To the Graduate Council:

I am submitting herewith a dissertation written by Robert B. Day entitled "Nod signal perception in the *Bradyrhizobium*-soybean symbiosis". I have examined the final 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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Associate Vice Chancellor and
Dean of The Graduate School

NOD SIGNAL PERCEPTION IN THE *BRADYRHIZOBIUM*-SOYBEAN SYMBIOSIS

A Dissertation

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Doctor of Philosophy

Degree

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For Noel

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ABSTRACT

The signal exchange mechanism mediating the onset of nodulation is a complex process involving the synthesis and perception of host-specific molecules bearing structurally distinct modifications. Central to the establishment of a symbiotic association between legumes and their rhizobial symbionts is the perception of rhizobial lipo-chitin Nod signals by the host plant. These signal molecules consist of a β-1,4-linked N-acetylglucosamine backbone of 3 to 5 residues modified in a species-specific manner at both the reducing and non-reducing termini.

This study focused on the *Bradyrhizobium*-soybean symbiosis. In an effort to define both the structural elements required for Nod signal perception, as well as to identify putative Nod signal binding proteins, a two-fold approach was used. Previous work (Minami *et al.*, 1996a) showed that an unsubstituted chitin oligomer could induce the expression of the early nodulin ENOD40. Therefore, a biochemical approach was used to identify and characterize a high-affinity binding site for *N*-acetylchitooligosaccharides localized in the plasma membrane of soybean. A 85 kDa binding protein was localized to the plasma membrane of both suspension-cultured soybean cells and root tissue from 5 day old seedlings. Inhibition analysis suggested a binding preference for larger (i.e., d.p.=8) *N*-acetylated chitin molecules, with a IC_{50} value of approximately 50 nM. *N*-acetyl-glucosamine and chitobiose showed no inhibitory effects at concentrations as high as 250 μM. Chitooligosaccharide specificity for induction of medium alkalinization and the generation of reactive

oxygen in suspension-cultured cells paralleled the binding activity of this protein. Taken together, the presence of the chitin binding protein in the plasma membrane, and the specificity and induction of a biological response upon ligand binding, suggest a role for the protein as an initial response mechanism for chitin perception in soybean, and not as a host-specific binding site for lipo-chitin Nod signals.

Recently, Etzler *et al.* (1999) reported the identification of a Nod signal binding protein present in the roots of the legume *Dolichos biflorus*. Therefore, we sought to identify orthologs of this protein in soybean. Two soybean cDNAs, termed GS50 and GS52, predicted to encode apyrase proteins of 50 kDa and 52 kDa, respectively, were isolated. Northern blot analysis indicate that GS52 mRNA is expressed in roots, hypocotyls, and flowers, whereas GS50 mRNA was expressed weakly in roots, yet abundantly in stems and flowers. Western blot analysis using antigen-purified antibodies to both GS50 and GS52 showed similar patterns of tissue localization. Furthermore, western blot analysis of sucrose gradient-fractionated membranes from root tissue revealed a differential cellular localization, with GS50 localizing to the Golgi, and GS52 to the plasma membrane.

GS50 was expressed constitutively in roots at a relatively low level under all conditions tested. However, GS52 mRNA levels in roots were significantly increased within six hours after inoculation with *Bradyrhizobium japonicum*. Therefore, GS52 can be classified as an early nodulin. Additionally, mRNA expression data also showed that GS52 was repressed in the presence of 20 mM nitrate, whereas expression of GS50 was not affected.

The differential expression pattern of GS50 and GS52 suggests that they likely have distinct cellular functions. The rapid induction of GS52 expression upon bacterial inoculation and the repression by nitrate suggest that this protein may have a function in the nodulation process.

TABLE OF CONTENTS

Page

CHAPTER 1 1
OVERVIEW OF THE NODULATION PROCESS 1
CHAPTER 2 3
LITERATURE REVIEW 3
SIGNAL EXCHANGE IN THE *RHIZOBIUM*-LEGUME SYMBIOSIS 3
Flavonoids signals and *nod* gene regulation 3
The lipo-chitin nodulation signals 7
Synthesis of the chitin backbone 10
Chemical modifications to the Nod signal 11
PLANT RESPONSES TO RHIZOBIAL INOCULATION AND INFECTION 12
ENOD40 14
ENOD5 and ENOD*12* 15
RIP1 17
OLIGOSACCHARIN PERCEPTION BY PLANTS 18
Oligoglucan 19
Oligogalacturonides 22
Chitin 24
Chitosan 25
Oligosaccharide Binding Proteins 26
β-Glucan 28
Chitin 30
Rice 30
Tomato 32
Lectins 33
Multiple recognition events? 35
Nod signal structure/function on soybean 37
Nod signal binding proteins 42
Apyrases 43
RATIONALE FOR PRESENT WORK 46

CHAPTER 3 49
MATERIALS AND METHODS 49
Chemicals 49
Bacterial stains and plasmids 49
Bacteria culture media and growth conditions 52
Plant germination and growth conditions 52
Enzymes and DNA primers 53
Membrane Chitin Binding Assays 53
Purification of plasma membrane enriched fractions from suspension-cultured cells and root tissue 53

Synthesis of ^{125}I-labeled aminophenyl derivatives of
N-acetylchitopentaose and *N*-acetylchitooctaose....56
Isolation of lipo-chitin Nod signals produced by
Bradyrhizobium japonicum USDA110....59
Binding assays and inhibition analysis....60
Affinity cross-linking....61
Reactive oxygen detection and quantitation....62
Alkalinization of soybean suspension cell medium in response
to perception of chitin oligosaccharides....63
ENOD40 induction by chitin oligomers....64
Thin layer chromatography of $(GN)_5$-APEA and $(GN)_8$-APEA....65
Nucleic Acid Isolation and Manipulation....66
Plasmid DNA isolation....66
Isolation of DNA fragments....67
Genomic DNA isolation....67
Isolation of *gs50* and *gs52* cDNA Clones....67
Isolation of total RNA and reverse transcription....67
TA cloning and DNA sequencing of PCR-amplified products....68
Extension of cDNA ends....69
Isolation of full-length cDNAs encoding *gs50* and *gs52*....69
Southern blot analysis....70
Northern blot analysis....70
***gs50* and *gs52* mRNA Expression Analysis**....71
Developmental expression....71
Tissue-specific expression of *gs50* and *gs52*....72
Bacterial inoculation assays....72
Nitrate expression assays....73
Generation of Antibodies to GS50 and GS52....73
Construction of plasmids expressing GST-GS50 and
GST-GS52 fusion proteins....73
Protein purification....74
GST-sepharose 4B affinity purification of GS50 and GS52....75
Immunization and antibody purification....76
Purification of antibodies by immuno-affinity chromatography....76
Construction of GST-GS50 and GST-GS52 antigen
affinity columns....77
Western Blot Analysis....77
Tissue-specific expression of GS50 and GS52....78
Fractionation of soybean root membranes on
sucrose density gradients....78
Membrane marker enzyme assays....80
Malachite green method of phosphate determination....81
Restriction Fragment-Linked Polymorphism (RFLP)
based mapping of GS52....82

CHAPTER 4.....83

RESULTS.....83

Section I: A high-affinity chitin binding site on the soybean plasma membrane.....83

