA, free and SA β-glucoside, essentially as described previously (Enyedi et al., 1992; Bowling et al., 1994). Ten microliters from a total volume of the final 80 μl or 150 μl of root and leaf methanolic extracts, respectively, was injected into a 5 μm C18 column (4.6 x 150 mm; Varian, Harbor City, CA). SA was separated isocratically with 30 % (v/v) methanol containing 1% (v/v) acetic acid at a flow rate of 1 ml min^{-1}. The temperature of the oven was 40ºC. Salicylic acid was detected with a Varian Prostar fluorescence detector using an excitation wavelength of 313 nm and an emission wavelength of 405 nm.

Nucleic acid isolation and manipulation

Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* and *A. tumefaciens* using the alkaline lysis method described in Sambrook et al. (1989) or by using the Wizard® Plus Miniprep DNA Purification System (Promega, Madison, WI) for automated DNA sequencing. DNA concentrations were determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA).

Isolation of DNA fragments

DNA restriction endonuclease fragments used in cloning were separated by agarose gel electrophoresis, as described by Sambrook et al. (1989). Gel purified fragments were isolated using the DNA Gel Extraction Kit (Qiagen, Valencia, CA).

Genomic DNA isolation

Genomic DNA was isolated from *Lotus japonicus* using the protocol described by Dellaporta et al. (1985). DNA concentrations were determined as described previously in Sambrook et al. (1989).

Isolation of total RNA

Total RNA was isolated from *L. japonicus* plants using the protocol described by Verwoerd et al. (1989). Briefly, plant material (approx. 100 mg) was ground in liquid nitrogen and 500 µl of hot (80ºC) extraction buffer was added (phenol-0.1M LiCl, 100

mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS) (1:1)) and homogenized by vortexing for 30 seconds. Next, 250 µl of chloroform-isoamyl alcohol (24:1) was added and the samples were vortexed again. The samples were centrifuged in a microfuge at 14,000 x g for 10 minutes and 4ºC. The waterphase was removed and mixed with one volume of 4M LiCl. RNA was then precipitated overnight and collected by centrifugation as described above. The resultant pellet was was dissolved in 250 µl nuclease-free water and 0.1 volume of 3M NaOAc, pH 5.2 and 2 volumes ethanol was added. The sample was incubated for 30 minutes at -80ºC and the RNA was collected by centrifugation as described above. Finally, the pellet was washed with 70% ethanol and allowed to air dry for 10 minutes before dissolving in 50 µl of sterile nuclease-free water. RNA quantity and integrity was determined by monitoring the optical density (OD) as 260 nm and by agarose gel electrophoresis, respectively.

DNA sequencing

Automated DNA sequencing was performed by Dr. Neil Quigley (The University of Tennessee, Molecular Biology Resource facility). Plasmid DNA sequencing was performed with the ABI Prism Dye Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer Inc., Foster City, CA). Both strands of two independent clones were sequenced to ensure the fidelity of sequences.

Southern blot analysis

Eight micrograms of genomic DNA from *Lotus japonicus* was digested with *Hind*III overnight at 37ºC, according to the supplier's specifications. Digests were

loaded onto a 0.7% agarose gel and separated overnight at 5v/cm. The DNA was then blotted onto ZetaProbe® Nylon membrane (BioRad Laboratories, Hercules, CA), according to the method of Sambrook et al. (1989). Hybridization and washing were performed according to the manufacturer's protocol. Gene-specific probes for *nahG* were labeled to a specificity of 10^8 cpm/μg DNA using random primer labeling (Promega, Madison, WI).

Results

Exogenous SA inhibits growth of Mesorhizobium loti.

Previously, Martínez-Abarca et al. (1998) reported that exogenously applied SA at a level of 25 μM would inhibit nodulation of alfalfa. Addition of exogenous SA to vetch roots showed a similar response with significant inhibition of nodulation occurring at levels > 5μM (van Spronsen et al., 2003). In contrast to these results, our own results and those of van Spronsen et al. (2003) showed that SA levels up to 100 μM had no apparent effect on nodulation of *Lotus japonicus* roots. However, preliminary results indicated that nodulation was significantly reduced when >100 μM SA was added (data not shown). Since these SA levels were in excess of those used by Martinez-Abarca et al. (1998) and van Spronsen et al. (2003), experiments were performed to test whether the response seen was due to detrimental effects on symbiont growth (see figure 2-1) or due to effects on the plant host. Initial experiments in rich media showed that the addition of SA levels up to 1mM had no detrimental effect on *M. loti* growth (data not shown).

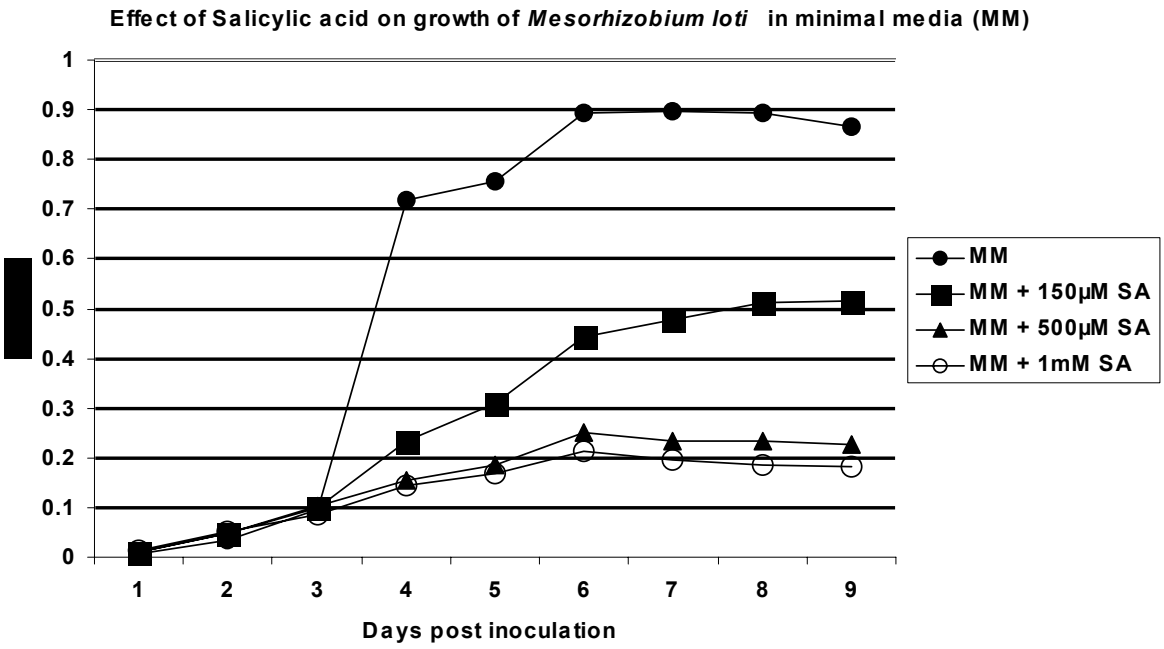


Figure 2-1. Effect of Salicylic acid on growth of *Mesorhizobium loti* in minimal media. The growth of a M*esorhizobium loti* culture in minimal medium was measured ($O.D._{600}$) in the absence of presence of various levels of salicylic acid. Closed circles represent the culture without added SA. Closed squares indicate the culture with 150µM SA. Closed triangles represent the culture with 500µM SA. Finally, the open circles represent the culture containing 1mM SA.

Therefore, we repeated these experiments by adding increasing levels of SA to *M. loti* growing in minimal medium. Figure 2-1 shows that *M. loti* growth (as measured by $O.D._{600}$) was adversely affected at all of the SA concentrations tested. This strong effect of SA addition on bacterial growth made it impossible to use this approach to accurately gauge the possible role of SA in modulating the nodulation response.

Construction of NahG transgenic Lotus japonicus

If SA is playing a role in nodulation, then the important parameter to be measured is not exogenous SA levels but endogenous SA levels. Indeed, both Martinez-Abarca et al. (1998) and Bililou et al., (1999) reported that endogenous SA levels significantly increased in alfalfa and pea roots, respectively, when inoculated with non-compatible rhizobia. However, this response was not found in vetch roots (van Spronsen et al., 2003). In order to modulate endogenous SA levels, transgenic *Lotus japonicus* plants were constructed in which the bacterial *nahG* gene was constitutively expressed from the strong CaMV 35s promoter. The *nahG* gene, originally from *Pseudomonas putida*, encodes salicylate hydroxylase, which catabolizes SA to catechol. Numerous authors have used transgenic expression of NahG to reduce endogenous SA levels. For example, Gaffney *et al*. (1993) showed that transgenic tobacco plants expressing NahG were unable to accumulate SA and failed to mount a plant defense response when challenged with tobacco mosaic virus.

Analysis of nahG copy number in transgenic plants

Transgenic *L. japonicus* plants, expressing *nahG,* were constructed by *Agrobacterium*-mediated hypocotyl transformation using published procedures (Stiller et al., 1997). Primary (T_1) transgenic plants were selfed and segregation of the transgene was analyzed in T_2 and T_3 generation seeds (Table 2-2). Segregation of the transgene was scored based on a comparison of seedling growth on medium with and without kanamycin. Copy number of the transgene (Table 2-2) was determined by Southern blotting and the results are shown in figure 2-2. With the exception of the nodulation assay, only lines confirmed to have one or two copies of the transgene were used exclusively for further analysis.

NahG expressing transgenic plants have increased nodulation

Transgenic plants, expressing NahG, were analyzed for their nodulation phenotype three weeks after inoculation with *M. loti.* Figure 2-3 presents the results from three independent experiments. Lines that are designated with an (a), (b), or (c) have mean nodule numbers that are statistically even, lower, or higher, respectively, than the mean nodule numbers on wild-type controls. Thirteen of the twenty-six lines tested exhibit a significantly higher mean nodule number, ranging from 22% to 78% higher than the wild-type control. It is notable that all of the lines that contained either a single or double copy of the *nahG* transgene, as listed in Table 2-2, were found in the statistical group with greater nodulation than wild type. Many of the lines that statistically fall in the even or lower groups were found to have three or more copies of the transgene. Therefore, we interpret these results to suggest that increasing transgene copy number,

Table 2-2. Segregation analysis of NahG transgenic *L. japonicus*.

Line	Ratio R:S (Resistant: Sensitive)	Segregation	χ^2	Transgene copy number (determined by Southern blot)
WT	0:157			
GI3.8 T_3	83:0	Homozygous		1
GK2.8 T_3	55:0	Homozygous		1
GB8.10 T_3	69:24	2.9:1	0	1
GD9.6 T_3	42:19	3.5:1	7.26E-77	1
GK1.9 T_3	68:0	Homozygous		1
GK6.7 T_3	34:0	Homozygous		2
GI1.1 T_3	32:0	Homozygous		2
GK6.6 T_3	77:0	Homozygous		2
GB3B T_3	53:0	Homozygous		3
GB8.19 T_3	91:0	Homozygous		3
GB3.14 T_3	82:0	Homozygous		3
GB8.9 T_3	29:0	Homozygous		3
GI3.15 T_3	70:0	Homozygous		4
GI3.11 T_3	80:0	Homozygous		4
GH3 T_2	50:0	Homozygous		4
GB3.8 T_3	31:0	Homozygous		3
GH7 T_2	37:0	Homozygous		4
GK6.1 T_3	49:0	Homozygous		2
GK1.13P T_3	73:0	Homozygous		2
GB4.9 T_3	64:1	64:1	0.89	2
GB3.13 T_3	38:0	Homozygous		2

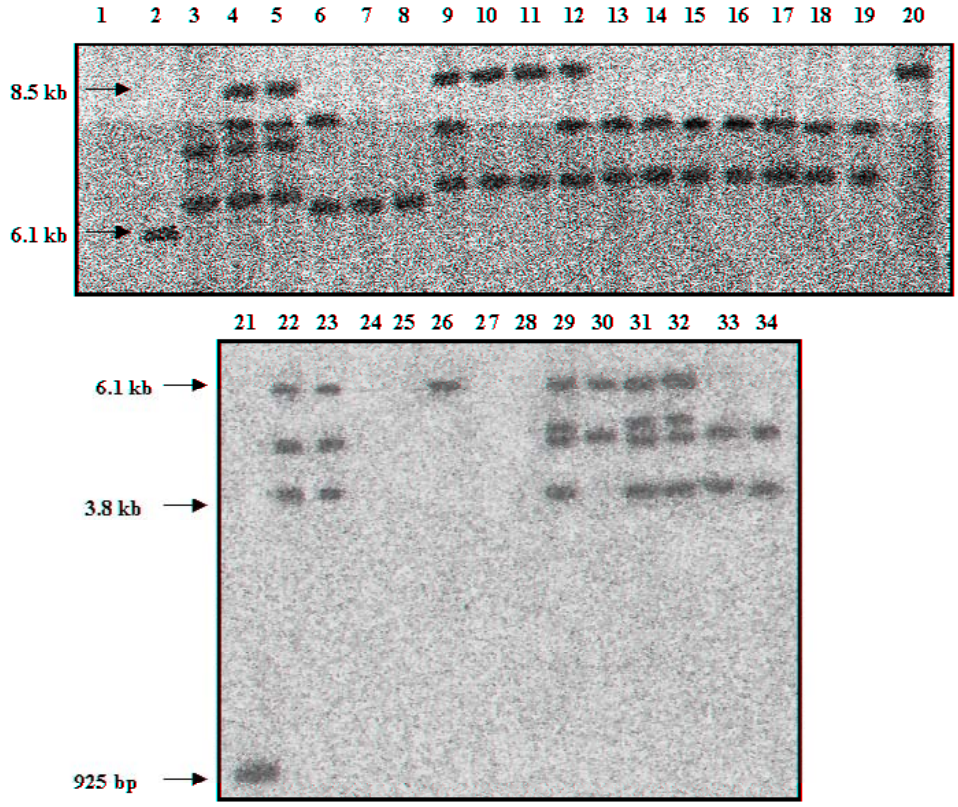


Figure 2-2. Southern blot analysis of *nahG* transgenic *L. japonicus*. Genomic Southern blot on selected transformants of *L. japonicus* lines. In all cases, DNA was digested with *Hind*III. A 925 bp *nahG* PCR product was used as probe and as a positive control. 1. WT; 2. GB8.10 T3; 3. GH4 T2 ; 4. GH6 T2; 5. GH7 T2; 6. GD14 T2; 7. GD9.6 T3; 8. GD4 T2; 9. GB3.8 T3; 10. GB3.13 T3; 11. GB4.9 T3; 12. GB3B T3; 13. GK1.13 T3; 14. GK3.11 T3; 15. GK1.9 T3; 16. GK2.8 T3; 17. GK6.7 T3; 18. GK6.1 T3; 19. GK3.6 T3; 20. GI3.8 T3; 21. 925 bp positive control; 22. GA8.19 T3; 23. GA3.14 T3; 24. GA6.1 T3; 25. GA6.3 T3; 26. GB8.10 T3; 27. GB7.8 T3; 28. GB3.5 T3; 29. GM3 T2; 30. GI1.1 T3; 31. GI3.11 T3; 32. GI3.15 T3; 33. GM3.6 T3; 34. GM6.6 T3. Lines GB8.10 T3, GD9.6 T3, GK2.8 T3, and GI3.8 T3 show single, independent insertions of the *nahG* gene into the plants genome.

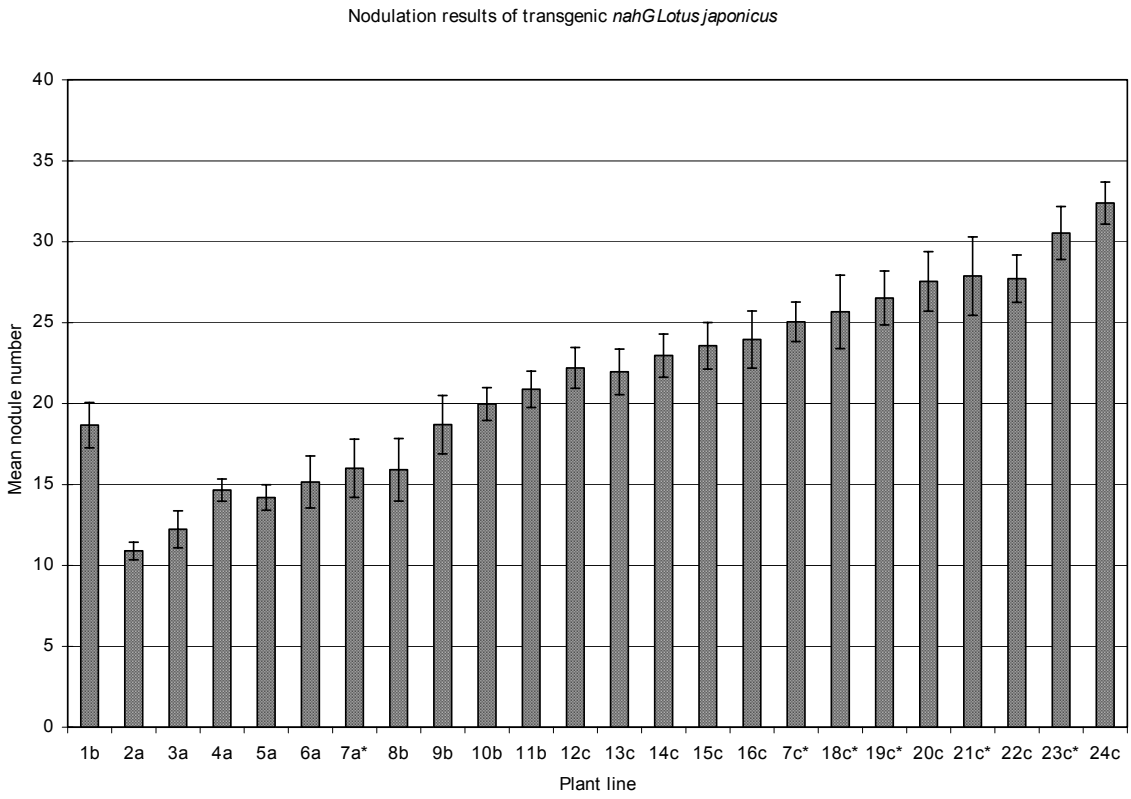


Figure 2-3. Nodulation results of the NahG transgenic *Lotus japonics* plants. Four-week-old plants were inoculated with 1 ml of a *M. loti* culture at an O.D. $_{600}$ of 0.1. The plants were harvested four weeks post inoculation and nodule numbers enumerated. Lines denoted 1a, WT; 2a, GK1.9T_3; 3a, GB8.9P T_3; 4a, GB3.14P T_3; 5a, GB3.13 T_3; 6a, GK6.1 T_3; 7a*, GA6.1 T_3; 8b, GD14 T_2; 9b, GH6 T_2; 10b, GB4.9 T_3; 11b, GI1.1 T_3; 12c, GI3.11 T_3; 13c, GB3B T_3; 14c, GB3.8 T_3; 15c, GI3.15 T_3; 16c, GK6.6 T_3; 17c*, GK2.8 T_3; 18c*, GD9.6 T_3; 19c*, GB8.10 T_3; 20c, GB8.19 T_3; 21c*, GI3.8 T_3; 22c, GK3.6 T_3; 23c*, GK6.7 T_3; 24c, GK1.13T_3. Plant lines marked with a, b, and c are statistically different groups. The lines marked with an (a) have a mean nodule number significantly lower than the lines marked with a b, α=0.05. The lines marked with a (b) have a mean nodule number significantly higher than the lines marked with an (a), but are still significantly lower than the lines marked with a c, α=0.05. And finally, the lines marked with a (c) have a mean nodule number statistically higher than the lines marked with a (b) or (c), α=0.05.

likely due to insertion position, was detrimental to nodulation. This effect may be the result of general detrimental effects on plant metabolism.