Preparation of radiolabeled chitin oligosaccharide ligands.....83

Identification of a high-affinity binding site for chitin in the soybean plasma membrane.....85

Scatchard and Hill plot analysis.....87

Structural specificity and inhibition analysis of chitin binding.....90

Purification of lipo-chitin Nod signals produced by *B. japonicum* USDA110.....92

The major lipo-chitin Nod signal from *B. japonicum* USDA110 inhibits ^{125}I-(GlcNAc)$_5$-APEA binding to the plasma membrane of soybean.....94

Affinity cross-linking of the GN$_8$-APEA to soybean plasma membrane.....94

Generation of reactive oxygen in suspension-cultured soybean cells by N-acetylchitooligosaccharides.....99

N-acetylchitooligosaccharides induce alkalinization of culture medium of soybean suspension cells in a size-dependent manner.....101

Accumulation of ENOD40 mRNA by *N*-acetylchitooligosaccharides is dependent upon the degree of polymerization of the chitin backbone.....103

Section II: Identification of two soybean orthologs, *gs50* and *gs52*, to a Nod signal binding protein from *Dolichos biflorus*....106

PCR products generated using DNA primers to GS50 and GS52 hybridize to genomic DNA from *G. soja*.....117

gs50 and *gs52* mRNA are differentially expressed in soybean tissues.....119

gs52 mRNA is induced in the presence of *B. japonicum* USDA110.....121

gs52 mRNA expression is repressed in the presence of nitrate.....126

Production and purification of GS50 and GS52 fusion proteins.....128

GS50 and GS52 proteins are differentially expressed in soybean tissues.....131

Anti-GS52 antibodies block nodulation of soybean by *B. japonicum* USDA110.....134

Subcellular localization of GS50 and GS52 using membrane separation in sucrose density gradients.....135
Genetic mapping of GS52 on an integrated genetic linkage map of the soybean genome.....137
CHAPTER 5.....143

DISCUSSION.....143

REFERENCES.....159

VITA.....197

List of Figures

Page

Figure 1: Proposed model of *nod* gene regulation in *Bradyrhizobium japonicum*.....5
Figure 2: Some examples of the lipo-chitin Nod signals produced by rhizobia.....9
Figure 3: Structures of the four major classes of oligosaccharin elicitor molecules.....20
Figure 4: Proposed model for lipo-chitin Nod signal perception in the *Bradyrhizobium*-soybean symbiosis.....41
Figure 5: Chemical structure of A) 2-(4-Aminophenyl)ethylamine and B) Iodogen.....58
Figure 6: Structure of ligands used in saturation analysis, inhibition analysis, and affinity cross-linking studies. A) β-1,4-linked *N*-acetylchitooligosaccharide. B) Chitin-APEA ligand.....84
Figure 7: Binding of ^{125}I-(GlcNAc)$_8$-APEA ligand to purified plasma membrane is saturable.....86
Figure 8: Scatchard analysis of ^{125}I-(GN)$_8$-APEA binding to soybean plasma membrane.....88
Figure 9: Hill plot analysis.....89
Figure 10: Inhibition of chitin oligosaccharide binding to a plasma membrane-enriched fraction from soybean proceeds in a size-dependent manner.....91
Figure 11: HPLC elution profile of the Nod signals produced by *B. japonicum* USDA110.....93
Figure 12: Inhibition analysis of ^{125}I-(GlcNAc)$_5$-APEA binding to soybean root plasma membrane.....95
Figure 13: Affinity cross-linking of ^{125}I-(GlcNAc)$_8$-APEA ligand to the plasma membrane-localized 85 kDa binding protein.....97
Figure 14: Affinity cross-linking of soybean plasma membrane.....98
Figure 15: Generation of reactive oxygen species in suspension-cultured soybean cells by N-acetylchitooligosaccharides.....100
Figure 16: Alkalinization of soybean suspension cell culture medium in response to chitin oligomers.....102
Figure 17: Size-dependent induction of ENOD40 mRNA by *N*-acetylchitooligosaccharides.....104
Figure 18: Induction of ENOD40 expression by chitin oligomers 40 hours post-inoculation.....105
Figure 19: Nucleotide and translated protein sequence of *gs50*.....107
Figure 20: Nucleotide and translated protein sequence of *gs52*.....110

Figure 21: Multiple protein sequence alignment of apyrase proteins from a variety of eukaryotic species....114
Figure 22: Phylogenetic analysis of apyrase proteins from plants and animals....115
Figure 23: Hydropathy analysis of GS50 and GS52....116
Figure 24: Southern blot analysis of *G. soja* genomic DNA....118
Figure 25: Reverse-transcriptase PCR analysis of *gs50* and *gs52* expression in various tissues from *G. soja*....120
Figure 26: RT-PCR analysis of GS50 mRNA developmental expression (-USDA110) and expression under conditions of rhizobial inoculation +USDA110)....122
Figure 27: Graphical analysis of total counts from developmental and *B. japonicum* inoculation expression of GS50 mRNA....123
Figure 28: RT-PCR analysis of GS52 mRNA developmental expression (-USDA110) and expression under conditions of rhizobial inoculation (+USDA110)....124
Figure 29: Graphical analysis of total counts from developmental and *B. japonicum* inoculation expression of GS52 mRNA....125
Figure 30: RT-PCR time course expression of *gs50* and *gs52* in the presence of 20 mM ammonium nitrate....126
Figure 31: Silver stain of SDS-PAGE analysis of GS50 and GS52 GST-fusion proteins expressed in *E. coli*....129
Figure 32: SDS-PAGE and silver stain analysis of urea extraction of GST-GS50 fusion protein from *E. coli* GEX50 inclusion bodies....130
Figure 33: Purified GST-fusion proteins....132
Figure 34: Western blot analysis of total protein extracts from various tissues and nodules from *G. soja*....133
Figure 35: Effect of anti-GS50 and anti-GS52 serum on nodulation of *G. soja* by *B. japonicum* USDA110....136
Figure 36: Sucrose membrane marker standards....138
Figure 37: Western blot analysis of sucrose-fractionated membranes isolated from 5-day-old soybean roots....139
Figure 38: Membrane localization of GS50 and GS52 based on sucrose density centrifugation of microsomal membrane fractions from *G. soja* roots....140
Figure 39: Genetic map of Major Linkage Group (MLG) J....142

List of Tables

Page

Table 1: Proposed function of the known nodulation (*nod, nol, noe*) genes....8
Table 2: Plant responses to rhizobial inoculation....13
Table 3: Biochemical and biological properties of the major classes of binding sites for oligosaccharide elicitors....27
Table 4: Bacterial strains and plasmids....50
Table 5: DNA primers used in this study....54

CHAPTER 1

OVERVIEW OF THE NODULATION PROCESS

Legumes possess the unique ability to overcome conditions of nitrogen stress by establishing a host-specific symbiotic association with bacteria of the family *Rhizobiaceae*. Gram negative soil bacteria belonging to the genera *Bradyrhizobium*, *Allorhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium* synthesize and secrete a complex mixture of phyto-active compounds known as Nod signals. Perceived by the developing root, these compounds trigger the onset of a physiological metamorphosis leading to the formation of a new organ, the nodule. It is within the nodule that the bacteria reside, housed within nitrogen-fixing compartments known as symbiosomes, providing a source of reduced nitrogen for the host plant. In turn, the bacteria feed from carbon resources provided by the host plant. The events leading up to the formation of the nodule and subsequent development of the symbiosome are marked by a number of molecular and physiological cues. It is the exchange of signals and the systematic induction of both plant and bacterial genes which lead to the successful establishment of a nitrogen-fixing symbiosis. In recent years, much attention has been focused on the molecular and biochemical aspects of the establishment of the rhizobial-legume symbiosis in terms of signal perception and transduction. Although a considerable amount is known with regard to the physiological responses of both the bacterial symbiont and

the host legume during the symbiotic association, much remains to be understood with regard to the mechanism of signal perception and the activation of the biochemical processes required for the successful establishment of a symbiotic association between rhizobia and legumes.

CHAPTER 2

LITERATURE REVIEW

SIGNAL EXCHANGE IN THE *RHIZOBIUM*-LEGUME SYMBIOSIS

Flavonoids Signals and nod Gene Regulation

Within the rhizosphere, communication between the legume host and the invading rhizobia must be established for successful infection and nodulation to occur. The microenvironment surrounding the legume root is the site of initial signal exchange. Here, the invading rhizobia come into contact with a mixture of plant exudates, which signal both the migration of the bacteria to the root surface and the induction of gene expression required for infection. The chemical composition of plant exudates which control both the activation and repression of rhizobial gene expression has been reviewed extensively (Peters *et al.*, 1986; Kosslak *et al.*, 1987; Hungria *et al.*, 1991; Bellato *et al.*, 1996; Mergaert *et al.*, 1997). Flavonoids present in plant exudates are the most potent inducers of the rhizobial nodulation genes, which are required for infection (Pueppke, 1996). Interestingly, analysis of plant exudates from numerous legume species has revealed a great deal of variability in flavonoid composition (Redmond *et al.*, 1986; Kosslak *et al.*, 1987; Hungria *et al.*,

1992; Phillips, 1992; Niebel *et al.*, 1998). This variability is thought to play an important role in host range determination (Zaat *et al.*, 1987; Peters and Verma, 1990; Sutherland *et al.*, 1990). Each *Rhizobium* is adapted to recognize the flavonoids exuded by its compatible host. In the case of *B. japonicum*, the isoflavones daidzein and genistein, found in soybean root exudates, have been shown to be the primary inducers of *nod* gene expression (Kosslak *et al.*, 1987; Banfalvi *et al.*, 1988; Göttfert *et al.*, 1988; Smit *et al.*, 1992).

The regulation of the *nod* genes appears to vary from strain to strain, but is always mediated by NodD. NodD belongs to a family of LysR-type transcriptional regulators which include *E. coli* LysR, CysB, and *S. typhimurium* MetR (Schell, 1993). Activation of the NodD-dependent pathway is believed to be mediated by the interaction of $NodD_1$ with plant-derived host-specific flavonoids, followed by the subsequent binding of the $NodD_1$ protein to the conserved *nod* box upstream of the *nod* operon (reviewed in Day *et al.*, 2000b) (Figure 1). Although direct binding of plant flavonoids to NodD has not been shown, mutational analysis of *nodD* has resulted in the extension of the *Rhizobium*-legume host-range (McIver *et al*, 1989), thereby suggesting that a correlation exists between the spectrum of flavonoids able to interact with NodD proteins and host-range determination. Thus, NodD appears to serve, in part, as a molecular interface between the invading *Rhizobium* and the host legume.

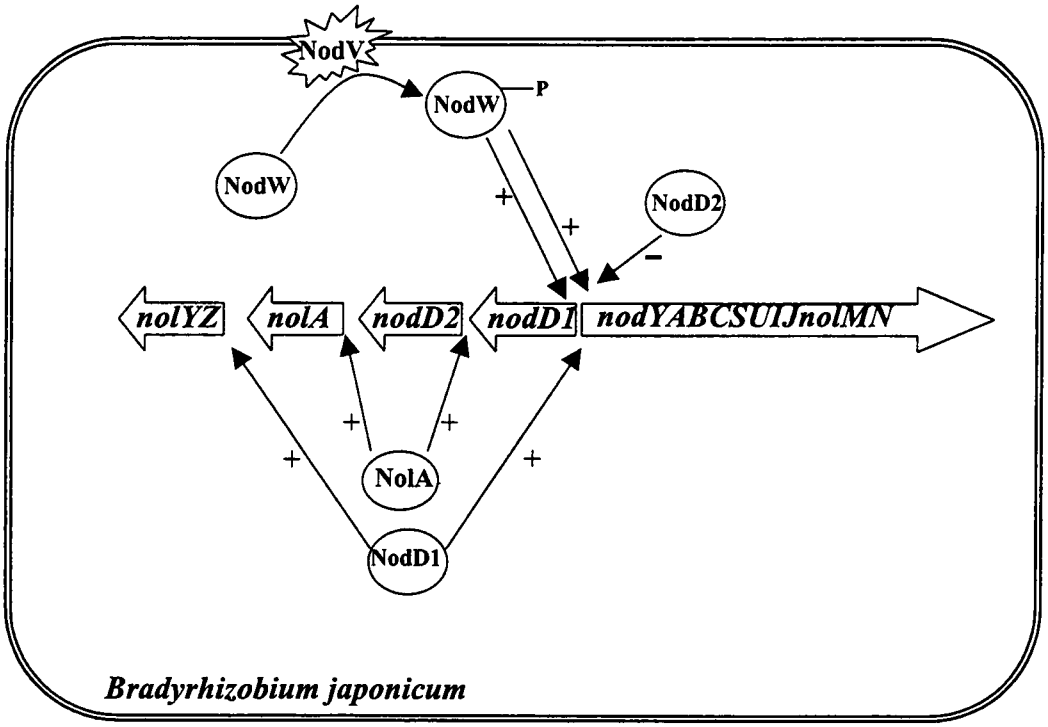


Figure 1: Proposed model of *nod* gene regulation in *Bradyrhizobium japonicum*. Open arrows indicate the open reading frames of the nodulation genes of *B. japonicum* USDA110. +, positive regulation; -, negative regulation; P, phosphorylation.

In the *Bradyrhizobium*-legume symbiosis, a second, species-specific regulatory system is also present. This mechanism involves NodV and NodW, a membrane-localized sensor and a transcriptional regulator, respectively. The *nod*VW genes were first identified by Göttfert *et al.* (1990) who demonstrated that NodV and NodW were essential for the nodulation of cowpea and siratro, alternative host plants of *B. japonicum*. The sensor, NodV, upon perception of the isoflavonoid signal (e.g., genistein), initiates a phosphotransfer reaction by autophosphorylation (Loh *et al.*, 1997). The phosphorylated NodV then phosphorylates the regulator protein, NodW, by transferring the phosphoryl group to the conserved aspartate (Asp70) residue of NodW. These results indicate that NodV and NodW comprise a second, in addition to NodD1, isoflavone recognition system in *B. japonicum* (Figure 1).

nod gene expression is also subject to negative regulation. A third component in the regulation of *nod* gene activity in the *Bradyrhizobium*-legume symbiosis involves NolA, a member of the MerR-type family of regulatory proteins (Summers, 1992). NolA was first reported as a genotype-specific nodulation factor (Sadowsky *et al.*, 1988). Subsequently, Dockendorff *et al.* (1994) suggested that NolA might function as a repressor of *nod* gene transcription. Negative control of *nod* gene transcription has also been reported in *Sinorhizobium meliloti* (Kondorosi *et al.*, 1989). A repressor, NolR, was identified and found to be present in most of the *S. meliloti* isolates. NolR represses the expression of the *nod*D and *nod*ABC genes by binding to a 26-bp site in the promoters of these genes. Unlike NolR, current information suggests that NolA does not act directly to repress *nod* gene expression.