NahG transgenics have reduced SA content

NahG expression should correlate with a significant decrease in endogenous SA content. Indeed, as shown in Table 2-3, SA levels, both the aglycone and β-glucoside form, were significantly reduced in root tissue of lines containing either a single or double copy of the transgene. The detection limit of our system is 98 ng/g FW. Therefore, those lines identified in Table 2-3 as ND (not detected) had SA levels <98 ng/g FW. This is in contrast to levels found in wild-type controls, which showed on average 824±124 ng/g FW.Representative chromatograms are shown in Figure 2-4. The HPLC chromatogram from wild-type extracted tissue is shown for comparison. The finding that the NahG lines had undetectable SA levels is consistent with the notion that reduced endogenous SA correlates with higher nodulation.

NahG lines have enhanced infection thread formation

As discussed, previous published reports suggested that a plant defense response could lead to an arrest of infection thread development or growth. If SA is involved in this phenomenon, then one would expect to see an increase in infection thread number or growth in *L. japonicus* plants with reduced levels of SA. Alternatively, the NahG lines might show the same number of total infections, but more of these infections could lead to nodule formation due to the lack of a defense response. To examine these possibilities,

Table 2-3. NahG plants have reduced SA content in Root and Leaf tissues

Line	Transgene copy number	Total SA content (leaves) (ng/g FW±SE)	Total SA content (roots) (ng/g FW±SE)	Nodulation result (Avg. nodule #/ plant ±SD)
WT	0	824 ± 124	386 ± 35	19 ± 6
GI3.8 T_3	1	ND	ND	28 ± 6
GK2.8 T_3	1	ND	ND	25 ± 6
GB8.10 T_3	1	ND	ND	27 ± 7
GD9.6 T_3	1	168 ± 16	ND	24 ± 8
GK6.7 T_3	2	ND	ND	31 ± 8

Four-week-old wild-type and NahG seedlings were frozen in liquid nitrogen. The frozen tissue was extracted in methanol and quantitated for total SA, free and β-glucoside. Extracts were analyzed for their SA content by C18 reverse phase chromatography as described in methods. ND indicates that the SA peak was below our detection limit of 98 ng/g FW, and therefore not detected (ND). These data are averages from four replicates.

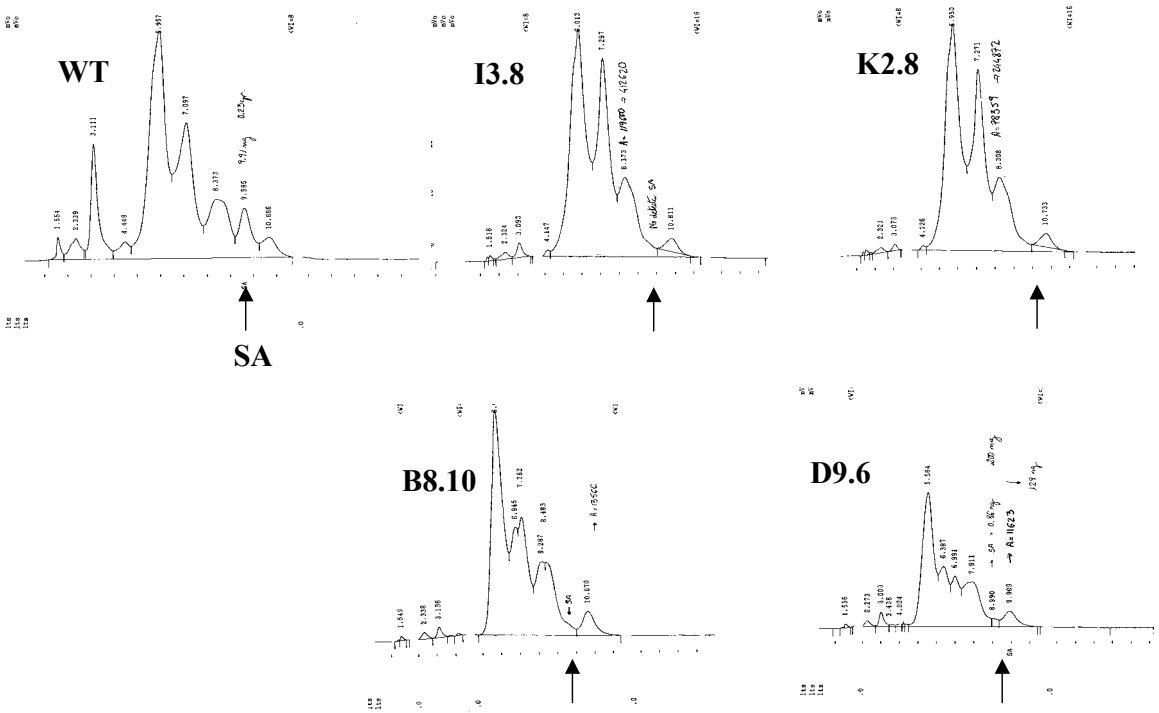


Figure 2-4. Representative chromatograms showing reduced SA content of NahG lines. WT, wild-type HPLC profile showing peak where SA elutes; I3.8, GI3.8 T3 line that has a single insertion; K2.8, GK2.8 T3 line that has a single insertion; B8.10, GB8.10 T3 line that has a single insertion; and D9.6, GD9.6 T3 line that has a single insertion. The X axis represents time and the Y axis represents absorbance using an excitation wavelength of 313 nm and an emission wavelength of 405 nm.

wild-type and *nahG* plants were inoculated with a *M. loti* strain carrying a *hemA-lacZ* fusion (Schauser et al. 1998). Subsequent staining of roots infected with this strain using 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) allowed visualization of the bacterial infection thread. A representative view seen through the microscope and used for scoring of infection thread numbers and growth is shown in Figure 2-5. Infection threads were counted on wild-type and NahG-expressing plants using a stereomicroscope and the results are shown in Table 2-4. When counting total infection threads/plant, the results show that the NahG-expressing plants have a significantly higher number of infection threads than the wild-type. In addition to infection thread numbers, the infection zone (i.e., area above the root tip at the time of inoculation, which is the primary area of infection on the root was measured; c.f. Calvert et al., 1985) of *nahG* transgenic plants was significantly larger. In addition to the size of the infection zone, the number of infection zones found on each plant is significantly increased in the *nahG* lines.

NahG lines have enhanced root growth

Keeping in mind the diverse roles of SA in plant physiology, including its role as a growth regulator (Lian et al., 2000; Gutiérrez-Coronado, 1998), we examined general root growth parameters of the NahG expressing plants. Figure 2-6 shows that representative plants from the NahG lines had significantly increased root size compared to wild-type controls. This phenotype was apparent with plants grown in nitrogen-free medium (allowing nodulation) and with 10 mM nitrate (where nodulation is inhibited). Furthermore, Table 2-3 shows root size data from 3 independent experiments. Compared

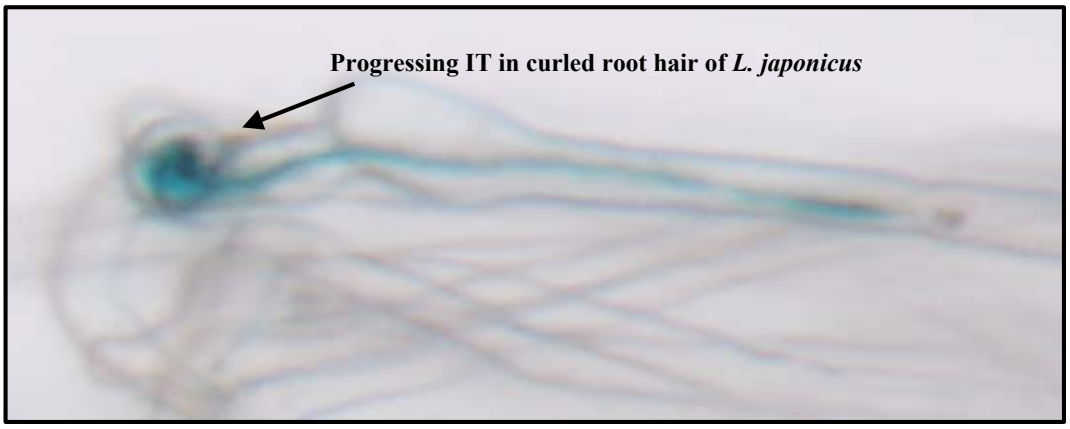


Figure 2-5. Typical bacterial infection thread. Three-week-old wild-type and NahG plants were inoculated with a *M. loti* culture carrying a *hemA-lacZ* fusion. 14 days post inoculation, roots were fixed in glutaraldehyde and stained with x-gal to detect infection events. Infection events were counted using a dissecting light microscope. IT, infection thread.

Table 2-4. NahG lines have enhanced infection thread formation.

Data	WT	GK6.7 T3 (2 copies)	GI3.8 T3 (1 copy)	GK2.8 T3 (1 copy)
Avg. lateral root (cm)	12.1[a]	27.88[b]	21.64[b]	22.7[b]
Avg. tap root (cm)	6.31[a]	12.7[c]	8.4[b]	10.7[c]
Avg. total root (cm)	18.41[a]	40.58[c]	30.04[b]	33.4[b]
Avg. lateral root nodule #	7[a]	10[b]	11[c]	9[b]
Avg. tap root nodule #	1[a]	1[a]	1[a]	1[a]
Avg. total nodule #	8[a]	11[b]	12[c]	10[b]
Avg. lateral root infections	154[a]	263[c]	197[b]	209[b]
Avg. tap root infections	14[a]	12[a]	22[b]	24[b]
Avg. total infections	168[a]	275[c]	219[b]	233[b]
Avg. infection zone length (mm)	6.4[a]	8.8[c]	7.7[b]	7.7[b]
Avg. # infections in infection zone	40.4[a]	66.7[b]	63[b]	67[b]
Avg. # infections/ Avg. infection zone length (mm)	6[a]	8[b]	8[b]	9[b]
Avg. total infections/ avg. total root (cm)	9[b]	7[a]	7[a]	7[a]

Data taken from 10 plants per line. Plants were germinated 7 days and then planted for 14 days before inoculation. Plants were harvested 14 days post inoculation and scored for infection events. The data in groups (a), (b), and (c) are statistically significant at $\alpha=0.05$ level.

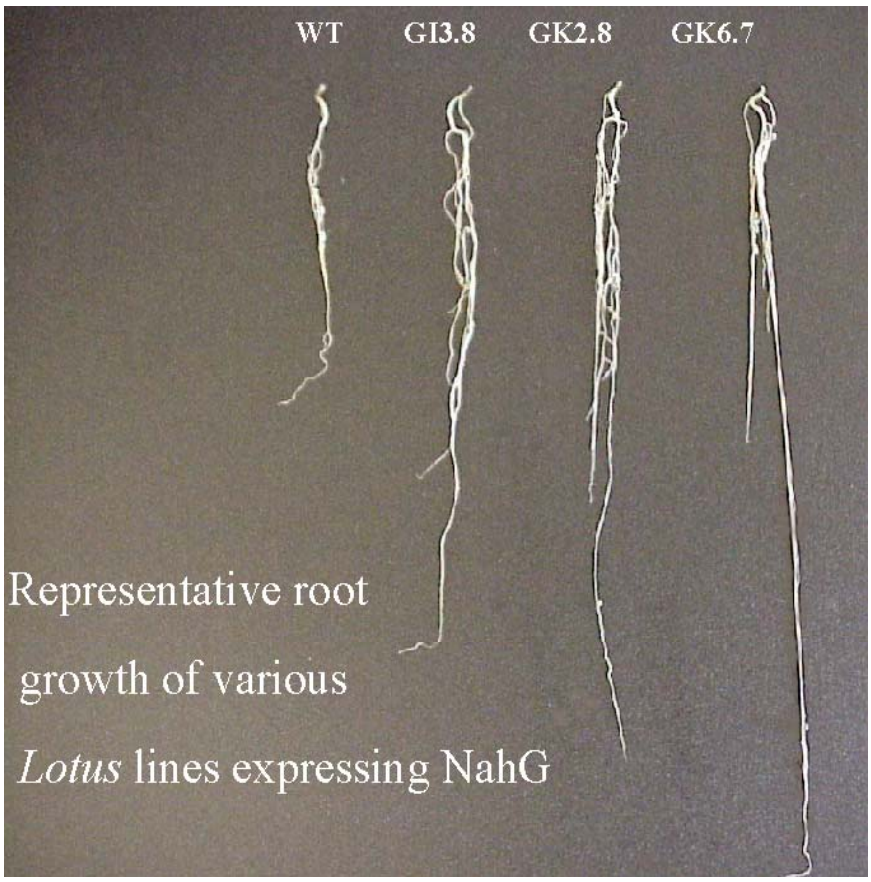


Figure 2-6. NahG lines have enhanced root growth. Five-week-old wild-type and NahG roots were harvested and measured for tap root size, lateral root number, and lateral root size. A. is a representative wild-type root and B-D are representative NahG roots (B. GI3.8, C. GK2.8, and D. GK6.7). Results are representative of three independent experiments.

to controls, the NahG lines had significantly increased tap root and lateral root length, as well as increased lateral root numbers/plant. Relative to root growth, the NahG plants did not have enhanced infection thread formation. Similarly, the increase in root length could also contribute to larger and more abundant infection zones on the NahG expressing plants. However, it is important to remember that only a certain portion of the root can be infected by rhizobia. Therefore, the infection-zone length, and not total root length, is perhaps a better parameter to measure when investigating the role of SA in nodulation. Indeed, the average number of infections per infection zone length is significantly greater on the NahG lines.

NahG plants are not altered in ethylene sensitivity

Ethylene is an important plant hormone that plays numerous roles in plants including regulation of nodulation and abscission. In addition, reports suggest that SA is required for some ethylene-induced processes (Rao et al., 2002). Therefore, we investigated the possibility that the *nahG* plants, with reduced SA levels, could be deficient in ethylene signaling or related pathways. To answer this question we analyzed the triple-response phenotype of the *nahG* plants compared to wild type plants. The triple-response is a widely publicized test for ethylene sensitivity in plants and takes advantage of the pivotal role of ethylene in plant growth and development. Plants that are insensitive to ethylene will have a triple-response phenotype, which includes an elongated, coiled, and crooked hypocotyl, when germinated in the dark and on media containing ACC (1-aminocyclopropane 1-carboxylic acid, an ethylene precursor). Figure 2-7 shows that the *nahG* plants are essentially identical to wild-type plants in their triple-

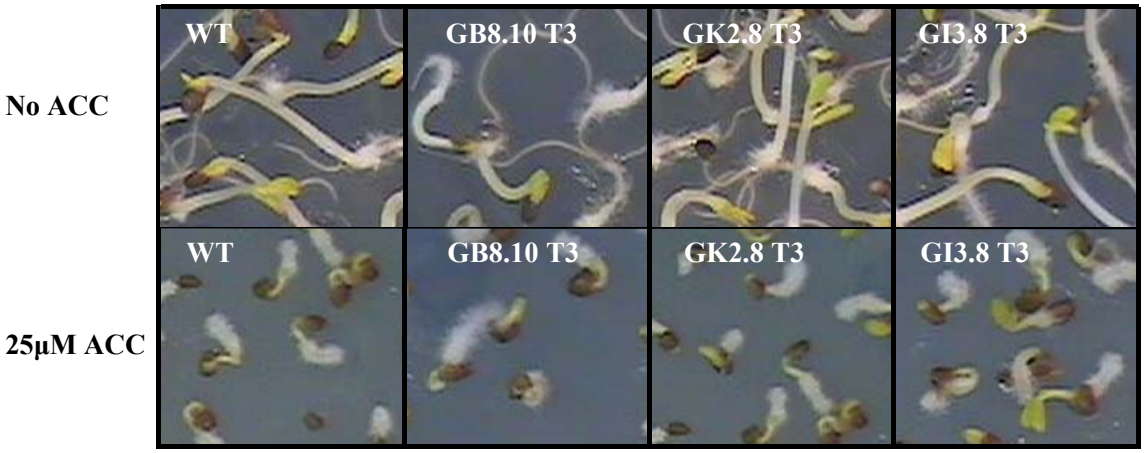


Figure 2-7. Triple response phenotype of wild-type and NahG *L. japonicus*. Seeds of respective plants were sterilized as described previously. Seeds were next germinated in the dark on Gamborg's B5 salt media ± 25 µM ACC (1-aminocyclopropane 1-carboxylic acid, an ethylene precursor) at 22°C and scored for their triple response phenotype one week post imbibition. WT, wild-type; GB8.10 T3, single copy NahG line; GK2.8 T3, single copy NahG line; GI3.8 T3, single copy NahG line.

response phenotype. When germinated in the dark on media containing ACC, neither the *nahG* nor the wild-type plants had elongated hypocotyls, roots, or exaggerated hypocotyls hook; Wopereis *et al*., 2000) suggesting that the nahG plants are not hampered in their ability to produce or sense ethylene.

Discussion

In the *Lotus japonicus*-*M. loti* model system, the results of adding exogenous SA clearly showed a detrimental effect on the bacterial symbionts. All SA levels tested inhibited bacterial growth in minimal medium in a dose-dependent manner. Therefore, in this system, addition of exogenous SA could not be used to examine the role of this signal molecule in nodulation. In order to modulate endogenous SA levels, transgenic *Lotus japonicus* plants expressing NahG were constructed.

Consistent with the postulated role of SA signaling in limiting nodulation, 13 out of 26 NahG expressing lines showed a significant increase in nodulation compared to wild-type controls. Since the majority of the remaining lines had three or more transgene insertions, we interpret their lack of nodulation response to the detrimental effects of multiple T-DNA insertions. Consistent with this theory, all of the single copy lines showed significantly higher nodulation. As expected, these NahG transgenic plants showed significantly lower levels of endogenous SA. The increased nodulation in the NahG expressing plants could be due to a larger number of infection events and/or to an increase in the number of successful infections. To examine these possibilities, we visualized infection by X-gal staining of roots infected with a *M. loti* strain expressing a *hemA*-*lacZ* fusion. These data showed that the *nahG* lines had roughly the same number

of successful vs. aborted infection threads. However, the total number of infection events on the NahG plants was significantly higher than on wild-type plants. These results support the hypothesis that a SA-mediated defense response may be involved in the plant's ability to autoregulate nodulation at the step of infection thread formation. Importantly, the root infection zone was also significantly increased in the NahG lines, suggesting that SA might play multiple roles in controlling nodulation. The increased infection zone in the NahG plants suggests that the presence of SA in *Lotus* can reduce the potential of the root for rhizobial infection.

It is widely accepted that plants have the ability to auto-regulate nodulation and it is clear that the nodulation process can be auto-regulated at different stages of the nodulation process (reviewed by Caetano-Anolles and Gresshoff, 1991). One possibility is that a localized defense response may be involved in limiting nodulation. It has long been known that few root hairs are infected and only a small percentage of those infected result in nodule formation (Dart, 1974). This indicates that the infection process, including infection thread and nodule formation, is a highly regulated step. Cytological studies in the alfalfa-*R. meliloti* symbioses showed that after initial nodule formation, subsequent infections were aborted. The aborted infections were accompanied by a HR-like defense response that included necrosis of infection thread cells and accumulation of phenolics and PR proteins (Vasse et al., 1993). Keeping in mind the importance of SA in the HR, the increased nodulation seen in the NahG expressing transgenic plants is consistent with the hypothesis that a SA-mediated, defense response is involved in limiting nodulation. Microscopy analysis would suggest that this SA-mediated response limits infection thread initiation and not infection thread growth.

Another intriguing possibility is that SA works in concert with ethylene to inhibit nodulation. SA and ethylene were shown to work synergistically in a number of stress-induced plant responses including ozone-induced cell death in *Arabidopsis* (Rao, M. et al., 2002). This report shows that SA and ethylene act in concert to influence ozone-induced cell death and more importantly that SA is required for ethylene production following ozone exposure. Is it possible that the reduced SA content of the NahG lines inhibits ethylene production or modifies ethylene sensitivity; thereby, allowing increased nodulation? It is widely known that ethylene is a key molecule in controlling nodulation. Ethylene production is triggered upon rhizobial inoculation and ethylene-insensitive mutants are hyperinfected by their rhizobial symbionts (Penmetsa and Cook, 2002). Ethylene is known to play a dual role in determining nodule number and positioning on *Medicago* roots. However, in contrast to the present results, this report did not find that the infection zone was enhanced in the ethylene-insensitive mutant, suggesting that ethylene does not play a role in the transience of root susceptibility to rhizobial infection. Rather, the nodulation results show that ethylene-insensitive mutants become more densely nodulated in a defined region of the root system, which is comparable to the region nodulated on wild-type plants.