This view stems from the observation that while NolA expression from a multicopy plasmid in *B. japonicum* resulted in significant reduction in *nod* gene expression, interposon mutations in *nolA* did not lead to elevated *nod* gene expression. The latter result suggested that NolA did not repress *nod* gene expression directly, but may affect repression through another protein. A likely candidate is the $NodD_2$ protein, which has been reported to repress *nod* gene expression in *Rhizobium* spp. NGR234 (Fellay *et al.*, 1998) and *Bradyrhizobium* spp. (*Arachis*) NC92 (Gillette and Elkan, 1996).

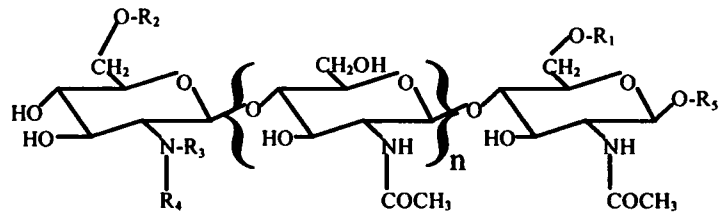
The lipo-chitin nodulation signals

It is now known that most of the nodulation genes code for the enzymatic machinery to synthesize unique lipo-chitooligosaccharides (LCO) molecules, the Nod signals (reviewed in Denarie *et al.*, 1992; Long, 1989, 1996; Mergaert *et al.*, 1997; Spaink, 1992) (Table 1). Nod signals consist of a β-1,4-linked *N*-acetylglucosamine (GlcNAc) backbone of three to five residues that is modified at both the reducing and non-reducing end in a species-specific manner (Figure 2). Active at sub-nanomolar concentrations, these phytoactive compounds can trigger the onset of nodule organogenesis when applied to the developing legume root.

Table 1: Proposed function of the known nodulation (*nod, nol, noe*) genes.

Gene	Proposed Function	Reference
Regulatory genes		
nodD$_1$	Transcriptional activator	Gottfert *et al.*, 1992
nodV	Two-component regulator	Loh *et al.*, 1998
nodW	Two-component regulator	Loh *et al.*, 1998
nolA	Transcriptional regulator	Sadowsky *et al.*, 1991
nolR	Repressor. DNA binding protein	Kondorosi *et al.*, 1989
SyrM	Transcriptional regulator	Hanin *et al.*, 1998
Nod signal core synthesis		
nodA	Acetyltransferase	Egelhoff and Long, 1985
nodB	Deacetylase	John *et al.*, 1993
nodC	Chitin synthase	Geremia *et al.*, 1994
nodM	D-glucosamine synthase	Baev *et al.*, 1992
Nod signal modifications		
nodE	β-Ketoacylsynthase	Demont *et al.*, 1993
nodF	Acyl carrier protein	Shearman *et al.*, 1986
nodH	Sulfotransferase	Ehrhardt *et al.*, 1995
nodL	Acetyltransferase	Berck *et al.*, 1999
nodS	Methyltransferase	Mergaret *et al.*, 1995
nodU	Carbamoyltransferase	Jabbouri *et al.*, 1995
nodP	ATP-sulfurylase	Schwedock *et al.*, 1994
nodQ	ATP-sulfurylase	Schwedock *et al.*, 1994
nodH	Sulfotransferase	Roche *et al.*, 1991
nodX	Acetyltransferase	Davis *et al.*, 1988
nodZ	Fucosyltransferase	Quinto *et al.*, 1997
nolK	NAD-dependent sugar epimerase	Mergaret *et al.*, 1996
nolL	O-acetyltransferase activity	Berck *et al.*, 1999
nolO	Carbamoyltransferase	Jabbouri *et al.*, 1998
nolXWBTUV	Cultivar-specific nodulation	Kovacs *et al.*, 1995
nolYZ	Type III Secretion	Viprey *et al.*, 1998
noeC	Arabinosylation	Mergaret *et al.*, 1996
noeD	Genotype-specific nodulation	Lohrke *et al.*, 1998
noeE	Sulfotransferase	Hanin *et al.*, 1997
noeI	2-O-methylation	Jabbouri *et al.*, 1998
noeJ	Phosphate guanyltransferase	Fellay *et al.*, 1995
noeK	Phosphomannomutase	Fellay *et al.*, 1995
noeL	Dehydratase	Fellay *et al.*, 1995
Transport		
nodI	ATP binding protein, Nod signal transport	Jabbouri *et al.*, 1996
nodJ	Integral membrane protein, Nod signal	Cardenas *et al.*, 1996
nodT	transport Outer membrane transport?	Rivilla *et al.*, 1995
nodO	Secreted, pore-forming protein	De Maagd *et al.*, 1992

A.



B.

Rhizobia	Host Plant	R_1	R_2	R_3	R_4	R_5	n
B. japonicum USDA110	Soybean (*G. max/soja*)	2-O-methyl fucose	H	H	C18:1/C16:0	H	3
USDA135		2-O-methyl fucose	H	H	C18:1/C16:0	H	3
S. meliloti	Alfalfa	Sulfate	H	H	C16:1/C16:2/ C16:3/C18 to C26	H	2, 3
R. tropici	*Phaseolus, Leucaena, Medicago, Macroptilium*	Sulfate, Hydrogen	H	Me	C18:1	Mannosyl	3
R. fredii	Soybean (*G. max*)	2-O-methyl fucose/fucose	H	H	C18:1	H	1-3
R. NGR 234	Broad host range (18 genera)	2-O-methyl- fucose/3-S-methyl- fucose/3 or 4 acetyl- methyl-fucose	Cb or H	Me	C18:1/C18:0	H	2, 3
R. leguminosarum bv *viciae*	Pea, Vetch	Hydrogen	Ac	H	18:1/C18:4	H	2, 3
R. leguminosarum bv *trifolii*	Clover	Acetyl	Ac	H	C18:1/C18:4	H	2, 3
R. etli	*Phaseolus*	4-*O*-acetyl-*L*-fucose	H	Me	C18:4	H	
A. caulinodans	*Sesbania*	Arabinose/Hydrogen	Cb	H	C18:1/C18:0	H	2, 3

Cb=carbamaoyl; Me=methyl; Ac=acetyl.

Figure 2: Some examples of the lipo-chitin Nod signals produced by rhizobia. A. Structure of the chitin backbone of the rhizobial Nod signal. B. Various modifications to the Nod signal.