In order to test the possible involvement of ethylene in the SA-mediated effects on nodulation, *Lotus* plants expressing NahG were grown in the presence of 25 μM ACC (1-aminocyclopropane 1-carboxylic acid, an ethylene precursor) and the triple response (short hypocotyl, short root, and exaggerated hypocotyls hook; Wopereis et al., 2000) measured in relation to the wild type (Figure 2-6). The results of this experiment indicated that the NahG-expressing transgenics responded similarly to the wild type to

higher ethylene levels. Therefore, based on these results, the *nahG* plants would appear to have a normal ethylene response making it unlikely that differing sensitivity to ethylene is responsible for the nodulation effects seen. However, an interesting phenotype was observed in NahG plants. It was noted that abnormal flower abscission occurred in NahG lines and resulted in bent seed pods (data not shown). This result is particularly interesting because of ethylene's widely accepted role in senescence and fruit ripening and suggests that the nahG plants could in fact be deficient in ethylene.

SA is a molecule that has a variety of effects in plant metabolism. For example, SA was shown to affect root and shoot growth in soybean (Lian et al., 2000 and Gutiérrez-Coronado et al., 1998) and abscission in peach and pepper leaves (Ferrarese et al., 1996). Therefore, we also examined the NahG expressing, transgenic *L. japonicus* plants for general effects on plant growth. The *nahG* transgenic *L. japonicus* plants clearly showed increased root growth compared to wild-type plants. If root size is taken into account, then the number of infection events per cm of root length was comparable between the *nahG* transgenic plants and the wild type. More rapid growth by the NahG expressing roots may also explain the larger infection zone found with these plants. Furthermore, the increase in the number of lateral roots on the NahG expressing plants may explain the increased number of infection zones/plant. Therefore, these pleotropic effects of NahG expression on root growth make it difficult to interpret the results relating to nodulation. Similar root growth effects were not observed in experiments with *Medicago* (personal communication, Soto, M.J.) and no mention was made of SA effects on root growth in vetch by van Spronsen et al. (2003). Therefore, available evidence points to the fact that indeterminate nodulating plants (e.g., alfalfa, vetch, and pea)

respond differently to exogenous SA in comparison to determinate nodulating plants (e.g., *Lotus,* common bean, and soybean). It may very well be, as suggested by van Spronsen et al. (2003), that this response reflects the differences in Nod signal structure and function. However, given the myriad effects of SA on plant growth and development, it is wise to be cautious in making stringent conclusions based on the data available. The availability of *Lotus japonicus* plants expressing NahG and the construction of similar transgenics in an indeterminate nodulating host would allow a more direct comparison of the effects of endogenous SA levels on nodulation.

CHAPTER 3: INVESTIGATION OF GS52, A SOYBEAN APYRASE THAT IS CLASSIFIED AS AN EARLY NODULIN.

Abstract

Primers designed to conserved apyrase motifs allowed the isolation of GS52, a 52 kDa protein from soybean, *Glycine soja*. Phylogenetic analysis indicated that *gs52* is an orthologue to a family of apyrases recently demonstrated to play a role in nodulation of leguminous plants. GS52 mRNA was found in roots and flowers, but not in stems, leaves, or cotyledons. Interestingly, inoculation of soybean roots with *Bradyrhizobium japonicum*, symbiont of soybean, resulted in the rapid (<6 h) induction of GS52 mRNA expression. Western blot (immunoblot) analysis did not mirror the results of mRNA analysis. In contrast to the GS52 mRNA analysis, GS52 protein was found in stems. Moreover, anti-GS52 antibody recognized a 50 kDa protein found only in nodule extracts. Treatment of roots with anti-GS52 antibody, but not preimmune serum, blocked nodulation by *B. japonicum*. Fractionation of cellular membranes in sucrose density gradients followed by subsequent Western blot analysis of the fractions revealed that GS52 co-localized with marker enzymes for the plasma membrane. These results demonstrate that GS52 is likely an ectoapyrase localized on the root surface and plays an interesting role in nodulation of soybean.

(Chapter 3 work was largely completed in conjunction with Dr. Robert Bradley Day)

Introduction

Apyrases are divided into two main classes: ectoapyrases and endoapyrases. Ectoapyrases have extracellular catatlytic domains, while endoapyrases have intracellular catalytic domains (Komoszynski and Wojtczak, 1996). Apyrases have been implicated as functioning in very diverse roles; such as neurotransmission (Edwards and Gibb, 1993), blood platelet aggregation (Marcus and Safier, 1993), and protein glycosylation in the golgi lumen (Abeijon et al., 1993; Gao et al., 1999). An interesting role for ectoapyrases in animals is at the synapse of nerve cells (Komoszynski and Wojtczak, 1996; Sarkis and Salto, 1991). In this case, an ectoapyrase degrades extracellular ATP to AMP. By modulating the level of ATP + ADP/ AMP, the apyrase releases the purinoreceptors due to their lower affinity for AMP. In addition, a 5'-nucleotidase enzyme is affected. This is an abundant enzyme, which is inhibited by ATP and activated by AMP, in the synaptic space of nerve cells and functions to convert AMP to adenosine. Adenosine can then penetrate cell membranes where it is phosphorylated and thus restores the cellular ATP pool. In addition, adenosine can stimulate adenyl cyclase, causing an increase in cyclic AMP (cAMP). Thus, apyrase activity can affect the synaptic junction in many ways.

In plants, an apyrase present in pea (*Pisum sativum*) has been studied for numerous years and characterized as a nuclei-localized apyrase that was originally thought to be involved in phytochrome responses (Chen and Roux, 1986; Chen et al., 1987). Subsequently, Hsieh et al. (1996) found that the differential expression pattern of the pea apyrase mRNA indicated expression in root tissues of light grown plants and also

in dark-grown plumules, stems, and roots, but not in leaves and stems of light-grown plants. Further, Thomas et al. (1999) demonstrated through functional analysis that transgenic *Arabidopsis thaliana* expressing the pea apyrase showed enhanced growth and augmented phosphate transport when supplied as inorganic phosphate or as ATP.

Etzler et al. (1999) reported characterization of a unique lectin (DB46) isolated from the roots of the legume *Dolichos Biflorus*. Sequence comparisons indicated that the DB46 lectin was likely an apyrase (i.e. NTPase). Indeed, Etzler *et al*. (1999) showed that DB46 did have ATPase activity that could be stimulated upon ligand binding. Moreover, this report demonstrated that DB46 could bind the lipo-chitin Nod signals produced by symbiotic rhizobia. DB46 had the highest apparent affinity for the major Nod signal produced *B. japonicum* and *Rhizobium* sp. strain NGR234, both natural symbionts of *D. biflorus*. The DB46 lectin was therefore termed a lectin-nucleotide phosphohydrolase (LNP). Immunolocalization studies revealed that DB46 was localized in root hair cells and that DB46 relocalized to the surface of the tips of root hair cells upon inoculation or treatment of the legume root with Nod signals (Kalsi et al., 2000). Moreover, pretreatment of *D. biflorus* roots with with anti-DB46 antibodies inhibited nodulation by its rhizobial symbiont. Taken together, these results strongly support the role of the DB46 apyrase in nodulation. In fact, Etzler et al. (1999) postulated that DB46 could play a role in recognition of the lipo-chitin Nod signal with transduction of the signal mediated via apyrase activity.

Due to their postulated role as Nod signal receptors, we initiated a study of the GS52 apyrase found in soybean (Day et al., 2000). Here, I worked very closely with Dr. R. Bradley Day to characterize the GS52 apyrase. The GS52 cDNA, encoding a 52 kDa

protein that is predicted to have ectoapyrase activity through sequence analysis, was isolated previously from soybean (Day et al., 2000) using a "touch-down"-PCR approach in combination with degenerate primers designed to conserved motifs in apyrase sequences. The mRNA encoding the GS52 cDNA is differentially regulated in soybean tissues. In addition, the GS52 mRNA is rapidly induced in roots inoculated by the soybean symbiont, *B. japonicum*. Antibodies were produced to recombinant GS52 and used to determine the cellular localization of the protein. Moreover, treatment of soybean roots with antibody directed against GS52 inhibited nodulation. These data support the hypothesis that GS52 plays a role in nodulation of soybean.

Materials and methods

Bacterial culture media and growth conditions

All strains of *E. coli* were grown and maintained on Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C. *B. japonicum* USDA 110 was grown and maintained on RDY medium (So et al., 1987) at 30°C. The antibiotics used for plasmid maintenance and selection in *E. coli* were as follows: ampicillin (200 μg/ml), kanamycin (100 μg/ml), and tetracycline (25 μg/ml).

Plant germination and growth conditions

G. soja (PI 468397) seeds were surface-sterilized in 20% hypochlorite for 20 min and then washed six times with sterile, distilled water. Seeds were scarified in concentrated H_2SO_4 for 5 min, followed by six washes with sterile, distilled water. Seeds

were germinated in petri dishes on sterile Whatman paper at 30°C for 2 days in the dark. Following germination, seeds were transferred to sterile plant growth pouches (Mega International, Minneapolis, MN, U.S.A.) and grown in a model CU-32L plant growth chamber (Percival Scientific, Boone, IA, U.S.A.) under a 16 h day/ 8 h night cycle at 28/22°C with 70 to 80% relative humidity.

Isolation of total RNA

Total RNA was isolated from 3- and 5-day-old roots of *G. soja* with Trizol Reagent (Gibco BRL, Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol. RNA quantity and integrity were determined by monitoring absorbance at A_{260} and by agarose gel electrophoresis, respectively.

Northern blot analysis

Total RNA was separated on formaldehyde denaturing gels in 1% agarose and blotted onto ZetaProbe Nylon membrane (Bio-Rad) according to the method of Sambrook et al. (1989). Hybridization and washing was performed as described for the Southern blots. In all cases, gene-specific probes were labeled to a specific activity of 10^8 cpm of DNA per μg with random primer labeling (Promega).

Developmental expression of gs52 mRNA

Root tissue was harvested at time points ranging from 2 days to 9 days postimbibition of *G. soja* seeds. In all *gs52* expression-analysis experiments, the zone of the first emergent root hairs on each plant was marked when plants were transferred to

growth pouches. To standardize the localization of mRNA expression, segments of the roots corresponding to 1 cm above and 1 cm below this mark were used in all expression studies. Total RNA was isolated and reverse transcribed as above.

To examine the tissue-specific expression of *gs52* mRNA, total RNA was isolated from roots, hypocotyls, cotyledons, stems, leaves, flowers, and nodules with Trizol Reagent (Gibco BRL). Total RNA was reverse-transcribed as follows and the following sets of primers were used: *gs52* forward full = 5'-GCCTCTAGAGCATGGTACTTGTGC-3'; gs*52* reverse full = 5'-GCCTCTAGACCCAGCATAAGC-3'. These primers, corresponding to the 5' and 3' full-length ends of *gs52* were used in PCR as follows: 5 µl of cDNA + 10 mM Tris-HCL (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 5 U *Taq* DNA Polymerase (Promega, Madison, WI, U.S.A.). DNA primers were added to a final concentration of 2.5 µM. PCR conditions were as follows: 25 cycles (94°C for 1 min, 55°C for 3 min, and 72°C for 1 min), with 10 min of extension time at 72°C. As an internal control, primers specific for ubiquitin were also included (ubiquitin forward = 5'-GGGTTTTAAGCTCGTTGT-3'; ubiquitin reverse = 5'-GGACACATTGAGTTCAAC-3').

After RT-PCR, PCR products were separated on a 1% agarose gel and blotted onto Zeta Probe nylon membrane (Bio-Rad) according to the manufacturer's specifications. Membranes were hybridized to full-length *gs52* cDNA probes that were ^{32}P-labeled with a random primer labeling kit (Promega).

To examine the expression of *gs52* mRNA in response to inoculation with B. japonicum, plants were grown in plastic growth pouches for 2 days, and then inoculated with 10^6 cells of *B. japonicum* USDA 110 resuspended in half-strength plant nutrient

solution (PNS) (Wacek and Brill 1976). At various time points (3 to 120 h postinoculation), root tissue was excised into liquid nitrogen and frozen at -80°C until further use. Total RNA was isolated and reverse transcribed as described above. PCRs were performed exactly as described above. As an internal control, primers specific for ubiquitin were also included, see above. Quantification of radioactive blots was performed with an Instant Imager Electronic Autoradiograph (Packard Instrument Company, Meriden, CT, U.S.A.).

Generation of antibodies to GS52

Construction of plamids expressing GST-GS52 fusion proteins

GS52 was expressed in *Escherichia coli* from the IPTG-inducible Tac promotor of the expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ). The plasmid used in expressing GS52 was constructed as follows: A *EcoR*I fragment containing *gs52* (1467 bp) was released from pDAY52new (plasmid containing *gs52*) and purified using Qiagen's gene clean kit (Qiagen, Valencia, CA). Orientation of the insert was determined by restriction analysis using *Hind*III digestion. In-frame ligation of *gs52* in pGEX-4T-3 was confirmed by automated DNA sequencing using the pGEX forward primer (Pharmacia Biotech, Uppsala, Sweden).

Protein purification

E. coli GEX52 (containing in-frame GST-GS52 fusion) was grown overnight in 50 ml of LB containing 200 µg/ml ampicillin at 37ºC and 200 rpm. This culture was then

subcultured into 8 liters of LB containing the same concentration of ampicillin and incubated under the same conditions. When an $O.D._{600}$ of 0.4 was reached, 0.25 mM of IPTG (Sigma, St. Louis, MO) was added to the cultures, and induced for 2 hours at 37ºC and 200 rpm. After induction, cells were centrifuged at 5000 rpm (Beckman JA-14 rotor, Beckman Instruments, Fullerton, CA). The cells were re-suspended into 40 ml of TN buffer (150 mM NaCl + 50 mM Tris, pH 8.2), and lysed at room temperature at 2000 p.s.i. using a French pressure cell press (American Instrument Company, Silver Spring, MD). Cells were processed 3 times using the cell press. The lysate was then centrifuged at 4ºC for 10 minutes at 5000 rpm (Beckman JA-14 rotor, Beckman Instruments, Fullerton, CA). The supernatant was transferred to a sterile centrifuge tube, and centrifuged at 15,000 rpm for 15 minutes. The supernatant was stored at -80ºC, and processed as described below. The resultant pellet was solubilized by a series of urea extractions (1M-10M). Extraction of expressed fusion protein was monitored by separation of proteins on a 12% SDS-PAGE gel (Laemmli, 1970), and visualized by silver staining. Urea extracts were dialyzed in TN buffer overnight at 4ºC to remove excess urea, and stored in 5 mL aliquots at -80ºC until further use.

GST-sepharose 4B affinity purification of GS52

Five milliliters of TN-dialyzed protein extract was applied to a 1-mL bed volume of GST-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) packed into a 10 mL disposable column (BioRad Laboratories, Hercules, CA), and allowed to enter the matrix. The column was rocked on a platform shaker overnight at 4ºC to insure mixing of the GST-matrix with the fusion protein. After overnight incubation, the column was washed

with TN buffer until the flow-through eluent had an $O.D._{280}$ of zero. The bound fusion protein was eluted with 12 mM glutathione (pH 8.2), and the protein content of eluted fractions was determined by monitoring the OD at 280 nm. Glycerol was added to the collected fractions to a final concentration of 15%, and fractions were stored at -80ºC until further use.

Immunization and antibody purification

For the first immunization, a solution containing 1.5 mg purified GST-GS52 protein was emulsified with an equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO), and injected subcutaneously at 4 sites into a female New Zealand white rabbit. Booster injections were administered 4 times at two-week intervals with 1.0 mg of the fusion protein emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO). Blood was collected 14 days after each injection. The pre-immune and immune sera were processed (Harlow and Lane, 1989) and stored at -20ºC in 500 µl aliquots.

Purification of antibodies by immuno-affinity chromatography

Absorption of anti-GST antibodies was performed using Immobilized Glutathione S-Transferase (GST) (Pierce, Rockford, IL). Five hundred microliters of immune serum was mixed with an equal volume of binding buffer (0.1M sodium phosphate + 0.15M NaCl, pH 7.2), and loaded onto an immobilized GST column, and allowed to enter the gel. The column was washed with 24 mL of binding buffer, and 1 mL fractions were collected. Eluted serum was further purified using ImmunoPure® Immobilized Protein A (Pierce, Rockford, IL), according to the manufacturer's specifications.

Construction of GST-GS52 affinity columns

Antigen purification of anti-GS52 IgG was performed according to the method of Tan-Wilson *et al.* (1976). Briefly, 0.4 grams of CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) was swollen for 15 minutes in 1mM HCl and washed on a sintered glass filter with the same solution. The gel was then washed with coupling buffer (0.2M $NaHCO_3$ + 0.5M NaCl, pH 8.5), and transferred to a 5 mL glass culture tube. The GST-GS52 fusion protein (1.5 mg) was dissolved in coupling buffer and added to the activated Sepharose 4B matrix and mixed end-over-end overnight at 4ºC. Following overnight incubation, the mixture was transferred to blocking buffer (0.2M glycine, pH 8.0), and again incubated overnight at 4ºC. The mixture was then transferred to a disposable, 10 mL chromatography colunm (BioRad Laboratories, Hercules, CA). Excess absorbed protein was removed by washing with 10 mL of coupling buffer, followed by acetate buffer (0.1M Sodium acetate + 0.5M NaCl, pH 4), followed by coupling buffer. Antigen-affinity purification of anti-GS52 antibodies was performed as follows: 1.5 mg of protein A purified IgG (see above) was dialyzed against TN buffer overnight at 4ºC. The sample was then loaded onto the Sepharose 4B-antigen affinity column constructed above, and washed with 20 mL of TN buffer. Antigen-specific antibodies were eluted with 5mL of elution buffer (100mM NaCl + 50 mM Tris, pH 7.0), and stored in 100µl aliquots at -80ºC.

Western blot analysis

Total protein from *G. soja* and *L. japonicus* tissues was isolated using similar protocols. The only difference was the age or treatment of tissue before harvest. Total

protein was isolated from 5-day-old roots, hypocotyls, cotyledons, stems, and leaves, from 12-week-old flowers, and from root nodules 15 days post-inoculation of *G. soja* plants. Total protein was isolated from roots and nodules 28 days post-inoculation of *L. japonicus* plants. Protein extracts were prepared as follows: 1.0 g of tissue was homogenized in extraction buffer (400 mM sucrose + 10% glycerol + 100 mM Tris (pH 8.0) + 10 mM EDTA + 10 mM KCl + 1 mM PMSF) using a mortar and pestle. Homogenized samples were transferred to sterile Eppendorf tubes and centrifuged in a microfuge at 14,000 rpm for 2 minutes. The resultant supernatant was removed and mixed with ¼ volume 5X Laemmli sample buffer (Laemmli, 1970), and stored at -80ºC until further use. The pellet was mixed with 500µl of 5X Laemmli sample buffer and boiled for 5 minutes. The sample was then centrifuged in a microfuge at 14,000 rpm. The supernatant was transferred to a sterile Eppendorf tube and strored at -80ºC until further use. Protein concentrations were determined using the BCA Protein Assay (Pierce Chemical, Rockford, IL), using bovine serum albumin standards. Total proteins were separated by 12% preparative SDS-PAGE. Proteins were stained with 0.05% Coomassie brilliant blue R-250 (BioRad Laboratories, Hercules, CA). Detection of GS52 was performed by immunoblotting using anti-GS52 antibodies. Separated proteins were electrophoresed onto Immobilon-P membranes (Bio-Rad). Following incubation of the membranes with anti-GS52 antibodies, the blots were incubated with goat anti-rabbit-IgG alkaline phosphatase conjugated antibody. GS52 was then visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) or nitroblue tetrazolium (NBT) as colorimetric substrates. Densitometry readings of GS52 Western blots were obtained with NIH Image Software (National Institutes of Health, Bethesda, MD, U.S.A.).