Synthesis of the chitin backbone

The common nodulation genes, *nodABC*, are conserved in all rhizobia and are involved in synthesis of the Nod signal molecule. The first step in the assembly of the Nod signal is the transfer of GlcNAc residues from UDP-GlcNAc to O4 of the nonreducing-terminal residue (Kamst *et al.*, 1999). This transfer reaction is performed by an *N*-acetylglucosaminyltransferase encoded by the product of the *nodC* gene (Debelle *et al.*, 1992). The NodC protein shares a significant degree of similarity to chitin synthase (e.g., >60%) (Bulawa, 1992). NodB, responsible for the next step in the assembly of the Nod signal, carries out the de-acetylation of the glucosamine residue, preparing the non-reducing end of the oligosaccharide for the addition of the acyl chain (John *et al.*, 1993; Kafetzopoulos *et al.*, 1993). The final step in the assembly of the chitin backbone is performed by NodA, an acyl-transferase, which catalyzes the addition of a fatty acid to the de-acetylated glucosamine at the non-reducing end of the oligomer (Atkinson *et al.*, 1994; Rohrig *et al.*, 1994; Kamst *et al.*, 1999). Both NodC and NodA are host-specific enzymes (Debelle *et al.*, 1996; Ritsema *et al.*, 1996). For example, NodC determines the chitin chain length (e.g., tetramer or pentamer), whereas NodA determines the addition of a specific acyl moiety.

Chemical modifications to the Nod signal

Host specificity in the symbiotic association between legumes and rhizobia is dictated, in part, by the modifications to the chitin oligosaccharide backbone of the Nod signal. The modifications are catalyzed by protein encoded by the host-specific nodulation genes (Table 1). In the case of *B. japonicum*, modifications of the Nod signal include the addition of a 2-*O*-methyl fucose moiety at the reducing end and a C18:1 fatty acid (vaccenic acid) at the non-reducing end (Table 2: Sanjuan *et al.*, 1992). In the case of the *S. meliloti*-alfalfa (*Medicago sativa*) symbiosis, the products of the *nodHPQ* genes function to add sulfate to the reducing end of the Nod signal (Denarie *et al.*, 1992). Mutations in any of these genes abolish the ability of *S. meliloti* to nodulate alfalfa, yet these mutants retain their ability to nodulate an alternative host, *Vicia sativa* (vetch) (Lerouge *et al.*, 1990; Roche *et al.*, 1991; Roche *et al.*, 1996). Transfer of the *S. meliloti nodHPQ* genes to *R. leguminosarum*, which normally nodulates pea, extends the host range to include alfalfa. More recently, variation in the *N*-acyl chain length has surfaced as a critical determinant in the specificity of the *S. meliloti* Nod signal (Demont-Caulet *et al.*, 1999). Demont-Caulet *et al.* (1999) demonstrated a correlation between fatty acid chain length and the ability to initiate root hair formation. This work suggests that the acyl chain may be involved in insertion of the Nod signal into the plant membrane, promoting lateral diffusion and subsequent interaction with a membrane receptor. In the case of *R. leguminosarum*, the specific *N*-acylation and degree of acetylation appear to be

critical determinants for the biological activity of the Nod signal (reviewed in Spaink, 1992).

PLANT RESPONSES TO RHIZOBIAL INOCULATION AND INFECTION

During the infection process, the systematic activation of plant genes associated with the onset and progression of nodulation is observed in response to the invading *Rhizobium*. Table 2 lists a number of events that have been characterized thus far. In addition to the induction of a number of physiological events within the developing root (e.g., root hair curling and calcium spiking), the transcriptional activation of plant genes believed to be directly involved in the nodulation process is also observed. Depending upon their time of activation, these genes can be classified as early or late nodulins. Classical examples of early nodulins include *enod2*, *enod12*, and *enod40* (Scheres *et al.*, 1990; Pichon *et al.*, 1992; Kouchi and Hata, 1993). Among those classified as late nodulins are leghemoglobin and uricase (Papadopoulou *et al.*, 1995; Heidstra *et al.*, 1997). Although nodulin genes were originally thought to be specifically expressed during the symbiotic association, a number are likely to participate in other plant developmental processes. In support of this hypothesis, several homologues have been identified in non-legume species (Crespi *et al.*, 1994; Cook *et al.*, 1995; Bauer *et al.*, 1996). Thus, the term nodulin might now be used as a broad classification of plant genes whose temporal expression pattern marks the onset and progression of nodule development. There remain,

Table 2: Plant responses to rhizobial inoculation. Note: These represent the collective data from numerous legume species, and should not imply that all responses are observed in all legumes.

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.*, 1995
2 minutes	Membrane Depolarization	Ehrhardt *et al.*, 1996
2-10 minutes	Calcium Spiking in root hairs	Ehrhardt *et al.*, 1996
5 minutes	Intracellular alkalization 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	Boulki *et al.*, (person. comm.)
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.* (1999b.)
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.*, 1996b
24 hours	Extensin mRNA	Arsenijevic *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
4-5 days	ENOD40 induction (specific)	Minami *et al.*, 1996b
5-6 days	ENOD2 induction	Minami *et al.*, 1996a
4 days	Development of nodule meristem	Turgeon and Bauer, 1982
4-10 days	Visible nodules	

however, a number of genes that are unique in that their expression pattern is regulated to some degree by the plant's perception of rhizobial signals, both secreted and otherwise.

ENOD40

First induced in the root pericycle following rhizobial inoculation, *ENOD40* temporal and spatial expression coincides with the progression of nodulation within the rapidly dividing cortical cells during nodule organogenesis. In addition to its postulated role in nodule initiation, *ENOD40* has also been implicated in the late stages of nodule differentiation (Yang *et al.*, 1993). With an increasing number of homologues having now been identified in both legumes and non-legumes, it has also been postulated that *ENOD40* may be a universal, endogenous regulator of plant growth and development. Sequence analysis of *ENOD40* transcripts isolated from both legume and non-legume species has revealed the presence of a short open reading frame (ORF) at the 5' end of the gene, which has the capacity to encode a peptide of between 10-13 amino acids (Kouchi and Hata, 1993; Yang *et al.*, 1993; Crespi *et al.*, 1994; Vijn *et al.*, 1995; Papadopoulou *et al.*, 1996). The second conserved region, which is located at the 3'-untranslated end of the gene, although lacking an ORF, has been postulated to also play a role in plant development. Because this region has a propensity to form a stable secondary structure characteristic of a biologically active RNA species, it was suggested that the *enod40*

transcript might act as a riboregulator involved in cell division and differentiation (Crespi *et al.*, 1994).

Recently, Kouchi *et al.* (1999) reported on the identification and characterization of *ENOD40* homologues from rice (*Oryza sativa*), and presented new evidence suggesting a role for *ENOD40* that extends beyond the proposed role in cortical cell division and the initiation of nodule primordia. Using *in situ* hybridization analysis it was shown that *OsENOD40* expression coincided with the early stages of basipetal differentiation of the vascular bundles. In legumes, during the late stages of nodule development, *ENOD40* expression is also abundant in the vascular bundles of the developing nodule. Thus, it appears that legume *ENOD40* and *OsENOD40* share a common feature in that their spatial expression patterns are localized to regions associated with cell differentiation of the vascular bundle. These findings imply that *ENOD40* has a primary function other than inducing cortical cell division, and may in fact play a role in cell differentiation.