Fractionation of soybean root membranes on sucrose density gradients

Approximately 200 g (fresh weight) of 5-day-old roots was used as starting material for membrane isolation. Tissue was homogenized in homogenization buffer (0.3 M sucrose, 50 mM 4-morpholineethanesulfonic acid [MES]-Tris [pH 7.6], 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 1 mM dithiothreitol [DTT], 4 mM salicylhydroxamic acid [SHAM], 2 mM PMSF, 2.5 mM $Na_2S_2O_5$, and 0.5% bovine serum albumin [BSA]) on ice for 30 min. After homogenization, cells were lysed at 1,000 lb/in^2 with a French pressure cell press (American Instrument Company). Following lysis, the homogenate was centrifuged at 4,000 x g for 10 min at 4°C. The supernatant was then subjected to an additional centrifugation at 4°C for 10 min at 15,000 x g. The resultant supernatant was then centrifuged at 100,000 x g for 1 h at 4°C in a Ti65 fixed angle rotor (Beckman Instruments). After ultra-centrifugation, the microsomal fraction was resuspended in microsomal fraction buffer (0.25 M sucrose plus 10 mM sodium phosphate, pH 7.8), and homogenized with a hand-held Potter homogenizer.

After isolation of the microsomal membrane fraction, the membranes were resuspended in 40 ml of gradient medium (50 mM 4-morpholinepropanesulfonic acid [MOPS] [pH 7.5] plus 2 mM EDTA plus 5 mM DTT). An aliquot (10ml) of each fraction was layered onto four, 20-ml linear 20-45% (wt/wt) sucrose density gradients in gradient medium for isopycnic centrifugation at 100,000 x g at 4°C for 3 h (SW-28 rotor; Beckman Instruments). Following centrifugation, 1.5-ml fractions were collected (bottom to top) with a Versitaltic Pump Plus peristaltic pump (Manostat, New York, U.S.A.) connected to a model 2110 fraction collector (Bio-Rad). The refractive index

was determined with an Abbe-3L refractometer (Bausch and Lomb, Rochester, NY, U.S.A.), and sucrose concentrations were determined from a standard curve prepared with known sucrose concentrations. Protein concentrations of membrane fractions were determined with the BCA protein assay (Pierce Chemical), with BSA as a standard.

The following marker enzyme assays were used to identify the membranes found in the various sucrose gradient fractions. For the endoplasmic reticulum (ER): localization of ER membrane fractions was confirmed with a monoclonal antibody specific for calnexin (Li *et al*., 1998). Calnexin monoclonal antibody was a gift from Heven Sze (University of Maryland, College Park, U.S.A.). For the Golgi, latent UDPase activity was determined as follows: 50 µg of membrane was added to 50 µl of assay buffer (3 mM UDP plus 3 mM $MnSO_4$ plus 30 mM MES-Tris, pH 6.5), and incubated at 37°C for 20 min. Released phosphate was determined by the malachite green method (described below). For the plasma membrane: vanadate-sensitive ATPase assays were conducted immediately after membrane fractination. 25 µl of membrane was added to 250 µl of ATPase assay buffer (40 mM Tris-MES [pH 6.75] plus 3 mM $MgSO_4$ plus 1 mM Na_2MoO_4 plus 10 mM EGTA plus 0.01% Triton X-100). The final volume of the reaction was adjusted to 450 µl with dH_2O and incubated at 30°C for 3 min. ATP (Tris salt, 3 mM final concentration) was added to the reaction mixture, and the samples were incubated for an additional 30 min at 30°C. The reaction was stopped by the addition of 50 µl of 5 N H_2SO_4. Released phosphate was determined by malachite green method (described below; Baykov et al., 1989). Vanadate- and nitrate-sensitive ATPase activities were calculated as the difference between the activities with and without the respective inhibitor.

The malachite green method of phosphate determination (Baykov et al., 1989) was as follows: to prepare the color reagent, 3 parts 0.045% (wt/vol in water) malachite green/HCl was mixed with 1 part 4.2% (wt/vol in 4 N HCl) NH_4-molybdate. The mixtures were covered with foil and stirred for 30 min and then filtered through two No. 1 Whatman filters into a polyethylene container. To 10 ml of this mixture, 200 µl of 2% (wt/vol) tergitol NP (Pierce Chemical) was added. Released phosphate was determined as follows: 800 µl of the above mixture was added to 50 to 100 µl of ATPase assay. The mixture was incubated at room temperature for 1 min. After incubation, 100 µl of 34% (wt/vol) sodium citrate-$2H_2O$ was added to the reaction, and the mixture was incubated for 1 h at room temperature. Following incubation, the absorbance was read at 630 nm. Phosphate standards were prepared containing 0 to 30 nmol Pi from potassium phosphate (monobasic).

Results

gs52 mRNA is differentially expressed in soybean tissues

The GS52 cDNA was originally achieved with the use of total RNA from root tissue of *G. soja*. However, this method isolated more than one apyrase sequence, and each had a high degree of similarity to the other. Therefore, to determine the expression pattern of gs52 mRNA, a reverse transcription (RT)-PCR approach, with gene specific primers, was utilized. As shown in figure 3-1, gs52 was found to be expressed in soybean roots, hypocotyls, and flowers, but not in cotyledons, stems, or leaves.

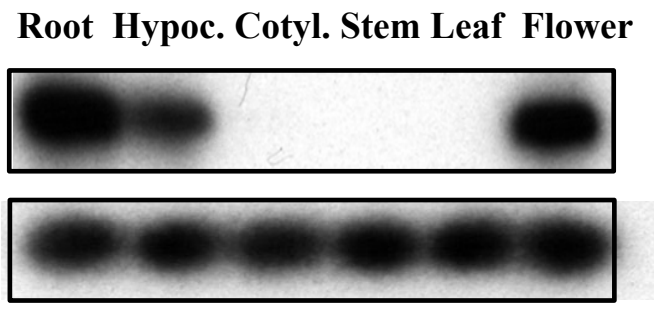


Figure 3-1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *gs52* mRNA in various tissues from *Glycine soja*. Root, root tissue from plants 5 days postimbibition (dpi); hypo., hypocotyls tissue from plants 5 days dpi; cotyl., cotyledon tissue from plants 5 dpi; stem, stem tissue from plants 5 dpi; leaf, leaf tissue from plants 5-7 dpi; and flower, flower tissue from plants 12 weeks post imbibition.

Interestingly, *gs52* mRNA was most abundant in root tissue, and coincided with that previously reported for DB46 from *D. biflorus* (Etzler et al., 1999).

gs52 mRNA is induced in the presence of B. japonicum

To determine whether *gs52* mRNA levels were significantly enhanced upon inoculation with *B. japonicum*, RT-PCR was performed with total RNA from root tissue corresponding to the zone of the developing root that is most susceptible to rhizobial infection (i.e., the zone surrounding the first emergent root hairs) (Bhuvaneswari et al., 1981). As shown in figure 3-2, the developmental expression pattern (-USDA 110) showed maximal expression 3 dpi and by 9dpi, expression had almost completely tapered off. However, in roots inoculated with *B. japonicum* 3 dpi, there was a marked increase in *gs52* mRNA levels within 6 hours postinoculation (Fig. 3-2A and B). The levels of *gs52* mRNA remained high until approximately 96 h postinoculation, when they returned to basal levels. These data clearly characterized *gs52* as an early nodulin gene.

Production and purification of GS52 fusion proteins

To generate large amounts of protein for antibody production, the *tac*-inducible expression vector pGEX-4T-3 was chosen for *Escherichia coli* expression of GS52. The full-length cDNA was cloned into the expression vector pGEX-4T-3 to generate pGEX52. This cloning resulted in translational fusion of *gs52* to the glutathione S-transferase (GST) gene. Cell extracts of *E. coli* harboring pGEX52 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (figure 3-3).

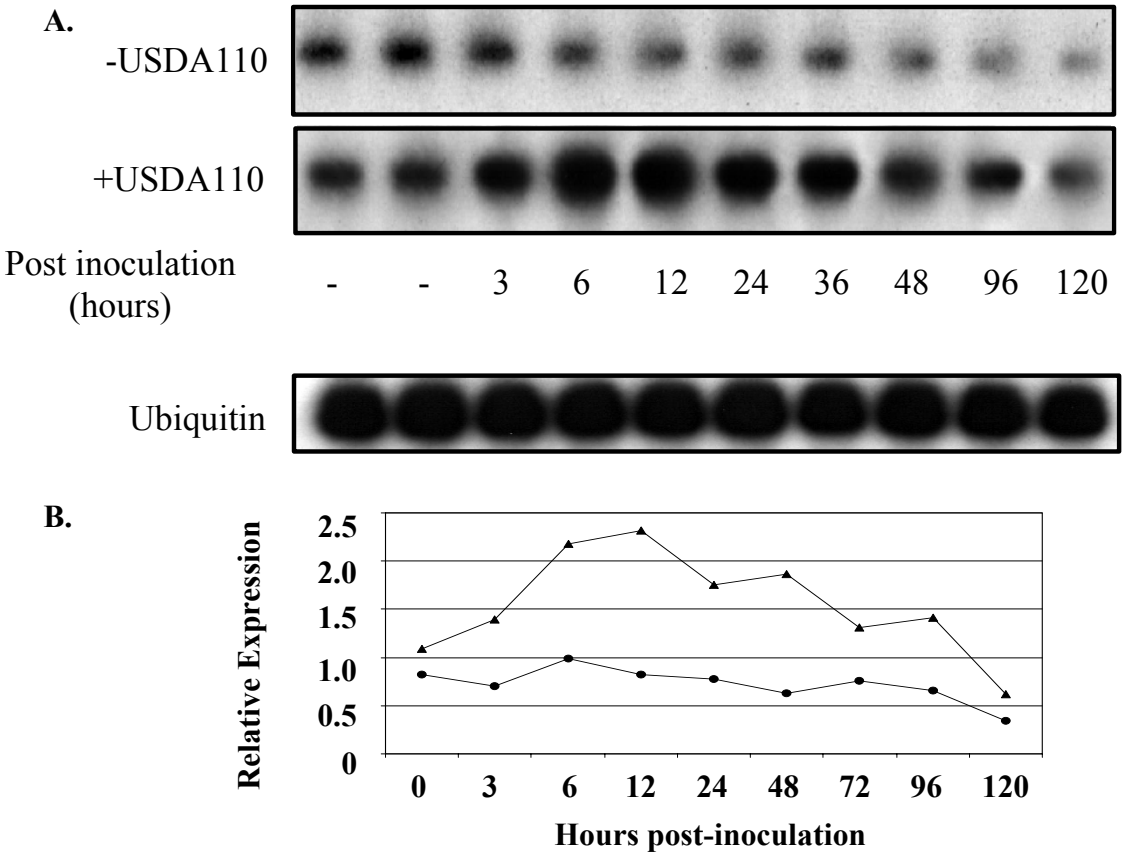


Figure 3-2. Induction of *gs52* in response to rhizobial inoculation. **A.** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *gs52* mRNA levels in *Glycine soja* roots uninoculated (-USDA 110) or inoculated (+USDA 110) with *Bradyrhizobium japonicum* USDA 110. First time point (left): roots from seedlings 2 days postimbibition (dpi). With the +USDA 110 treatment, roots were inoculated at 3 dpi (second time point from left). After this time, roots were harvested as shown (i.e., hours post-inoculation). To control for loading and PCR conditions, an internal control (ubiquitin) was included and is shown at the bottom. **B.** Graphical representation of relative expression of *gs52* as shown in Figure 3-2A. Relative expression was calculated as follows; relative expression = total counts (±USDA 110)/ total counts ubiquitin control. Relative expression of *gs52* in uninoculated roots (-USDA 110; ●) and inoculated roots (+USDA 110; ▲).

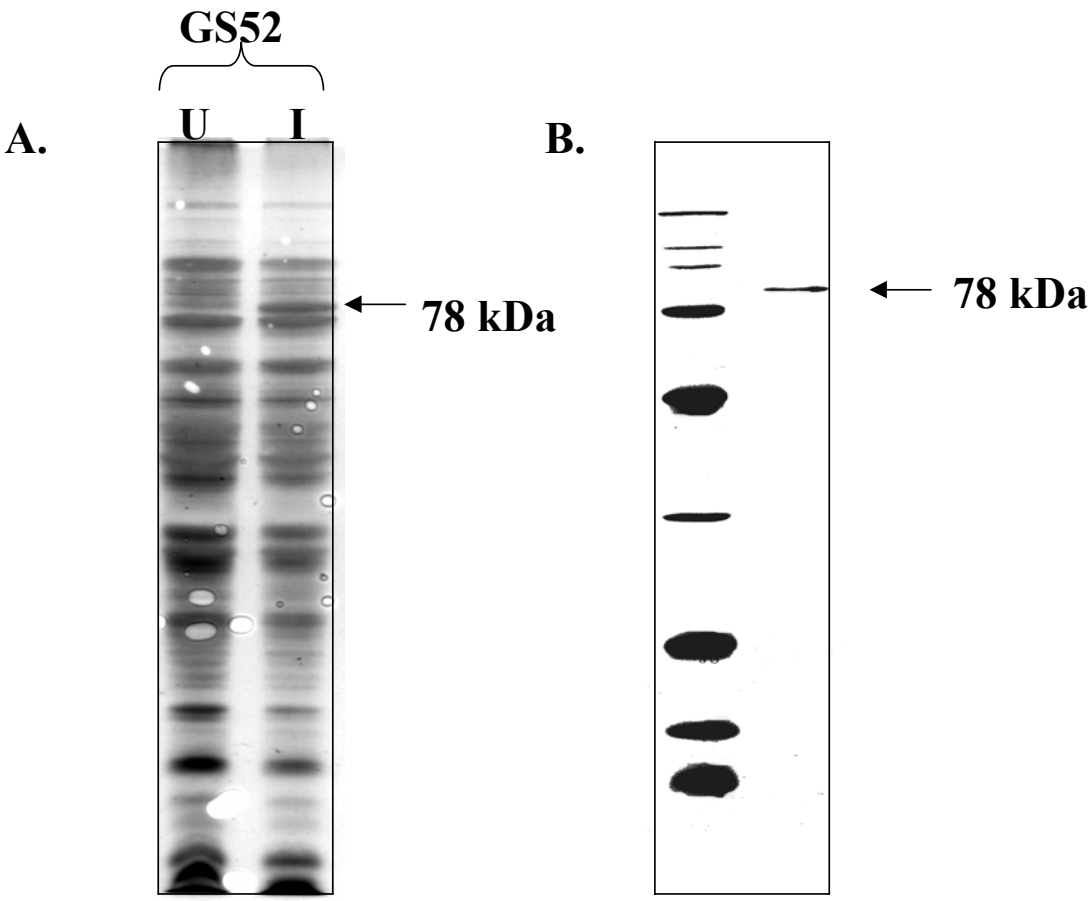


Figure 3-3. Purification of GS52-GST fusion proteins. **A,** Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of GS52 glutathione S-transferase (GST)-fusion proteins expressed in *Escherichia coli*. Log phase cultures of *E. coli* were induced with 500 μM IPTG for 1 h. Total protein was separated on a 12% SDS-PAGE gel. U = Uninduced culture; I = Induced culture. **B.** Purified GST-GS52 fusion proteins. Molecular mass of the protein reflects the apyrase protein plus the 26 kDa GST fusion tag. Protein molecular mass markers are shown on the left.

As shown in figure 3-3A, dominant bands of 78 kDa were observed in the lanes corresponding to the induced fractions (Fig. 3-3A, lanes marked I). This polypeptide band had a molecular mass consistent with that predicted for the GST-GS52 fusion protein (e.g., GST = 26 kDa, GS52 = 52 kDa). In the absence of the IPTG inducer, no fusion protein was detected (Fig. 3-3A, lanes marked U). Immunoblot analysis with anti-GST antibodies confirmed the SDS-PAGE results (data not shown). The majority of the GS52 fusion protein was present in inclusion bodies. Therefore, the protein was solubilized with urea, dialyzed, and affinity purified on a GST-Sepharose 4B column. Figure 3-3B shows the presence of a single band corresponding to the 78 kDa GS52 fusion protein following purification of the protein on GST-Sepharose 4B. Immunoblot analysis with anti-GST antibodies to the eluted proteins confirmed the presence and purity of the eluate. The purified fusion protein was used to immunize rabbits for the generation of anti-GS52 antibodies. After affinity purification, the antibodies produced were shown to specifically recognize only their cognate protein (data not shown).

The GS52 protein is differentially expressed in soybean tissues

The mRNA expression analysis described shows that GS52 has a distinct pattern of localization in *G. soja*. To investigate whether the RT-PCR data was representative of the protein localization of the apyrase, total protein extracts were prepared from various tissues of *G. soja* and were used in immunoblot analysis to more accurately determine protein localization. As shown in Figure 3-4, the tissue localization revealed that the epitope was abundant in root and flowers, similar to the transcript abundance detected with RT-PCR (Figure 3-4). GS52 protein was most abundant in developing roots of

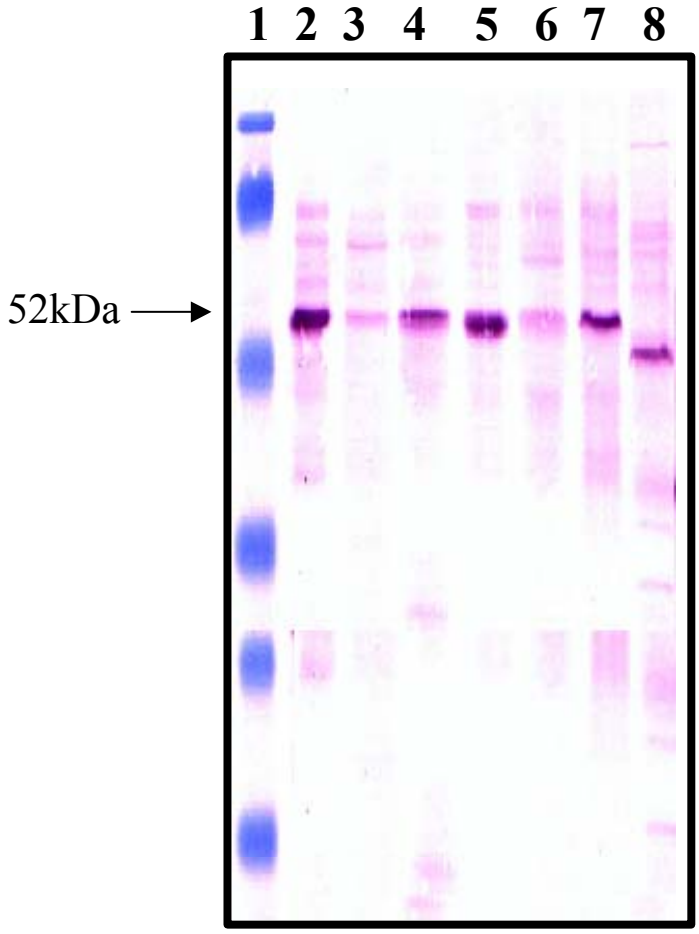


Figure 3-4. Western blot (Immunoblot) analysis of total protein extracts from various tissues and nodules from *Glycine soja*. Immunoblot was performed with antigen-purified anti-GS52 IgG antibody. Lane 1, Broad range molecular weight markers; lane 2, roots (5 days postimbibition(dpi)); lane 3, hypoctoyls (5 dpi); lane 4, cotyledon (5 dpi); lane 5, stem (5dpi); lane 6, leaf (5-7dpi); lane 7, flower (12 weeks post imbibition); and lane 8, nodule (15 days postinoculation).

soybean. However, in contrast to the RT-PCR results, GS52 protein was detected in the cotyledon and stem at significant levels. The failure of RT-PCR to detect *gs52* in these tissues suggests that *gs52* mRNA is rapidly turned over in this tissue, thereby limiting the ability of PCR to detect the mRNA. However, we cannot rule out the possibility of a cross-reacting paralogue present in the cotyledon and stem. Perhaps the most interesting result was the detection of a smaller protein (approximately 50 kDa) in nodule extracts. Considering the specificity of the antibodies used, we favor the hypothesis that this protein may be a posttransitionally modified form of GS52 that is specific to nodule tissues. This idea is supported by the absence of this band in the other samples (e.g., root, stem, and flower). However, we also cannot rule out the possibility that the anti-GS52 antibodies are reacting with a nodule-specific GS52 paralogue.

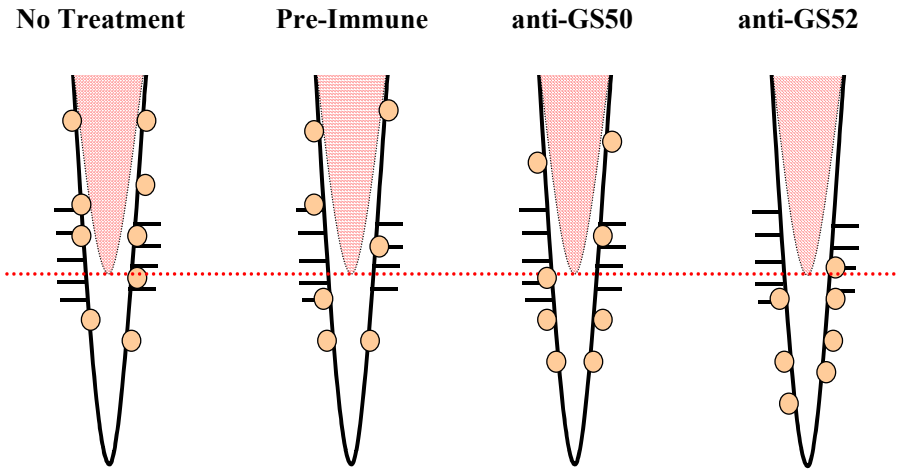
Anti-GS52 antibodies block nodulation of soybean by B. japonicum

The studies of DB46 by Etzler et al. (1999) demonstrated the ability of anti-DB46 antiserum to block nodulation of *D. biflorus* by *B. japonicum* 24A10. These results prompted us to investigate whether anti-GS52 antiserum could affect the ability of *G. soja* to be nodulated by *B. japonicum*, its natural symbiont. To answer this question, 2-day-old seedlings were incubated for 1 h in antiserum raised against GS52. As controls, plants were incubated in the presence of preimmune serum or left untreated. As demonstrated in Figure 3-5, treatment of plants with anti-GS52 antiserum significantly inhibited nodulation by *B. japonicum*.

To answer this question, 2-day-old seedlings were incubated for 1 h in antiserum raised against GS52. As controls, plants were incubated in the presence of preimmune serum or left untreated. As demonstrated in Figure 3-5, treatment of plants with anti-GS52 antiserum significantly inhibited nodulation by *B. japonicum*. This inhibition of nodulation occurred in the region of the root that was exposed to the antiserum (i.e., the region of the root above the root tip at the time of treatment and inoculation = upper zone). Accordingly, control plants (untreated and preimmune serum) did not affect the ability of *G. soja* to the nodulated. In addition, antibodies towards another *G. soja* apyrase, did not affect nodulation (Figure 3-5).

Subcellular localization of GS52 with membrane separation in sucrose density gradients

Numerous reports on plant apyrases indicated that they are likely associated with cellular membranes (e.g., Chen and Roux 1986; Abeijon et al., 1993; Hseih et al., 1996; Etzler et al., 1999). Indeed hydropathy analysis of GS52 suggested the presence of an N-terminal membrane-spanning domain. Accordingly, the membrane localization of GS52 was examined by immunoblot after separation of soybean root membranes by ultracentrifugation on linear 20-45% (wt/wt) sucrose density gradients. As controls, the distribution of three marker enzymes/proteins commonly separated by sucrose gradients are shown in Figure 3-6. The localization of the marker proteins correlated with sucrose density gradients previously published for these plant membrane markers (Leonard and Vanderwoude, 1976; Giannini et al., 1991). Shown in Figure 3-7 is the relative



	n=8	n=16	n=19	n=17
upper zone	6 ± 1	4 ± 1	3 ± 1	1 ± 1
new zone	3 ± 1	3 ± 2	5 ± 3	6 ± 3

Figure 3-5. Effect of anti-GS52 antiserum on nodulation of *Glycine soja* by *Bradyrhizobium japonicum* USDA 110. Hatched area represents root at the time of antibody treatment and inoculation. Black-bounded, unhatched area represents new root growth untreated with antibody. No treatment, control treatment with water; Preimmune, control treatment with preimmune sera; anti-GS50, treated with anti-GS50 antibody; anti-GS52, treated with anti-GS52 antibody. N, number of plants in each treatment; upper zone, number of nodules (± standard error) formed in upper zone of root in each treatment; lower zone, number of nodules (± standard error) formed in lower zone of root in each treatment. Data are from a single experiment. Replicate experiments yielded similar results.

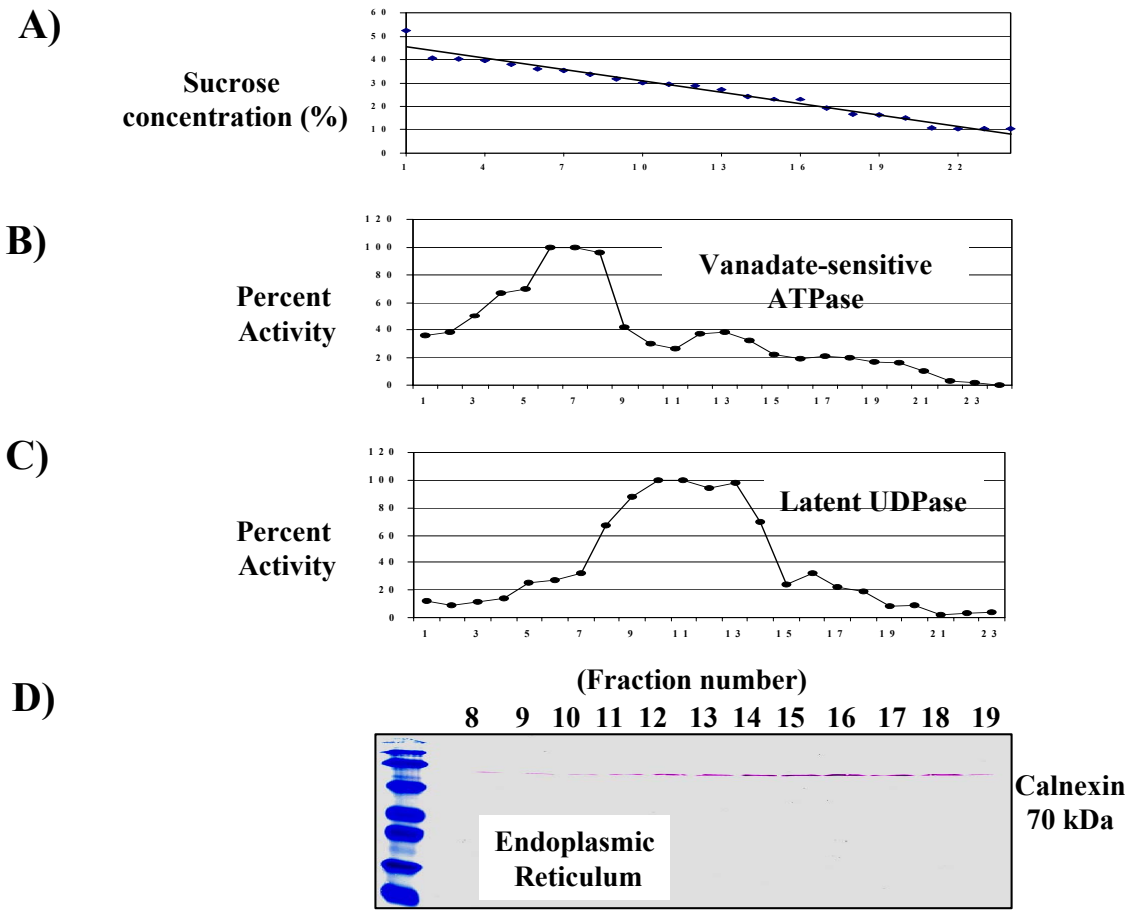


Figure 3-6. Localization of marker protein after sucrose gradient fractionation. **A,** Sucrose concentrations of various gradient fractions based on refractive index. **B,** Plasma membrane marker, vanadate-sensitive ATPase-activity. **C,** Golgi membrane marker, latent UDPase activity. **D,** Endoplasmic reticulum (ER) membrane marker, Western blot (immunoblot) of protein in gradient fractions with monoclonal antibodies to oat calnexin (only selected fractions are shown). Numbers above represent gradient fraction number. Molecular weight markers are shown in the far left lane.

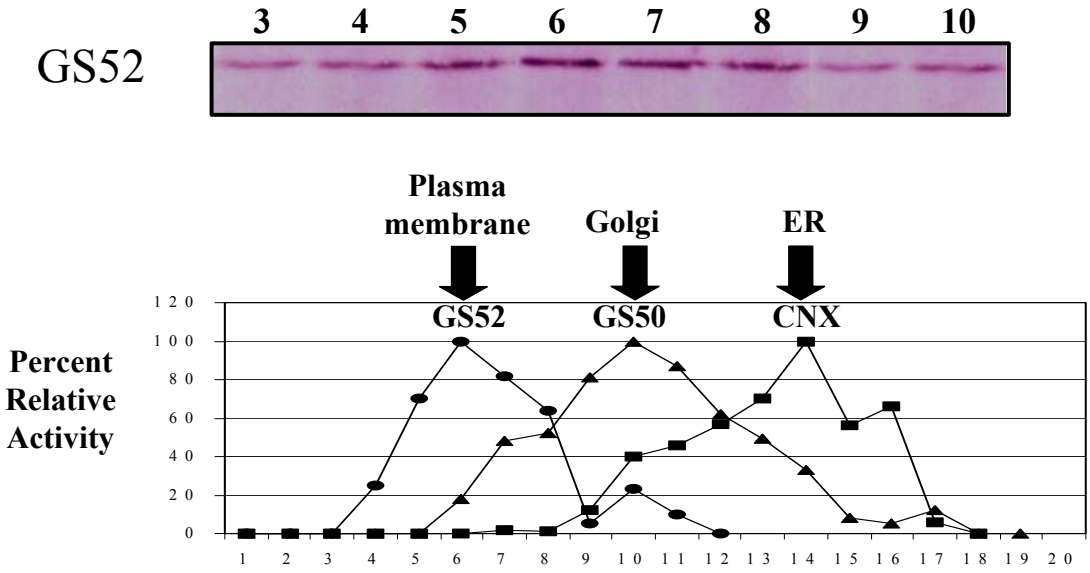


Figure 3-7. Western blot (immunoblot) analysis of sucrose gradient fractions with antigen–purified, anti-GS52 antibody. Numbers above represent gradient fraction number. Only selected fractions are shown. Sucrose gradient localization of GS52 relative to membrane marker proteins. Percent relative activity was determined based on densitometry analysis of Western blots of protein in sucrose gradient fractions (e.g., Figure 3-6). Peak fractions containing plasma membrane marker (vanadate sensitive ATPase), Golgi membrane marker (latent UDPase), and ER membrane marker (calnexin), as determined in figure 3-6, are shown above by arrows. ■ = Densitometry scan of calnexin Western blot, ● = Densitometry scan of anti-GS52 Western blot, ▲ = Densitometry scan of anti-GS50 Western blot.

density gradients previously published for these plant membrane markers (Leonard and Vanderwoude, 1976; Giannini et al., 1991). Shown in Figure 3-7 is the relative distribution of GS52 in comparison to the corresponding sucrose density fractions characterized in figure 3-6. GS52 localized to membrane fractions corresponding to the peak of H^+-ATPase activity, a marker enzyme for the plant plasma membrane (i.e., ATPase activity peaked in fraction 6 (Figure 3-6), as did GS52 Western localization (Figure 3-7).

Discussion

In contrast to animals, very little is known about plant receptors. Therefore, identification of a true Nod signal receptor would be a great advance in plant biology and allow further studies to characterize the cellular signaling mechanism that leads to nodule organogenesis. Identification of DB46, a unique apyrase isolated from the roots of *D. biflorus*, with the ability to specifically bind the lipo-chitin Nod signals produced by a variety of rhizobia (Etzler et al., 1999), was a very significant finding. Legume lectins have also long been implicated in the nodulation process, but to date, a precise role for these proteins has not been defined. In an effort to further characterize the role of soybean apyrases in nodulation, we characterized the GS52 soybean apyrase. We chose G. soja for our investigation because this plant was previously shown to respond well to Nod signal addition(Sanjuan et al., 1992; Stacey et al., 1994). Considering the orthology to the unique lectin nucleotide phosphohydrolase (i.e., DB46) we predict that GS52 plays a very similar biological role in the plant, which could include a role in Nod signal recognition.

g*s52* mRNA showed differential expression in various soybean tissues. g*s52* mRNA was found to be maximally expressed in the roots of soybean, with a reduced expression in the hypocotyl. Further, *gs52* mRNA is also found abundantly in flowers. This result is expected as Roberts et al. (1999) recently reported that DB46 LNP was also abundant in flowers of *D. biflorus*. We specifically chose to further examine *gs52* mRNA expression in root due to the possible role of apyrases in nodulation. In uninoculated roots, *gs52* mRNA levels accumulated to maximal levels within 3 dpi. By 9 dpi, expression of *gs52* mRNA was decreased significantly. The expression of the GS52 apyrase appeared to parrallel the susceptibility of the developing root to rhizobial infection. This hypothesis is supported by reports demonstrating that the differentiating epidermal cells lying just behind the growing root tip are the region of the root most susceptible to rhizobial infection (reviewed in Caetano-Anollés and Gresshoff, 1991). In addition to analyzing the mRNA expression pattern, the protein expression pattern was also analyzed using antibodies raised against GS52 protein.

Numerous plant genes responsive to rhizbial inoculation have been characterized for their temporal and spatial expression patterns during the infection and nodulation process. The early nodulins are among the best characterized of these host-responsive genes. For example, Minami et al.(1996) showed the rapid accumulation (within 48 h) of *enod40* mRNA upon inoculation of soybean roots with *B. japonicum* cells or treatment with Nod signal. In this study, RT-PCR analysis was used to determine if *gs52* mRNA levels would increase upon inoculation of soybean with *B. japonicum*. Interestingly, *gs52* mRNA rapidly increased within 3 h postinoculation, with sustained increased expression detectable beyond 120 h postinoculation. It may seem counterintuitive that

gs52 is rapidly induced by Nod signal or inoculation if it is in fact a bona fide Nod signal receptor. However, the induction of *gs52* is consistent with a number of reports from various biological systems demonstrating agonist-induced expression of membrane-localized receptors (Ng et al., 1997; Perera et al., 1999). Indeed, the described expression data on *gs52* justifies its classification as an early nodulin, and further supports the possibility that this protein is involved in nodulation.

Interestingly, the *gs52* mRNA expression pattern did not directly match the protein expression pattern. Using antibodies to GS52, we examined the protein expression pattern in various tissues of *G. soja.* In general, the mRNA expression pattern did match the protein expression pattern. However, Western blot analysis clearly showed expression of GS52 in 5-day-old cotyledons from *G. soja*, while *gs52* mRNA was not detected in cotyledons by RT-PCR. One possible explanation for this is that gs52 mRNA has a high turnover rate in this tissue, and therefore RT-PCR failed to detect a signal. An additional possibility is that the anti-GS52 antibodies are detecting an additional apyrase paralogue present in the cotyledon of soybean.

Another interesting observation was the reactivity of anti-GS52 antibodies with a protein of a smaller molecular mass (e.g., 50 kDa) in nodule extracts. One reason for this may be that the GS52 protein is postranslationally modified (e.g., cleaved signal sequence) in nodule tissues. The potato apyrase exists in both soluble and insoluble states that differ in their catalytic properties, isoelectric points, and molecular weights (Handa and Guidotti, 1996). Likewise, reports suggest that both the pea apyrase and DB46 (LNP) exist in both an insoluble and soluble state (Chen and Roux, 1986, Etzler et al., 1999). Interestingly, the smaller form of GS52 was only detected in nodule extracts,

indicating that this form of the apyrase is specific to nodulation. An alternate explanation for this observation is that the anti-GS52 antibody is cross-reacting with an unidentified paralogue to GS52. However, we do not favor this hypothesis because this cross-reacting band is present only in nodule extracts, suggesting that the paralogue is not present in any other soybean tissue. Indeed, even if it is not the favored hypothesis, we cannot rule out this idea because data suggests that multiple (e.g., = 5) apyrase proteins are present in *Medicago truncatula* (Cohn et al., 2000).

The report from Etzler et al. (1999) that antibodies to DB46 (LNP) had the ability to block nodulation of *D. biflorus* by *B. japonicum* led to the hypothesis that anti-GS52 antibody might block nodulation of soybean. Indeed, treatment of 2-day-old soybean roots with anti-GS52 antiserum resulted in a marked decrease in nodule formation. As expected, control plants (e.g., no treatment or treatment with preimmune sera) showed normal nodulation patterns. The inhibition of nodulation observed by pretreatment of soybean roots with anti-GS52 suggests that GS52 plays a critical role in soybean nodulation. These data, together with mRNA induction data, suggests that GS52 has a role in the early stages of the infection or nodulation process.

To date, the subcellular localization of numerous apyrases has been characterized. Two apyrases in yeast, GDA1 and YND1, have been identified as Golgi-localized apyrases (Abeijon et al., 1993; Gao et al., 1999). In contrast, the pea (*Pisum sativum*) apyrase is nuclear-localized. Pertinent to nodulation is the observation that DB46 (LNP), the Nod signal-binding protein, was immunolocalized to the surface of root hairs in *D. biflorus*. However, it remains to be determined whether DB46 is membrane-associated. The current investigation demonstrated that GS52 is clearly localized in sucrose gradient

fractions that are enriched in plasma membrane. Indeed, this finding is in agreement with the role of GS52 as a membrane-associated Nod signal receptor.

Despite the interesting data that clearly implicates GS52 in nodulation, more experiments are required to elucidate the precise role played by this protein. Based on sequence homology, GS52 is likely an active apyrase. However, initial attempts failed to isolate an active GS52 protein, likely due to denaturing purification procedures. It will be critical to define the enzymatic function of GS52 as well as determine whether or not this activity is modulated by addition of Nod signals.

CHAPTER 4: INVESTIGATION OF THE ROLE OF APYRASES IN NODULATION.

Abstract

The soybean apyrase, i.e., GS52, was previously characterized as an early nodulin that is expressed in roots and localized to the plasma membrane. Treatment of roots with anti-GS52 antibodies inhibited nodulation of soybean, suggesting a critical role for this protein in nodulation. To further investigate the function of the GS52 apyrase in nodulation, transgenic *Lotus japonicus* plants were constructed expressing *gs52* in the sense and antisense orientation. Segregation and Southern blot analysis identified three single copy sense lines, several double copy sense lines, and one double copy antisense line for further analysis. The sense *gs52 L. japonicus* lines showed enhanced nodulation that correlated with expression of the transgene. In addition, the sense transgenic lines exhibited increased infection thread formation and enhanced infection zone length when infected by *Mesorhizobium loti*, the natural symbiont of *L. japonicus.* Surprisingly, expression of the soybean GS52 apyrase in the sense orientation allowed *Bradyrhizobium japonicum* to infect the root hairs of *L. japonicus,* although this infection did not progress further. . The data presented supports a crucial role for the Gs52 apyrase in nodulation and in the control of infection host specificity.

Introduction

Lectins are important proteins in the plant kingdom that play numerous roles in plant physiology, including: plant development (Hirsch et al., 1995), defense (Sequeira and Graham, 1977; Shibuya et al., 1987; Knibbs et al., 1991; Peumans and Van Damme, 1995), cytokinin interactions (Gregg et al., 1992), and more recently, a hypothesized role as Nod signal binding proteins (Etzler et al., 1999). The possibility that lectins are involved in nodulation has been investigated for many years. Many reports demonstrate that lectins are intimately involved in the nodulation process, but supporting data remain ambiguous and their exact role in nodulation is unclear. These interesting proteins were first implicated in the nodulation process as early as 1973, when Hamblin and Kent first provided evidence for a role for a lectin as a determinant for host-specificity in the rhizobium-bean (*Phaseolus vulgaris*) symbiosis. Further evidence came when Dazzo and Hubbell (1975) postulated a role for a lectin as a host-specificity determinant in the rhizobium-clover symbiosis.