ENOD5 and ENOD12

A detailed analysis of the action of Nod signals requires the identification of plant genes that can serve as molecular markers for the developmental processes that occur during the nodulation process. In addition to ENOD40 described above, an increasing number of plant genes whose temporal and spatial expression patterns signal discrete developmental processes during the progression of nodulation have

been identified. For example, *enod5* and *enod12* encode (hydroxy) proline-rich proteins, and are the earliest nodulin genes to be induced upon perception of rhizobial Nod signals. In the presence of rhizobia, *enod12* is rapidly induced in the root epidermis (Scheres *et al.*, 1990). Although an exact role for ENOD12 during nodulation has not been demonstrated, expression studies in alfalfa (*M. varia*) have demonstrated a highly-specific, localized zone of expression within the developing root (Journet *et al.*, 1994). Using an ENOD12-GUS fusion, a series of activation assays were undertaken with a variety of *Sinorhizobium meliloti* Nod signals. In this study, it was shown that the maximal level of GUS activity was restricted to a zone localized in the differentiating epidermal cells that lie just behind the rapidly growing root tip. These data support the hypothesis that the region of the root which is most susceptible to rhizobial infection lies between the root tip and the zone of first emergent root hairs (reviewed in Caetano-Anollés and Gresshoff, 1991). Therefore ENOD12 expression might function to make the root more susceptible to *Rhizobium* infection. However, an exact role for ENOD12 during nodule organogenesis has not been determined.

Like *ENOD12*, *ENOD5* also encodes a proline-rich protein hypothesized to be a cell wall component involved in the infection process (Bauer *et al.*, 1994). The differential expression pattern of *ENOD12* and *ENOD5* provide molecular markers correlating gene expression and Nod signal-induced morphological changes in the developing root. Vijn *et al.* (1995) undertook an extensive analysis of the differential

expression patterns of *ENOD5* and *ENOD12* in vetch (*Vicia sativa*). They found that in *Rhizobium*-induced primordia there is a significant difference in the spatial distribution of cells which are expressing either *ENOD5* or *ENOD12*. Interestingly, *ENOD12* transcripts were found in all cells, yet *ENOD5* transcripts were localized only in cells which were infected by bacteria, and thus were in close proximity to the rhizobial Nod signals. These data suggest the possible involvement of a diffusable, secondary signal molecule that is generated upon Nod signal perception. One possible candidate for such a secondary messenger is uridine (stele factor), which has been shown to complement the action of Nod signal-induced cortical cell divisions in vetch (Schlaman *et al.*, 1997).

RIP1

The perception of host-specific Nod signals dictates the establishment of a successful *Rhizobium*-legume symbiosis. However, it has been suggested that the establishment of a host-specific *Rhizobium*-legume symbiosis is not solely determined by the perception of Nod signals, but also by the ability of the invading rhizobia to suppress the host plant's defense response (Baron and Zambryski, 1995). In terms of the signal transduction mechanism that is initiated upon perception of fungal pathogens by plants, the generation of an oxidative burst is among the best-characterized responses (reviewed in Wojtaszek, 1997). Recently, Cook *et al.* (1995) reported on the identification of a *Rhizobium*-induced-peroxidase (*rip1*) that is

induced within 3 hours of rhizobial inoculation. Interestingly, inoculation of plants with an incompatible *Rhizobium* (i.e., a non-symbiont of the legume) does not result in *rip1* induction. The spatial expression pattern of *rip1* also suggests a possible involvement in the establishment of a symbiotic association. *In situ* hybridization of *M. truncatula* roots showed *rip1* was localized within the differentiating epidermal cells in the root zone subsequently infected by *Rhizobium*. More recently, Ramu *et al.* (1999) showed that *rip1* induction parallels the spatial distribution of the oxidative burst response. Induction of the oxidative burst was dependent upon structural determinants of the *R. meliloti* Nod signal. These data suggest that the induction of *rip1*, and the subsequent elicitation of a localized oxidative burst response in alfalfa, serve to potentiate the onset of a compatible *Rhizobium*-legume symbiosis. The exact function of the oxidative burst response in the *Rhizobium*-legume symbiosis is largely undefined. However, one hypothesis is that the generation of reactive oxygen species may serve as a second messenger to recruit other genes for the purpose of nodulation (Cook *et al.*, 1995).

OLIGOSACCHARIN PERCEPTION BY PLANTS

Among the earliest elicitors of a plant defense response to be characterized in greatest detail were the oligosaccharidic polymers that constitute the outer cell walls of pathogenic microorganisms. To date, four major classes of oligosaccharides have

been characterized as having the ability to elicit a defense-related response in a number of plant species: oligochitin, oligochitosan, oligoglucan, and oligogalacturonide. An increasing amount of evidence now suggests that oligosaccharides are important signal molecules that have the capacity to mediate a variety of cellular processes, including growth and development, as well as to mediate defense responses in a number of plant species.

Oligoglucan

The elicitor activity of branched 1,3-1,6-β-glucans was identified by Anderson-Prouty and Albersheim (1975). This study was the first to demonstrate the ability of a fungal cell wall component to induce a defense-related response in plants. These elicitors are composed of 3-, 6-, and 3,6-linked β-glucosyl residues, a composition similar to the glucans that are major constituents of the cell wall of the soybean pathogen *Phytophthora sojae* (Ayers *et al.*, 1976). The smallest active elicitor oligoglucoside purified from the complex mixture of oligoglucosides generated by partial acid hydrolysis of mycelial cell walls is a branched hepta-β-glucoside. The molecule has a backbone of five (1→6)-linked β-glucosyl residues with two terminal β-glucosyl residues attached at C-3 of the second and fourth backbone glucose rings (Figure 3A). These phytoactive compounds are able to induce the rapid accumulation of antimicrobial phytoalexins and thus serve as a