More recently, Diaz et al. (1999) demonstrated that the pea seed lectin (PSL) was involved in host-specificity through the use of transgenic plants. They found that transgenic clover plants expressing PSL could be nodulated by *Rhizobium leguminosarum* b.v. *viciae.* This was an outstanding finding because *Rhizobium leguminosarum* b.v. *viciae* is the natural symbiont of garden pea, and does not normally nodulate clover. However, this work was criticized because *R. leguminosarum* b.v. *viciae* can nodulate wild-type clover (i.e., not expressing

PSL) at very low levels. Therefore, this work was not considered as unequivocal evidence for the role of lectins in host range determination.

This subject was again addressed when van Rhijn et al. (1998) used a similar approach to demonstrate a role for the soybean seed lectin, SBL, in host range determination. Transgenic *Lotus corniculatus* plants expressing the SBL gene exhibited an extended host range that included nodulation by *B. japonicum.* This finding was of considerable interest because *B. japonicum* does not nodulate wild-type *L. corniculatus* at any level. Therefore, together these findings suggested that lectins are responsible, at least in part, for host-range determination in legumes.

Unlike the above studies that focused on classical seed lectins,Etzler et al. (1999) identified a unique, non-agglutinin lectin, DB46, from the legume *Dolichos biflorus.* DB46 was found to have apyrase (i.e. NTPase) activity. DB46, with dual activity as a lectin and apyrase, was termed a lectin nucleotide phosphohydrolase (LNP). As discussed below, LNPs have been implicated in the nodulation process.

Apyrases have been observed to play diverse roles, as might be expected of enzymes that can change the ratios of key energy carriers (e.g. ATP), inorganic phosphorus, and signaling molecules (e.g. GMP, cyclic AMP). Some of the diversity of apyrase function is also due to the fact that the catalytic domains may be located both within cells or organelles (endoapyrases) or outside of cells (ectoapyrases) (Komoszynski and Wojtczak 1996, Day et al. 2000). In animals, the roles of apyrases include modulation of neurotransmission (Edwards and Gibb

1993) and blood platelet aggregation (Marcus and Safier 1993). In yeast, two apyrases facilitate the glycosylation of N- and O-linked oligosaccharides in the Golgi lumen(Abeijon et al. 1993; Gao et al. 1999).

In plants, apyrases were suggested to facilitate phosphate transport and mobilization (Thomas et al. 1999). A pea apyrase expressed transgenically in *Arabidopsis thaliana* produced plants with increased growth rates and phosphate transport when supplied with either inorganic phosphate or ATP. In the case of animal neurotransmission, ecotapyrases recycle and diminish the hormonal activity of extracellular ATP/ADP, which act through purinergic receptors (reviewed in Clark et al. 2001). A similar function for apyrases in plants was suggested by work of Lew and Dearnaley (2000) who showed that extracellular ATP (~ 1mM) would depolarize the membrane potential of growing *Arabidopsis thaliana* root hairs. This response was pronounced with ATP or ADP and the authors suggested that these molecules could be acting as secondary messengers, perhaps through an unidentified plant purinergic receptor. In order to act in this way, physiological levels of extracellular ATP would need to exist in plants. Indeed, Thomas et al. (2000) suggested that plants could use the ATP gradient across the plasma membrane to drive symport through a multiple drug resistance (MDR) transporter. Consistent with this hypothesis, transgenic expression of either PGP1 (an MDR) or ectoapyrase resulted in enhanced resistance to cycloheximide, presumably due to symport-mediated extrusion of this antibiotic. Thus, plants may indeed have a means to transport ATP to the extracellular space where it could be a substrate for ectoapyrases. More recently, Tang et al. (2003)

reported that extracellular ATP (>1 mM) inhibited root gravitropism and polar auxin transport. Extracellular ATP also increased the sensitivity of roots to exogenous auxin.

Membrane depolarization, hormonal activity, and root hair growth are all relevant to legume nodulation in response to inoculation with rhizobia (reviewed in Cohn et al. 1998). Therefore, some excitement was generated by the publication of the Etzler et al. (1999) report showing that the DB46 ecotapyrase could bind the lipo-chitin Nod signal produced by rhizobia. The Nod signal is essential for the induction of a nodule structure in response to rhizobial infection. The ATPase activity of the *Dolichos* apyrase was stimulated upon binding the Nod signal, as well as other related ligands. It was suggested that Db-LNP could act as a Nod signal receptor and induce a signal cascade through hydrolysis of ATP. Indeed, treatment of roots with antibody directed against either Db-LNP (Etzler et al. 1999) or a homologous soybean apyrase (Day et al. 2000) inhibited nodulation. Subsequently, Kalsi and Etzler (2000) reported the immunolocalization of Db-LNP to *Dolichos* root hairs, the site of rhizobial invasion. Indeed, inoculation with compatible, but not with incompatible, rhizobia led to a redistribution of Db-LNP to the root hair tips. Cohn et al. (2001) reported that inoculation of the roots of *Medicago truncatula* with *Sinorhizobium meliloti* rapidly induced expression of two apyrase genes, *Mtapy1* and *Mtapy4* (termed *Mtapy1_1* and *Mtapy1_4* in this paper). However, the interpretation of these results has recently been questioned (Navarro-Gochicoa et al. 2003). Although a similar induction of apyrase expression was found, this was attributed

to an unidentified stress response and was not correlated with inoculation with either rhizobia or Nod signal. Still, the earlier Cohn et al. (2001) study is consistent with the finding of apyrase induction (i.e., Gs52) in soybean roots upon inoculation with *Bradyrhizobium japonicum* (Day et al., 2000). Similar induction of apyrase expression has been reported in pea in response to fungal infection or treatment with fungal elicitors(T. Shiraishi, pers. commun.). Therefore, precedent exists to believe that apyrases may respond to rhizobial infection, as well as to biotic (e.g., fungal) and abiotic stress.

Previous phylogenetic work on this gene family (Roberts et al. 1999; Cohn et al. 2001) identified a group of apyrases that do not have close homologs in *Arabidopsis* (which is in Rosid II, sister to the legume-containing Rosid I superorder). This potentially legume-specific clade contains apyrase homologs from *Lotus*, *Dolichos*, *Pisum*, *Glycine*, and *Medicago*. This work also described sequence clades that contain apyrase homologs from legumes, other dicots, and monocots (Roberts et al. 1999; Cohn et al. 2001). Further, Cohn et al. (2001) described several apyrase-containing BACs from *Medicago*, as well as several predicted apyrase gene sequences identified in these BACs. Recently, Cannon et al. (2003) described the finished sequences of apyrase-containing BACs from *Medicago*, *Glycine*, and *Lotus*. These results allowed an opportunity to integrate phylogenetic patterns and genomic context. These data support the previous claim of Roberts et al. (1999) and Cohn et al. (2001) for a clade of orthologous apyrases, probably arising early in legume evolution and therefore found only in the legumes.

In order to further investigate the role of the soybean apyrase, GS52, in nodulation, we opted to create transgenic *Lotus japonicus* plants expressing the GS52 apyrase. Consistent with previous studies implicating this protein in nodulation, sense expression of GS52 in *L. japonicus* significantly increased nodulation by *Mesorhizobium loti.* This increase in nodule number correlated with an increase in infection foci and an increase in the root infection zone. Surprisingly, GS52 expression allowed *B. japonicum* to infect *Lotus* root hairs, although these infections did not progress further.

Materials and Methods

Chemicals and enzymes

Na-^{32}Phosphate was purchased from ICN (Costa Mesa, CA). Protease inhibitor phenylmethylsulfonyl flouride (PMSF) was obtained from Sigma Chemical Company (St. Louis, MO). Unless specified, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA). Most enzymes in this study were purchased from Promega (Madison, WI) or Fisher Scientific (Pittsburgh, PA).

Bacterial strains and plasmids

Bacterial strains, plasmids, and plant material are listed in Table 4-1.

Table 4-1. Bacterial strains and plasmids used in this study.

Bacteria, plasmid, or plant material	Relevant characteristics	Source or reference
Escherichia coli, JM109	EndA1, recA1, gyrA96, thi, hsdR17, (r_k^-, m_k^+), relA1, supE44, Δ(lac-proAB), F', traD36	Messing, 1983
Mesorhizobium loti, NZP2235	*HemA-lacZ* fusion, Tc^r	Schauser, 1998
Bradyrhizobium japonicum USDA 110	*Npt-lacZ* fusion, Cm^r	
Agrobacterium tumefaciens, LBA 4404	Cb^r	Stiller, 1997
pGA941	Km^r	
Lotus japonicus ecotype 'Gifu'		Stiller, 1997

Bacterial culture media and growth conditions

Escherichia coli strains were grown and maintained on LB medium (Sambrook et al., 1989). Where appropriate, ampicillin (200 μg/ml) was added to the *E. coli* medium for plasmid maintenance and selection. *Bradyrhizobium japonicum* USDA 110 was grown and maintained on RDY medium (So et al., 1987) at 30°C. Minimal medium (MM) (Bergersen, 1961) was used for growth of *B. japonicum* USDA 110 for nodulation assays. Minimal medium consists of 0.3 g/L KH_2PO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 1 mL of 1000X trace elements solution, 0.5 g/L NH_4NO_3, 0.4% glycerol, 0.0002 g/L biotin, 0.0001 g/L thiamine, and 0.0001 g/L calcium pantothenate, pH 7.0. *Mesorhizobium loti* NZP2235 was cultivated on yeast mannitol broth (YMB) medium (Handberg et al., 1992) at 30°C. *Agrobacterium tumefaciens* cultures were grown at 30°C on yeast extract peptone (YEP) medium (Vervliet et al., 1975). Antibiotic concentrations were 2 μg/ml tetracycline and 100 μg/ml carbenicillin for *M. loti* NZP2235 and *A. tumefaciens* LBA4404, respectively. Antibiotic concentrations for *B. japonicum* carrying the *npt-lacZ* fusion were 25 μg/ml tetracycline and 50 μg/ml chloramphenicol.

Construction of GS52 transgenic Lotus japonicus

The *gs52* cDNA was isolated and cloned previously as described in Day et al. (2000). Subsequently, the entire GS52 sequence was subcloned into the plant binary vector pGA941. This vector was then electroporated into *Agrobacterium tumefaciens* strain LBA4404 by procedures described in Sambrook et al. (1989).

Agrobacterium-mediated hypocotyl transformation of *L. japonicus* was carried out based on the protocol described by Stiller et al., (1997).

Seed germination and sterilization

Wild-type and transgenic *Lotus japonicus* seeds were first scarified by rubbing briefly between two sheets of fine 150 grain sandpaper. This was done until the seed coat was visibly roughened. The scarified seeds were then soaked in 3% hydrogen peroxide (H_2O_2), 95% ethanol (EtOH) for fifteen minutes with shaking at room temperature. The seeds were germinated in square petri dishes on a 0.5 cm stack of sterile Whatman #1 filter paper soaked in sterile, distilled, de-ionized water. The petri dishes were sealed with parafilm and placed in a Percival model CU-32L (Percival Scientific, Boone, IA) incubator for one week with a light cycle of eight hours, 22°C day and 16 hour, 20°C night. The relative humidity was 70-80%.

Segregation analysis of transgenic Lotus japonicus

One-week-old germinated seedlings were transferred to ½ x B5 (Gamborg's B5 medium; Gamborg, 1970) agar plates containing 5 μg/ml G418 (Sigma, CA) for segregation analysis. The seedlings were allowed to grow on selection medium for 4 weeks before scoring for antibiotic resistance. Antibiotic resistance was based on growth phenotype compared to wild-type plants that were obviously sensitive to the antibiotic. Resistant plants had enhanced root growth compared to wild-type sensitive plants and in most cases the roots of resistant

plants grew in a curled and/or wavy pattern. In addition to an obvious root phenotype, resistant plants had healthy, green leaves, while sensitive plants appeared chlorotic.

Nucleic acid isolation and manipulation

Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* and *A. tumefaciens* using the alkaline lysis method described in Sambrook *et al.* (1989) or by using the Wizard® Plus Miniprep DNA Purification System (Promega, Madison, WI) for automated DNA sequencing. DNA concentrations were determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA).

Isolation of DNA fragments

DNA restriction endonuclease fragments used in cloning were separated by agarose gel electrophoresis, as described by Sambrook et al. (1989). Gel purified fragments were isolated using the DNA Gel Extraction Kit (Qiagen, Valencia, CA).

Genomic DNA isolation

Genomic DNA was isolated from *Lotus japonicus* using the protocol described by Dellaporta et al. (1985). DNA concentrations were determined as described previously in Sambrook et al. (1989).

Isolation of total RNA

Total RNA was isolated from *L. japonicus* plants using the protocol described by Verwoerd et al. (1989). Briefly, plant material (approx. 100 mg) was ground in liquid nitrogen and 500 µl of hot (80ºC) extraction buffer was added (phenol-0.1M LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS) (1:1)) and homogenized by vortexing for 30 seconds. Next, 250 µl of chloroform-isoamyl alcohol (24:1) was added and the samples were vortexed again. The samples were centrifuged in a microfuge at 14,000 x g for 10 minutes and 4ºC. The water phase was removed and mixed with one volume of 4M LiCl. RNA was then precipitated overnight and collected by centrifugation as described above. The resultant pellet was was dissolved in 250 µl nuclease-free water and 0.1 volume of 3M NaOAc, pH 5.2 and 2 volumes ethanol was added. The sample was incubated for 30 minutes at -80ºC and the RNA was collected by centrifugation as described above. Finally, the pellet was washed with 70% ethanol and allowed to air dry for 10 minutes before dissolving in 50 µl of sterile nuclease-free water. RNA quantity and integrity was determined by monitoring the optical density (OD) at 260 nm and by agarose gel electrophoresis, respectively.

DNA sequencing

Automated DNA sequencing was performed by Dr. Neil Quigley (The University of Tennessee, Molecular Biology Resource facility). Plasmid DNA

sequencing was performed with the ABI Prism Dye Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer Inc., Foster City, CA). Both strands of two independent clones were sequenced to ensure the fidelity of sequences.

Southern blot analysis

Eight micrograms of genomic DNA from *Lotus japonicus* was digested with *Hind*III overnight at 37ºC, according to the supplier's specifications. Digests were loaded onto a 0.7% agarose gel and separated overnight at 5v/cm. The DNA was then blotted onto ZetaProbe® Nylon membrane (BioRad Laboratories, Hercules, CA), according to the method of Sambrook et al. (1989). Hybridization and washing were performed according to the manufacturer's protocol. Gene-specific probes for *gs52* apyrase were labeled to a specificity of 10^8 cpm/μg DNA using random primer labeling (Promega, Madison, WI).

Northern blot analysis

gs52 transgenic *L. japonicus* plants were sterilized and germinated in the dark. Etiolated seedlings were ground in liquid nitrogen and total RNA isolated as described below. Total RNA 20 (μg) was separated on formaldehyde denaturing gels in 1% agarose and blotted onto ZetaProbe® Nylon membrane (BioRad Laboratories, Hercules, CA) according to the method of Sambrook et al. (1989). Hybridization and washing were performed according to the manufacturer's protocol. In all cases, gene-specific probes were labeled to a

specific activity of 10^8 cpm/μg DNA using random primer labeling (Promega, Madison, WI). A 925 bp. PCR product complementary to *gs52* was used as a probe to detect *gs52* mRNA. Futher, a soybean actin probe was used as a control for RNA loading.

Nodulation assays

Lotus seeds were sterilized and germinated as described above. One-week-old seedlings were planted in four-inch plastic pots containing sterile vermiculite and given B and D nutrient solution (Broughton and Dilworth, 1971). After planting, the seedlings were allowed to grow for two additional weeks (as described above) and then inoculated with a *M. loti* culture as described above. The plants were placed back into the growth chamber and allowed to grow for an additional four weeks (as described above). At four weeks post-inoculation, the plants were harvested and scored for nodule number.

Infection thread assays

Lotus seeds were sterilized and germinated as described previously. After one week, the germinated seedlings were transferred to sterile Leonard jars containing sterile vermiculite for further growth and nodulation assays. To ensure sterility, all containers, vermiculite, and water and/or nutrient solutions used in these experiments were autoclaved. The plants were watered with B and D nutrient solution as described by Broughton and Dilworth, (1971) and grown for an additional two weeks as described above.

Next, the three-week-old plants were inoculated with 1ml each of either a three day *M. loti* (*hemA-lacZ*) or *B. japonicum* USDA 110 (*npt-lacZ)* culture washed with sterile water and diluted to an $O.D._{600}$ of 0.1. The culture inoculant was manually applied to each plant with a pipette. The plants were allowed to grow for an additional 2 weeks post-inoculation before the roots were harvested, fixed, and stained for visualization of infection threads.

At the time of harvest, the plants were removed from the Leonard jars by flooding gently with water. The roots were further washed gently in water and subsequently detached from the plant using a razor blade. The detached roots were immediately fixed in glutaraldehyde and stained for LacZ (β-galactosidase) expression as described by Boivin et al. (1990). Roots were stored in the dark in sterile, distilled water at 4°C until use.

The roots were measured and photographed using a stereoscope (Olympus SZX12) equipped with a Nikon DXM1200 digital camera. The infection zone was defined as an area on the root showing the most abundant infections (c.f. Calvert et al., 1985). The edges of the infection zone were arbitrarily determined as the point where no additional infections were apparent within 3 mm.

Results

Construction of GS52 transgenic Lotus japonicus

Transgenic *Lotus japonicus* plants were constructed in which the *G. soja gs52* apyrase gene was constitutively expressed from the strong *CaMV 35s* promoter in either the sense or antisense orientation.

Analysis of gs52 copy number in transgenic plants

Primary (T_1) transgenic plants were selfed and segregation of the transgene was analyzed in T_2 and T_3 generation seeds (Table 4-2). Segregation of the transgene was scored based on a comparison of seedling growth on medium with and without G418. Segregation analysis suggested that numerous lines were either single copy (segregating 3:1) or homozygous (copy number unknown) for the transgene. Copy number of the transgene was determined by Southern blotting. Figure 4-1 shows the results of the southern blot when probed with a 1.4 Kb probe complementary to the *gs52* cDNA. Three single copy sense, lines 45WT_2, 45B1K T_3, 45D5 T_3, and one double copy sense, line 45B77 T_3, were identified and used for further analyses. In addition, a double copy antisense line, 44HH T_3, was identified and used as a control in further analysis. With the exception of the nodulation assay, these lines were used exclusively in further experiments.

Table 4-2. Segregation analysis of *gs52* transgenic plants.