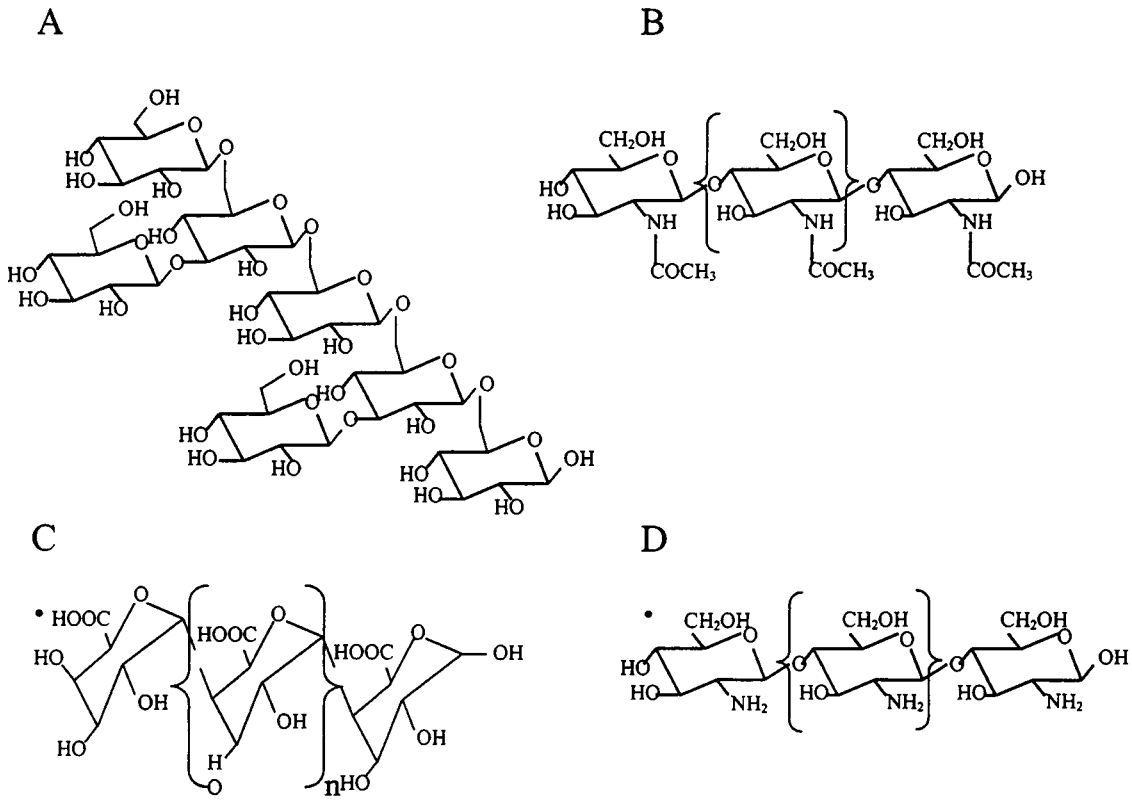


Figure 3. Structures of the four major classes of oligosaccharin elicitor molecules. A) 1,3-1,6-β-glucan. B) *N*-acetylglucosamine (chitin). C) 1,4-linked α-D-galactosyluronic acid. D) Chitosan.

primary signal for the cascade of events in the establishment of a host-specific defense response.

The mutual signaling between plants and microbes in the host-specific defense response extends beyond the initial perception of microbial-derived elicitors. Recent work has demonstrated that the soybean symbiont, *B. japonicum* USDA110, also synthesizes cyclic 1,3-1,6-β-glucans during the symbiotic interaction (Rolin *et al.*, 1992; Gore and Miller, 1993). Interestingly, β-glucans produced by the bradyrhizobial symbiont do not induce the accumulation of phytoalexins at physiological concentrations (1 μM), yet β-glucans produced by a *Rhizobium* which does not infect soybean induces phytoalexin accumulation (Mithöfer *et al.*, 1996). These findings suggest that the establishment of a successful plant-symbiont interaction may occur through a novel mechanism whereby the rhizobial symbiont suppresses the plant's defense response. Recently, Bhagwat *et al.* (1999) examined the structure of the glucan synthesized by *B. japonicum* strain AB-1, and determined that the cell membrane consists of cyclic cyclodecakis-(1→3)-β-D-glucosyl, a β-(1→3)-linked decasaccharide in which one of the residues is substituted in the 6 position with β-laminaribiose. Cyclodecakis-(1→3)-β-D-glucosyl did not suppress the fungal β-glucan-induced plant defense response in soybean cotyledons and had much lower affinity for the putative membrane receptor protein than cyclic β-(1→3),β-(1→6)-glucans produced by wild-type *B. japonicum*. This is consistent with

the hypothesis presented previously that the wild-type cyclic β-glucans may function as suppressors of a host defense response.

Oligogalacturonides

In addition to the β-glucan elicitors released from the fungal cell wall, lytic enzymes secreted by the invading pathogen promote the release of polysaccharide fragments from the host plant that play an important role in signaling a defense-related response. Oligogalacturonides, 1,4-linked α-D-galactosyluronic acid (Figure 3C), released from the pectic component of plant cell walls via the action of polygalacturonase and pectate lyase are capable of initiating a broad range of plant defense responses (Bishop *et al.*, 1984; Ryan and Farmer, 1991). Unlike the β-glucan fragments described above, as well as the chitin oligosaccharide fragments (described below), the pectic fragments generally require a much greater degree of polymerization (e.g., d.p. =10-20) in order to initiate a biological response (Darvill *et al.*, 1992). Defense responses include the synthesis of phenylalanine ammonia-lyase and chalcone synthase, the induction of chitinases, as well as the activation of a number of protease inhibitors (reviewed in Côte and Hahn, 1994). The exact nature of the plant defense response(s) induced by oligogalacturonide elicitors varies from species to species. For example, oligogalacturonides have been shown to induce the accumulation of phytoalexins in soybean, castor bean, pea, and parsley (Walker-

Simmons *et al.*, 1984; Davis *et al.*, 1986; Davis and Hahlbrock, 1987). In *Lithospermum* spp., oligogalacturonides induce the biosynthesis and accumulation of the anti-microbial compound shikonin (Tani *et al.*, 1992). Other responses include the depolarization of the plasma membrane (Thain *et al.*, 1990; Mathieu *et al.*, 1991; Thain *et al.*, 1995), extracellular alkalinization (Mathieu *et al.*, 1991), as well as the increase of free calcium in the cytosol (Farmer *et al.*, 1991).

A signaling mechanism by which oligogalacturonides may activate the expression of defense-related genes in plant cells has been proposed (Eberhard *et al.*, 1985; Marfa *et al.*, 1991). The proposed pathway involves mediation of hormone levels via the action of oligogalacturonic acid. Bellincampi *et al.* (1993) showed that the addition of exogenous oligogalacturonides to tobacco leaf explants could inhibit the formation of roots. By increasing the amount of auxin in the medium, this effect could be overcome, thereby suggesting that oligogalacturonides may act in an antagonistic manner to mediate the action of auxin in plant development (Bellincampi *et al.*, 1993, 1996). The role of oligogalacturonides in both the plants ability to mount a defense response, as well as to modulate the action of endogenous hormones during plant development, support the hypothesis that oligosaccharides could be a new class of endogenous signal molecules.

Chitin

Chitin perception by plants in response to microbial invasion plays an integral role in cell signaling during pathogenesis (Shibuya *et al.*, 1996; Stacey and Shibuya, 1997). Conversely, modified chitin oligosaccharides (e.g., Nod signals) play a central role in the establishment of a host-specific symbiosis between legumes and their rhizobial symbionts. Although much work has been done to elucidate the numerous responses evoked upon ligand recognition, relatively little is known regarding how these signals are perceived by the host plant. However, it is apparent that chitin perception by diverse plant species does have some similarity (reviewed in Stacey and Shibuya, 1997). For example, tomato, soybean, carrot, wheat, and rice have the ability to respond to the application of chitin oligosaccharides (Baureithel *et al.*, 1994; Minami *et al.*, 1996a, 1996b; De Jong *et al.*, 1993; Barber *et al.*, 1989; Shibuya *et al.*, 1993). These responses include the induction of mRNA expression in soybean (Minami *et al.*, 1996c, 1998), the depolarization of the rice plasma membrane (Kuchitsu *et al.*, 1993), as well as an effect on embryogenesis and re-initiation of meristematic cell division (Kragh *et al.*, 1996; Baldan *et al.*, 1997). Thus, it is likely that most plants possess a common chitin recognition system.