Line	Resistant: Sensitive	Segregation	Southern Results
45W T_2	34:9	3.8:1	1S
45D5 T_3	68:2	2.8:1	1S
45B1K T_3	51:1	3.4:1	1S
45B77 T_3	62:0	Homozygous	2S
45A7 T_3	75:0	Homozygous	2S
45B38 T_3	64:0	Homozygous	2S
45B1.2 T_3	92:0	Homozygous	2S
45B1H T_3	60:0	Homozygous	2S
45B35 T_3	22:0	Homozygous	2S
45B1I T_3	100:0	Homozygous	2S
45B16 T_3	53:0	Homozygous	2S
45B37 T_3	73:0	Homozygous	2S
44HH T_2	99:0	Homozygous	2AS

Plants were germinated on filter paper and then transferred to selection media (see methods) and then scored for resistance or sensitivity. Segregation was confirmed by southern blotting (see figure 4-1) and is listed in column 3 of the table. S, Sense copy of *gs52*; AS, antisense copy of *gs52.*

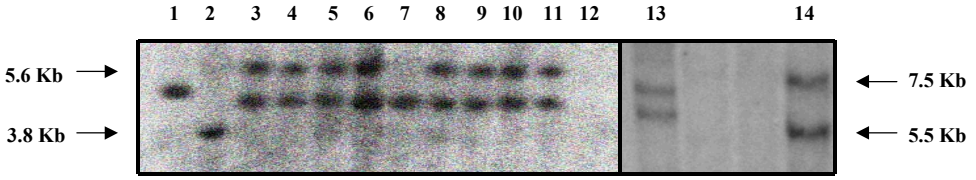


Figure 4-1. *gs52* Genomic southern blot. 8 µg genomic DNA was digested with *Hind*III and separated by agarose gel electrophoresis. A 1.4 Kb PCR product complementary to the *gs52* cDNA was used as a probe. 1. 45WT_2 (single copy line); 2. 45D5 T_3 (single copy line); 3. 45B1.2 T3; 4. 45B1HT_3; 5. 45B35 T_3; 6. 45B1I T_3; 7. 45B1K T_3; 8. 45B16 T_3; 9. 45B37 T_3; 10. 45B38 T_3; 11. 45A7 T_3; 12. Wild type *L. japonicus*; 13. 44HH T_2 (double copy antisense line); and 14. 45B77 T_3 (double copy sense line). The enzyme *Hind*III cuts once within the t-DNA of pGA941 (but not within the *gs52* sequence) therefore each band represents an independent insertion into the plant genome.

gs52 expression analysis of transgenic L. japonicus lines

In order to determine if the *gs52* gene was successfully expressed in the transgenic lines, Northern blot analysis was performed. As seen in Figure 4-2, Northern blot analysis demonstrated that the three single copy lines had varying levels of *gs52* expression. The double copy *gs52* line, 45B77 T_3, had strong *gs52* mRNA expression. In addition, the Northern blot indicated that neither the wild-type *L. japonicus* nor the antisense *gs52* line, $44HHT_2$, had detectable expression of *gs52* mRNA. Since a double-stranded DNA probe was used for these hybridizations, the results indicate that the $44HHT_2$ line does not express significant levels of antisense Gs52 RNA.

gs52 sense plants have enhanced nodulation

Figure 4-3 shows the nodulation results of 12 lines of transgenic *L. japonicus* in comparison to the nodulation results of wild-type plants. These experiments demonstrated that *gs52* expression in *L. japonicus* signficantly enhanced nodulation. Sense *gs52* lines had anywhere from a 30% - 50% increase in nodule numbers compared to wild-type or $44HHT_2$ controls.

gs52 sense plants have enhanced infection thread formation

The single copy transgenic *L. japonicus* plants had significantly enhanced nodulation compared to wild-type and antisense *L. japonicus* plants.

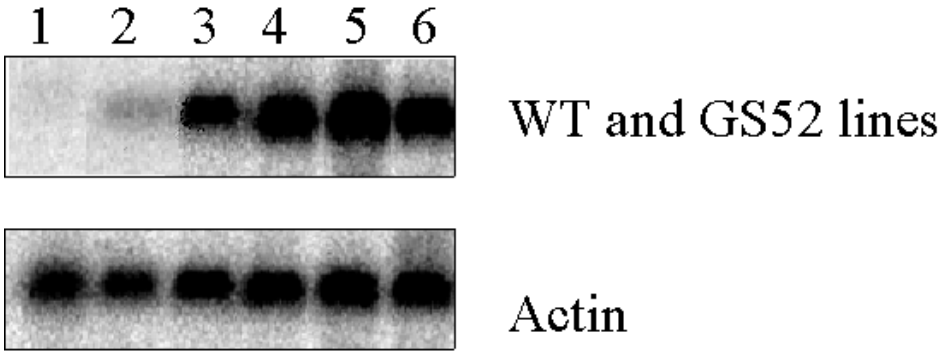


Figure 4-2. Northern blot analysis of wild-type and *gs52* transgenic *L. japonicus*. A 1.4 Kb PCR product complementary to *gs52* cDNA was used to detect *gs52* mRNA expression levels (top). Lanes 1-6 represents the results of 20 µg total RNA from each sample. 1. Wild-type; 2. 44HH T_2 (double copy antisense line); 3. 45D5 T_3 (single copy sense line); 4. 45W T_2 (single copy sense line); 5. 45B1K T_3 (single copy sense line); and 6. 45B77 T_3 (double copy sense line). A soybean actin probe was used as a RNA loading control (bottom).

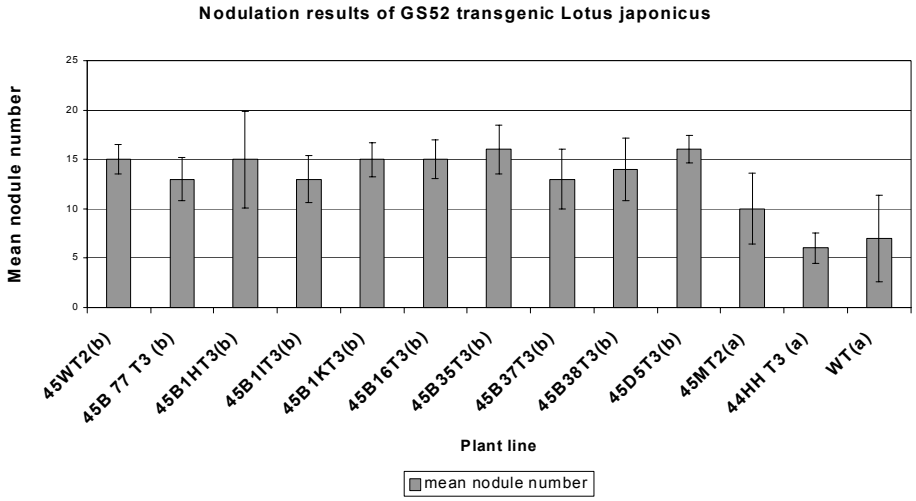


Figure 4-3. Nodulation results of *gs52* transgenic *L. japonicus* plants. Compared to wild-type and antisense *gs52* plants, sense *gs52* plants have enhanced nodulation. Seeds from each line were sterilized, germinated, and grown as described in methods. Three-week-old plants were equally inoculated with *M. loti* NZP2235 and then grown for an additional four weeks. Four weeks post inoculation the plants were scored for their nodulation phenotype. Data are the mean results from at least 30 individual plants (n=30) and are representative results from 3 independent experiments. Groups marked (a) and (b) are statistically significant at $\alpha = 0.05$.

In order to understand the mechanism of the enhanced nodulation, the frequency of infection thread formation was measured on infected *L. japonicus* roots. *gs52* sense and antisense lines and wild-type *L. japonicus* plants were inoculated with *M. loti* NZP2235 harboring a *hemA-lacZ* fusion. This strain carries a marker (constitutive *lacZ* fusion) allowing detection of bacterial infection events via staining with X-Gal. Infection thread analysis of the transgenic plants demonstrated that the enhanced nodulation phenotype of the *gs52* plants correlated with enhanced infection thread formation. Further, as seen in Table 4-3, the level of infection thread formation that correlated with the expression level of *gs52* mRNA. These data support the idea that expression of the soybean apyrase, GS52, leads to enhanced infection thread formation resulted in increased nodule numbers.

It is well known that legume roots have defined regions, i.e. infection zones, that are most susceptible to infection (Calvert et al., 1985). When the infection zone of the Gs52 transgenic plants wasmeasured, it was clear that these plants had significantly larger infection zones than those found on either wild-type or $44HT_2$ control plants (Table 4-3). Further, the GS52 transgenic plants had greater infection thread numbers per unit infection zone length (mm). In other words, the GS52 plants had enhanced infection thread formation, i.e. infection threads were more abundant inside the infection zone. Figure 4-4 shows a representative view of the infection zone of wild-type plants compared

Table 4-3. Infection thread analysis of GS52 plants.

Data	WT	44HH T_2	45D5 T_2	45W T_2	45B1K T_2	45B77 T_3
Copy #	0	2AS	1S	1S	1S	2S
Avg. total Root length (mm)	210.2[a]	144.4[b]	231.8[a]	195.2[a]	185.4[a]	182.3[a]
Avg. nodule # / plant	8[a]	7[a]	12[c]	13[c]	11[c]	9[c]
Avg. tap root infection threads/ plant	83[a]	50[b]	144[c]	229[d]	316[e]	207[d]
Avg. lateral root infection threads/ plant	55[a]	42[b]	137[c]	164[d]	270[e]	123[c]
Avg. infection threads/plant	138[a]	92[b]	281[c]	393[d]	586[e]	330[c]
Avg. root infection zone length (mm)	11[a]	7[b]	17[c]	20[d]	20[d]	13[c]
Avg. root infections / (mm) in the avg. infection zone	0.65[a]	0.64[a]	1.21[b]	2.01[c]	3.16[d]	1.81[c]

Groups marked (a),(b), (c), (d), or (e) are statistically significant at α=0.05.

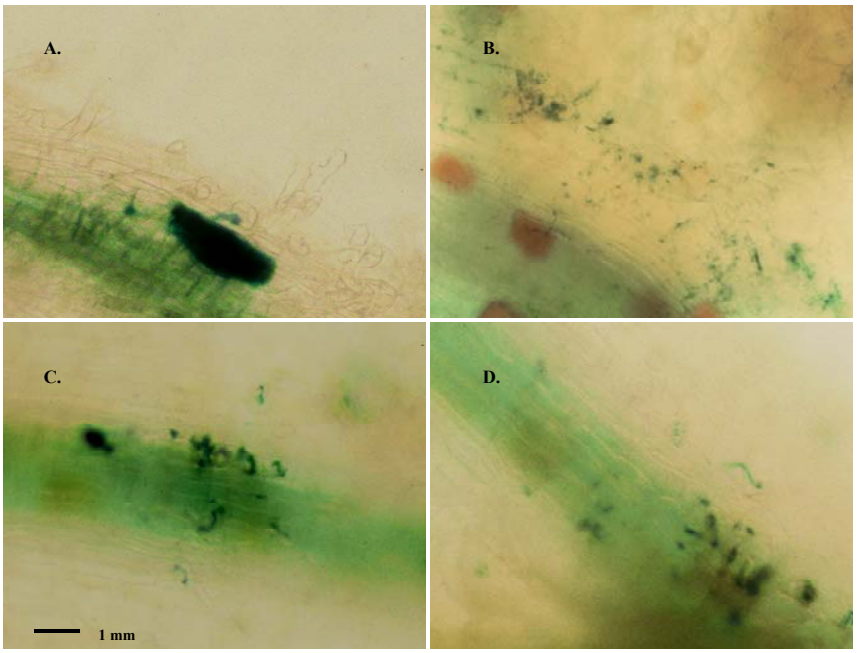


Figure 4-4. Infection thread analysis of GS52 transgenic *L. japonicus* plants. A. WT, wild-type; B. 45W T_2; C. 45D5 T_3; D. 45B1K T_3. Wild type and GS52 *Lotus* plants were inoculated with *M. loti* NZP2235 *hemA-lacZ*. Two weeks post inoculation, roots were stained with X-Gal to visualize bacterial infection. 10 plants per line were examined. Pictures are representative results from three independent experiments.

to a representative view of the infection zone of the three single copy GS52 transgenic lines. If the nodulation results of Table 4-3 are compared to the results shown in figure 4-3, there are obvious discrepancies between the average nodule number per plant. This difference can be explained by the time course of the nodulation assay. Nodules in figure 4-3 were counted 4 weeks post inoculation whereas in Table 4-3 the results were recorded only two weeks post inoculation.

B. japonicum USDA 110 can infect gs52 sense L. japonicus lines

B. japonicum, symbionts of soybean, normally does not infect *L. japonicus* roots. Therefore, this bacterium was used to examine whether transgenic Gs52 apyrase expression could expand the host range of *L. japonicus.* Inoculation of Gs52 expressing plants with *B. japonicum* did not result in nodule formation. However, when plants were inoculated with a *B. japonicum* strain carrying an *npt-lacZ* fusion, infection thread formation was observed. Figure 4-5 shows representative infection threads seen when *gs52* transgenic plants were inoculated with *B. japonicum*. Neither the wild-type nor the $44HHT_2$ control plants were infected by *B. japonicum*. Infection threads were seen inside the root hair cell but did not appearto progress further into the root cortical cells. Table 4-4 shows the results when infection threads were counted on wild-type, and sense and antisense *gs52* plants (10 plants were counted per line). The frequency of *B. japonicum* infections was not equivalent to that seen after *M. loti* inoculation of transgenic GS52 plants.

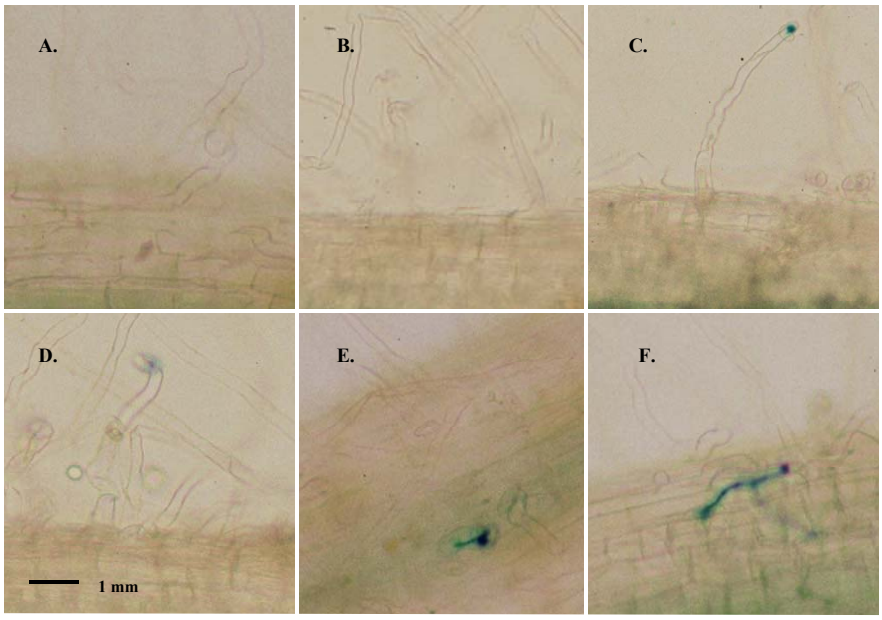


Figure 4-5. GS52 transgenic *L. japonicus* plants are infected by *Bradyrhizobium japonicum.* A. WT, wild-type; B. 44HHT2 (double copy antisense); C. 45W T2 (single copy sense line); D. 45D5 T3 (single copy sense line); E. 45B1K T3 (single copy sense line); F. 45B77 T3 (double copy sense line). GS52 and wild-type plants were inoculated with *Bradyrhizobium japonicum npt-lacZ* and subsequently stained with X-Gal to visualize bacterial infection.

Table 4-4. *B. japonicum* infection data on GS52 transgenic plants.

Data	WT	44HH T_2	45D5 T_3	45W T_2	45B1K T_3
Avg. total infection threads/ plant	0	0	21	28	39

Data is representative results of a single experiment with 10 plants counted per line.

Discussion

Transgenic *Lotus japonicus* plants were created expressing the soybean Gs52 apyrase geneAnalysis of the resultant transgenic lines showed that a variety of lines were either segregating 3:1 for the transgene (indicating a single insertion into the plant genome) or homozygous for the transgene (not indicating insertion number). We chose to investigate further lines containing a single insertion of the *gs52* transgene Lines 45D5 T_3, 45W T_2, 45B1K T_3 all showed a single, independent insertion of the T-DNA as visualized by Southern analysis. The southern blot also revealed multiple lines that had two independent insertions of *gs52* into the plant genome during *Agrobacterium* transformation. We randomly chose one (line 45B77T_3) of those double copy sense lines for further analysis. This choice was random, however, the Southern blot results indicated that each of the double copy sense lines is in fact genetically identical. Therefore, these lines are likely siblings arising from a single transformation event. As a control, we

generated lines constructed to express *gs52* in the antisense orientation. 44HH T_2 was chosen as an antisense line for further analysis. This type of antisense control was constructed with the idea that antisense expression can cause silencing of the transgene and in some cases silencing of endogenous genes that are homologous to the inserted transgene. Moreover, antisense expression can cause endogenous gene silencing and thus create a knockout mutation in homologous genes. However, line 44HHT_2 showed only marginal expression of Gs52 RNA. Therefore, although endogenous *L. japonicus* apyrase expression was not measured, it is unlikely that gene silencing is occurring in this line. Instead, for the purposes of our experiments, line 44HHT_2 served as a non-expressing, transgenic control.

Northern blot analysis revealed that the three single-copy lines (expressing sense *gs52*) had various levels of *gs52* mRNA expression. 45W T_2 strongly expressed *gs52*, however, it had the lowest level of expression compared to the other single-copy sense lines. The other single-copy sense lines, 45D5 T_3 and 45B1K T_3, had medium and maximum levels of *gs52* expression, respectively. It is interesting to note that *gs52* expression levels in the transgenic lines correlated with the infection and nodulation phenotypes observed and thus provides strong support that the phenotypes seen are due to Gs52 expression.

Expression of *gs52* in the sense orientation in *L. japonicus* increased nodulation anywhere from 30-50%, compared to the wild type and 44HHT_2 control lines. These observations are in accordance with the hypothesis that the GS52 apyrase is important to infection and/or nodulation. This increase in nodule

number appears to be the result of increased infection in the roots of transgenic plants. This enhancement of infection in the sense *gs52* lines was apparent even when root length was taken into account. In part, this increase in infections was due to an increase in the infection zone in the transgenic roots. Taken together, the infection thread and infection zone data indicate that the GS52 apyrase likely plays a role in a very early step in in the nodulation process leading to enhanced infection.

As discussed earlier, Diaz et al. (1999) and van Rhijn et al. (1998) showed that transgenic expression of classical seed lectins could extend the host range of that legume species to include the bacterial symbiont of the source lectin host. This extension of host range was measureable by the formation of true nodules. In contrast, transgenic expression of the GS52 apyrase did not permit nodule formation by *B. japonicum,* normal symbionts of soybean. However, infection threads were formed that quickly aborted in the root hair cell. These data suggest that the apyrase leads to more promiscuous infection but this does not negate other host-specific signaling steps essential for nodule formation

In addition to the hypothesized role in host range determination, lectin nucleotide phosphohydrolases (LNP apyrases) were hypothesized to play a role as Nod signal binding proteins (Etzler et al., 1999 and Day et al., 2000). Our experiments did not directly address this hypothesis. However, it seems possible that expression of a Nod signal receptor, i.e. GS52, could lead to increased infection and nodulation. In addition, root hair susceptibility to rhizobial infection could be altered by expression of a Nod signal receptor. Kasi et al.

(2000) localized the DB46 LNP protein to the surface of root hairs. The expression of this protein was found to be greatest in young emergent root hairs, which are most susceptible to rhizobial infection, and found to taper off as the root hairs matured. Therefore, the expression pattern of DB46 LNP correlated with root hair susceptibility to infection. This finding in conjunction with the finding that transgenic expression of *gs52* apyrase leads to increased infection zone length strongly supports the hypothesis that GS52 plays a role in root hair infection, perhaps as a Nod signal receptor.

If GS52 is not playing a role as a bona fide Nod signal receptor, then what other functions could explain the phenotype seen? There are many described roles for lectins in plant physiology including a role in plant defense. For example, Kiba et al. (2003) demonstrated that an NTPase / peroxidase complex in the pea plant cell wall is the regulated by the *Mycosphaerella pinodes* fungal elicitor and suppressor. NTPase activity was modulated by addition of the suppressor and elicitor, which in turn modulated the activity of the peroxidase enzyme. This finding demonstrated that the NTPase could indirectly affect the plant defense response through perception of the fungal elicitor or suppressor. With this in mind, it seems possible that the legume apyrase, i.e. DB46 (LNP) and GS52, could modulate a localized plant defense response at the site of root hair infection. It has long been hypothesized that successful symbiotic infection requires suppression of the plant defense response (Baron and Zambryski, 1995). Therefore, rhizobia must have evolved mechanisms to suppress the plant defense response. Could the rhizobial Nod signal be a suppressor of plant defense? Does

GS52 indirectly suppress the plant defense response and thus allow rhizobia to infect the root hair? In accordance with this hypothesis, is it possible that the GS52 apyrase acts as a Nod signal receptor and upon perception of the Nod signal, apyrase activity is stimulated (Etzler et al., 1999)? Apyrase activity could in turn modulate the activity of the plant peroxidase, which would have significant effects on the plant defense response (Kiba et al., 2003). Indeed, it is known that either rhizobial inoculation or treatment of roots with Nod signals induces expression of a plant peroxidase, termed rhizobial induced peroxidase 1(*rip1*) and induction of this peroxidase is required for successful nodulation (Cook et al., 1995). Modulation of the plant peroxidase, which is responsible for the enzymatic destruction of superoxide radicals such as hydrogen peroxide (H_2O_2), would have a direct effect on the plant defense response. Hydrogen peroxide is a key molecule of the plant defense response and it has been shown to have a direct role in programmed cell death associated with the hypersensitive response (HR), induce defense related gene expression, and induce salicylic acid (SA) accumulation (Shirasu et al., 2000). Therefore, activation of peroxidase would suppress the plant defense response.

As attractive as some of these ideas seem, further experiments are required to determine the exact mechanism of GS52's role in the infection and nodulation process. Determination of GS52's Nod signal binding capabilitites along with enzyme kinetic studies will be helpful to characterize whether or not GS52 is a bona fide Nod signal receptor. In addition, experiments to elucidate the effect of GS52 apyrase activity on plant peroxidases are required to determine what effect,

if any, GS52 might have on the plant defense response. With advancing techniques in generating antisense constructs (Thakur et al., 2003), another approach would be to construct effective antisense *gs52* plants in which the endogenous apyrase was successfully silenced. Investigation of the infection and nodulation phenotype of these plants could give further insights as to the role of GS52.

CHAPTER 5: DISCUSSION

The research described focuses on understanding the early events which allow the infection of legume root hairs by rhizobia. It is widely accepted that salicylic acid (SA) is a key molecule in the plant defense response (Draper, 1997; Gaffney et al., 1993). Therefore, transgenic *Lotus japonicus* plants that express the bacterial gene, *nahG*, were constructed to study the role of the plant defense response in early nodulation. NahG encodes salicylate hydroxylase, an enzyme that can degrade SA to inactive catechol. Plants expressing NahG cannot accumulate SA and hence cannot mount a successful plant defense response. Gaffney et al. 1993 used a similar *nahG* construct to demonstrate the important of SA accumulation in the resistance of tobacco plants to infection with tobacco mosaic virus (TMV). Plant expressing NahG and, therefore, unable to accumulate SA were unable to mount the defense response, including the hypersensitive response (HR) and systemic acquired resistance (SAR).

Similar to the case of Gaffney et al. (1993), the NahG expressing, transgenic *L. japonicus* plants were exhibited significantly reduced levels of SA. Interestingly, this lack of SA correlated with increased nodulation, suggesting a role for a SA-mediate plant defense response in controlling nodulation..

Increased nodule numbers can result from either an increase in the number of infections or due to a greater ability of existing infections to lead to nodule formation. To examine these two possibilities, we analyzed the NahG plants for infection thread formation after inoculation with *M. loti* NZP2235 harboring a *hemA-lacZ* fusion. The NahG plants had enhanced infection thread formation suggesting, at least in part, that the increase in nodule numbers was due to increased infection. However, in addition, the

NahG plants also showed an increased infection zone on the root, i.e. an increased region on the root that was susceptible to infection. These results are all consistent with the idea that SA mediates a defense response that reduces the number of infections, resulting in a smaller infection zone. In the latter case, one possibility is that SA plays a role in the transient susceptibility of root hairs to rhizobial infection.

However, the NahG plants also showed an increased root growth phenotype that complicated the interpretation of our results. Was the increased in the infection zone in the NahG expressing plants due merely to more rapid growth or, as hypothesized, due to a reduction in a SA-mediate defense response? We were unable to unequivocally answer this question. However, if total infections were counted the NahG plants did have increased infection thread formation suggesting that SA likely has a direct role on rhizobial infection. In retrospect, the observed root growth phenotype was not surprising, given because the numerous roles that SA plays in plant growth and development and the complex interactions between plant growth regulators (Gutiérrez-Coronado et al., 1998). Relative to nodulation, criticism of the results included the idea that the increased root growth led to increased nodulation. Increased root growth also could have resulted in the increased infection zone length that was observed in the NahG plants.

Apyrases have received much attention due to their postulated role as Nod signal binding proteins (Etzler et al., 1999) and we sought to further characterize the role of the GS52 apyrase in nodulation. If GS52 plays a critical role in nodulation, then we hypothesized that transgenic expression of *gs52* in *L. japonicus* could lead to an alteration in nodulation phenotype. Indeed, it was found that GS52 expression in transgenic *L. japonicus* plants resulted in a significant enhancement of nodulation by *M. loti.* Similar to

the results using the NahG expressing plants, this increase in nodule formation correlated with an increase infection thread formation, as well as an increased infection zone length. However, notably different from the NahG expressing plants, the transgenic root expressing GS52 did not exhibit increased root growth. Therefore, the data appear to support a strong conclusion that transgenic expression of the GS52 apyrase directly enhances root infection and nodule formation..

Ectopic expression of a Nod factor receptor could be expected to alter the host range of the plant. Indeed, root hairs of transgenic *L. japonicus* plants expressing GS52 could be infected by a *B. japonicum npt-lacZ* strain. *B. japonicum* is not a natural symbiont of *L. japonicus*, but does nodulate the host plant (soybean) from which the *gs52* gene is derived. Although requiring further confirmation, these results support the idea that GS52 plays a role in host range determination. However, since inoculation of *B. japonicum* onto the roots of the GS52 plants, did not result in cortical cell division or subsequent nodule development, it is clear that Gs52 alone does not determine nodulation host specificity. Indeed, the data would argue that GS52 plays a very early role in host range determination, which allows entry of the bacterium into the root hair cell, but does not overcome additional barriers allowing infection thread penetration into the cortex or subsequent nodule development.

As discussed above, NahG, through its degradation of SA, reduces the ability of the plant to mount a defense response against invading pathogens. Apyrases, such as GS52, have also been implicated in the plant defense response (Shirashi, pers. Commun.)). Shirashi demonstrated that the apyrase present in the pea (*Pisum sativum*) cell wall responds to fungal elicitors and suppressors. The elicitor and suppressor

modulated activity of the apyrase and the apyrase activity, in turn, modulated activity of a peroxidase enzyme in the plant cell wall. Antibody against the pea apyrase co-immunoprecipitated the peroxidase, suggesting that the two proteins interact in a complex in the pea cell wall. The model proposed is that the pea apyrase modulates the production of reactive oxygen species through its effects on the peroxidase enzyme.

Reactive oxygen species are known to be important in early nodulation. For example, Cook et al. (1995) demonstrated that inoculation of alfalfa plants with *Sinorhizobium meliloti* (compatible symbiont of alfalfa) induced expression of a peroxidase enzyme, called rhizobium-induced-peroxidase (*rip1*). Interestingly, inoculation of alfalfa roots with incompatible rhizobia did not result in *rip1* induction. Is it possible that legume apyrases recognize the Nod signal and, in turn, modulate peroxidase activity to suppress the plant defense response? This idea seems reasonable. However, the data available are tenuous and clearly much research remains to be done.

One can also postulate a role for reactive oxygen species in explaining the enhanced infection phenotype of the NahG plants. For example, Durner et al. (1996) demonstrated that SA could inhibit catalase (Durner et al., 1996). This activity was hypothesized to modulate the plant defense response by allowing the buildup of hydrogen peroxide. When NahG is present, SA would not accumulate, catalase would not be inhibited, and hydrogen peroxide would be rapidly destroyed suppressing the defense response activated by reactive oxygen species.

One of the most intriguing results to come from the current research was the demonstration that transgenic expression of GS52 allowed heterologous infection of *Lotus* roots by *Bradyrhizobium japonicum.* These results clearly implicate the GS52

apyrase in controlling infection specificity and are consistent with the idea that apyrases may be an important Nod Signal receptor. . However, support for such a claim would by necessity include biochemical evidence that Gs52 can bind with high affinity and specificity to the Nod Signal. After repeated efforts, we were unable to isolate active GS52 apyrase after expression in *E. coli.* Therefore, we were unable to pursue these biochemical studies. Future efforts should be directed toward isolating the enzyme directly from soybean tissue so that its enzymatic and Nod Signal binding properties can be studied.

It is now well accepted that Nod Signal recognition involves multiple (likely two) recognition events (perhaps two receptors) with differing chemical specificity and biological activity. This idea was first reported by Ardourel et al. (1994) who used *S. meliloti* mutants, producing Nod Signals of differing chemistry, to show that different plant responses (e.g. infection thread initiation and cortical cell division) had different Nod signal structural requirements. They proposed a 'two-recognition event' model in which a low-stringency "signaling" receptor mediated such events as cortical cell division, while a second, more stringent, high-affinity "entry" receptor was necessary to trigger rhizobial infection thread initiation. Similar results were obtained by Felle et al. (1995) who examined the ability of various Nod signals to trigger either cytosolic alkalinization or membrane depolarization in alfalfa root hairs. Sulfated Nod signals, which were biologically morphogenic on alfalfa, were able to induce membrane depolarization, and rapid intracellular alkalinization in root hairs. Non-sulfated Nod signals induced an intracellular pH change but in the absence of membrane depolarization. Thus again, this study demonstrated the presence of two Nod signal

recognition events in alfalfa that are independently coupled to cellular events associated with nodulation.

In the case of soybean, Stokkermans et al. (1995) showed that only four [i.e. Nod*Bj*V(C18:1Δ11, MeFuc), Nod*Bj*V(C18:1Δ9, MeFuc), Nod*Bj*V(C16:0, MeFuc), and Nod*Bj*V(C16:0)] of the various synthetic Nod signals tested could elicit a morphogenic response on soybean roots. This study demonstrated that both fucosylation and chitin chain length were the most important determinants for Nod signal specificity on soybean. This work was extended by Minami et al. (1996) who investigated the ability of various Nod signals to induce the expression of the early nodulins, ENOD40 and ENOD2. Expression of ENOD40 mRNA was induced transiently by a simple, non-acylated chitin pentamer. Sustained ENOD40 expression, however, required one of the soybean-specific Nod signals listed above. Subsequently, Minami et al. (1996) showed that addition of any single Nod signal to soybean roots failed to induce ENOD2 expression. In contrast, when a mixture of Nod signals was added, ENOD2 expression was induced. Further analysis of the mixture showed that addition of a non-acylated chitin pentamer, along with the addition of any of the four above-mentioned soybean specific Nod signals, was sufficient to elicit expression of ENOD2. Therefore, taken together, these studies demonstrated the presence of at least two Nod signal recognition events that vary in chemical specificity and are uncoupled to cellular events. In addition, ENOD40 and ENOD2 expression is known to mark different stages of nodule development. Thus, these results suggest that different Nod signal events are required to induce the temporal development of the nodule.

Figure 5-1 shows a model depicting what is known about Nod signal receptors to date. In addition to the possible involvement of the apyrase in conjunction with peroxidase, a very recent candidate gene encoding for a Nod Signal receptor was reported (J. Stougaard, T. Bisseling, ISMPMI Meeting, St. Petersburg, Russia). In both cases, the gene was identified by positional cloning starting with plant mutants defective in early nodulation. In one case, this mutant allele was *sym2,* first identified in pea (Guerts et al., 1997). The Sym2 mutant restricts nodulation of *R. leguminosarum* strains lacking the *nodX* gene that encodes for acetylation of the Nod signal. Thus, due to the chemical specificity of the phenotype, the Sym2 protein is a good candidate for a Nod signal receptor. The genes cloned by the Stougaard and Bisseling laboratories encode proteins with a cytoplasmic protein kinase domain, a transmembrane domain and an external LysM domain (Bateman and Bycroft. 2000). The LysM domain first found in bacterial proteins is involved in binding murein (peptidoglycan) found in bacterial cell walls. Peptidoglycan, a polymer of N-acetylmuramic acid and N-acetylglucosamine, is structurally similar to chitin and, therefore, the presence of the LysM domain on the Sym2 protein suggests a role in binding the lipo-chitin Nod Signal. Sym2 pea mutants recognize the Nod Signal, induce cortical cell division, and infection threads are initiated but do not extend beyond the epidermis (Heidstra and Bisseling, 1996 New Phytol. 133: 25-43). With regard to infection thread growth, the phenotype of the Sym2 mutant and *B. japonicum* inoculated, transgenic *Lotus* expressing Gs52 is similar. Heidstra and Bisseling (1996) equated the putative Sym2 Nod Signal receptor to the “entry” receptor postulated by Ardourel et al. (1994). Heidstra and Bisseling (1996) proposed a model in

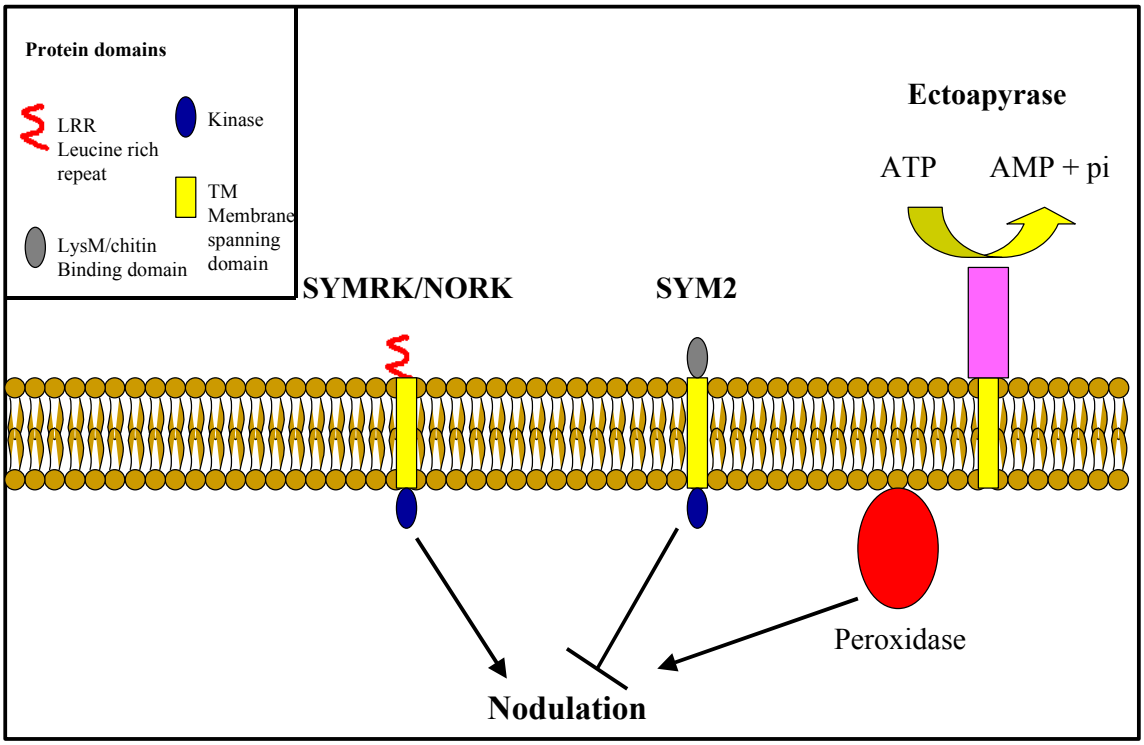


Figure 5-1. Model depicting known Nod signal receptors. SYMRK/NORK, Symbiotic Receptor Kinase/ Nodulation Receptor Kinase;LRR, leucine rich repeat; TM, transmembrane.

which Sym2 in its unbound state acted to repress infection and this repression was relieved upon binding the Nod Signal. Given the similarity in phenotype it is possible that the apyrase acts in conjuction with the LysM domain receptor kinase. Indeed, destruction of ATP through the action of the apyrase could relieve the repressive effects of the Sym2 receptor even in the absence of Nod Signal. These ideas clearly suggest additional experiments to resolve the role of apyrases in nodulation. In addition, figure 5-1 depicts the SYMRK/NORK Nod signal receptor. *Medicago* nodulation mutants were found to have insertion mutations in a receptor kinase that is essential for nodulation. These mutants were infected but not nodulated by rhizobia. The insertion mutation caused a loss of function of SYMRK and thus nodulation was inhibited, therefore, it was hypothesized that SYMRK is a positive regulator involved in modulation of nodulation.

With regards to the NahG plants, there are several experimental approaches that can be taken to further characterize the role of SA in nodulation. It is pertinent to design experiments that can separate the enhanced infection and nodulation phenotype from the enhanced root growth phenotype. With this in mind, it would be very interesting to test the NahG plants for heterologous nodulation. If the plant defense response is involved in inhibition of infection thread formation and/or nodulation, then it seems reasonable to hypothesize that the NahG plants might be infected or nodulated by non-compatible rhizobia. In fact, there are reports in the literature that demonstrate that the plant defense response inhibits infection by heterologous rhizobia. In addition, compatible rhizobia that are defective or altered in Nod signal synthesis have been shown to induce SA accumulation in alfalfa roots. Therefore, it would also be interesting to test the NahG

plants for their ability to be infected and nodulated by mutant rhizobia strains that are altered in Nod signal synthesis.

On the other hand, microscopic analysis of the NahG plants following inoculation could help to separate the infection and nodulation phenotype from the root growth phenotype. The NahG plants could be analyzed microscopically for the oxidative burst and phenolic compound accumulation, both of which are signatures of the plant defense response. Another approach to separate the root growth phenotype would be to spot inoculate the NahG roots. NahG plants could be grown in petri dishes on solid medium. The NahG roots could then be spot inoculated with a liquid culture and the spot marked. The infections and/or nodules that form could then be scored within a certain region around the spot of inoculation on NahG and wild type plants. This type of approach could remove the concerns associated with the enhanced root growth phenotype.

Data from the Roux lab provides interesting data characterizing the role of apyrases in plant physiology. For example, the pea apyrase was found to be nuclear-localized and light-regulated. Apyrase expression was found in dark grown leaves of pea plants but inhibited in light grown leaves. Interestingly, light is a factor that regulates nodulation. Further, the pea apyrase was found to be involved in phosphate transport. *Arabidopsis* plants expressing the pea apyrase had enhanced phosphate transport and increased sensitivity to auxin. Auxin is a plant hormone hypothesized to play a role in nodule development. In addition, phosphate nutrition can also affect nodulation. Therefore, it appears that apyrases may play numerous roles affecting nodulation, however, a more thorough investigation is required to determine the precise role(s) that apyrases play in the infection and nodulation process.

In conclusion, the current studies on the NahG and GS52 plants implicated the involvement of the plant defense response in nodulation. In addition, the GS52 plants further characterize the role of legume apyrases in the infection and nodulation process. Further, the generation of the transgenic NahG and GS52 plants should provide valuable tools for further characterization of the role of the plant defense response and the role of legume apyrases in the nodulation process, respectively.

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VITA

Crystal Bickley McAlvin was born in Knoxville, Tennessee on October 25, 1976. After graduating from Farragut High School in Farragut, Tennessee in 1994, she enrolled at Pellissippi State Technical Community College of Knoxville, Tennessee, where she majored in Biology. After two years, Crystal transferred to the University of Tennessee, Knoxville and continued her education in Microbiology. During her junior year at UT, she began working as an undergraduate volunteer in the lab of Dr. Gary Stacey and she continued to volunteer until she received her B.S. in Microbiology in December, 1998. In January of 1999, Crystal entered graduate school in the Department of Microbiology at UT, as a doctoral student in the lab of Dr. Gary Stacey. On May 29, 1999 Crystal married her husband, Jesse McAlvin. In December 2003, Crystal received her Ph.D. from the University of Tennessee. She will continue her interest in scientific research by starting a postdoctoral position at Oak Ridge National Laboratory where she will investigate the carbon cycling pathway of the environmentally important *Nitrosomonas* bacteria.