Chitosan

Virtually identical to the *N*-acetylchitooligosaccharides described above, the last class of oligosaccharin molecules exhibits a strikingly different activity in terms of perception and host specificity. The de-*N*-acetylated β-1,4-linked glucosamine polymers (chitosan) (Figure 3D) are not active in those plants where the chitin oligomers are active, and thus appear to involve a distinct signaling mechanism. Chitosan elicitors were first implicated in host-parasite interactions when they were identified in the pea-fungus interface by immuno-histological methods (Hadwiger *et al.*, 1981). Chitosan-like polymers are released from the fungal cell wall when in contact with plant tissue and in the presence of β-glucanase and plant chitinases. Chitosan elicitors induce such responses as phytoalexin accumulation (Hadwiger *et al.*, 1994) and the expression of disease resistance response proteins in pea (Hadwiger *et al.*, 1995).

The induction of a defense response by chitosan oligomers has been more extensively studied in wheat. Partially *N*-acetylated, polymeric chitosans elicited both phenylalanine ammonia lyase (PAL) and pathogen induced peroxidase (POD) activities in wheat (Vander *et al.*, 1998). Maximum elicitation was observed with chitosans of intermediate degrees of acetylation. Interestingly chitosans fragments also induced the deposition of lignin, the appearance of necrotic cells exhibiting yellow auto-fluorescence under ultraviolet light, and macroscopically visible

necroses. In contrast, addition of *N*-acetylated chitin fragments induced no visible patterns of necrosis. These results suggest that different mechanisms are involved in the elicitation of POD activities by GlcNAc oligomers, and of PAL and POD activities by partially *N*-acetylated chitosan polymers.

Oligosaccharide Binding Proteins

The perception of oligosaccharide signals by plants has revealed a complex communication network which has evolved as a mechanism for plants to sense, as well as to interact with, their environments. As described above, four general classes of oligosaccharin signal molecules have been classified as having the ability to stimulate a number of developmental and defense-related responses in a broad range of plant species. To date, a number of membrane-localized binding sites specific for oligosaccharin elicitors have been identified in a variety of plant species (Table 3). However, none of these binding sites have been classified as a specific receptor for a given oligosaccharin elicitor. In order for a binding protein to be classified as a biological receptor for a particular ligand, it must first satisfy a set of widely accepted criteria (Venis, 1985; Hulme and Birdsall, 1992). The criteria are as follows:

1. Binding should be saturable, reversible, and of high-affinity.

Table 3. Biochemical and biological properties of the major classes of binding sites for oligosaccharide elicitors.

Elicitor	Origin	Active Concentration	Polymerization	Binding Protein				Biological Effect
				Plant	Location	Size	K_d[1]	
β-Glucan[a]	Fungal, Bacterial	EC_{50}=10 nM	>6 mer	Soybean	PM	75kDa	1 nM	Defense
Oligogalacturonide[b]	Endogenous	10^{-4}-10^{-7} M	Variable (2-20)	?	?	?	?	Defense/Development
Chitin[c]	Fungal, Insect	Nanomolar range	7-8 mer	Rice	PM	75kDa	29 nM	Defense
			8 mer	Soybean	PM	85kDa	40 nM	Defense/Development
			4-5 mer	Tomato	MF	?	23 nM	Defense
					Intact cells	?	1.4 nM	
Chitosan[d]	Fungal	50-2000 nM	8 mer					
Nod signal[e]	Rhizobial	Sub-nanomolar	4 mer-5 mer	*Medicago*				Symbiosis
				NFBS1	MF	?	72 nM	
				NFBS2	MF	?	2 nM	
				Dolichos				
				LNP	PM (root hairs)	46kDa	?	

PM, plasma membrane; MF, microsomal fraction. [1]Relative affinity as determined by Scatchard analysis. Relevant references: [a] Cote and Hahn, 1992; [b] Farmer *et al.*, 1991; [c] Ito *et al.*, 1997; Baureithel *et al.*, 1994; Stacey and Shibuya, 1997; [d] Vander *et al.*, 1998; [e] Bono *et al.*, 1995; Demont-Caulet *et al.*, 1999; Etzler *et al.*, 1999.

2. The range of saturation for binding should correlate with the concentration range over which the biological response saturates.
3. Binding specificity must be demonstrated. (e.g., using a pharmacological approach, the affinities of various structural analogs of the ligand should come into agreement with the abilities of those analogs to induce a biological response).
4. Binding of the ligand by the binding protein should result in a ligand specific biological response, such as growth, differentiation, and gene expression.
5. The functionality and relationship of binding and signal transduction must be demonstrated.

β-Glucan

β-glucan elicitor molecules are active at nanomolar concentrations and possess the structural specificity to either induce or suppress a defense response depending upon their microbial origin. Therefore it is likely that plants possess a high-affinity, reversible, and specific binding site for β-glucan. A series of structure-function studies defined the minimum degree of polymerization of the β-glucoside elicitor required to induce a biological response (Fugedi *et al.*, 1987; Birberg *et al.*, 1989; Hong and Ogawa, 1990; Cheong *et al.*, 1991). These data demonstrated that

three of the non-reducing terminal glucosyl residues in the hepta-β-glucoside elicitor are essential for the ability of the molecule to elicit phytoalexin accumulation in soybean. In addition to the minimum degree of polymerization required, these results established that coupling of aromatic moieties to the reducing end of the molecule resulted in no loss of biological activity. Subsequently, solubilization and affinity cross-linking of plasma membrane-enriched fractions from soybean identified the presence of a binding complex consisting of at least 3 proteins (Cosio *et al.*, 1990; Cosio *et al.*, 1992; Cheong *et al.*, 1993). Recent work employing affinity purification using a ^{125}I-labeled β-glucoside resulted in a 9000-fold enrichment of the 75 kDa protein. Therefore, this protein is likely the main component of the membrane-localized binding site (Mithöfer *et al.*, 1996). The membrane-localized β-glucan binding protein exhibited a high degree of specificity with respect to the oligoglucoside interaction. Binding of radiolabeled hepta-β-glucoside to root membrane preparations from soybean was saturable over a concentration range of 0.1 to 5 nM (Cheong and Hahn, 1991). Scatchard analysis suggested the presence of a single class of binding site of relatively low abundance ($B_{max} \approx 1$pmol/mg protein) with a binding affinity (K_d) of 1nM (Cheong and Hahn, 1991). Given the high degree of structural specificity exhibited by this class of oligosaccharin binding proteins, as well as the correlation between binding activity and the induction of a defense-related response, it is widely accepted that the β-glucan binding protein is responsible for the

recognition and activation of cellular events involved in fungal pathogen perception in soybean.

Chitin

Rice

Shibuya *et al.* (1993) reported on the identification of a high-affinity binding site for *N*-acetylchitooligosaccharides present in membrane preparations from rice. This study represented the first identification of a site for the chitinaceous elicitor molecules derived from the cell wall of fungal pathogens. Moreover, it provided physical evidence for the presence of binding site from which the induction of a number of defense-related responses might arise. In pathogen defense responses, such as those evoked by the rice fungal pathogen *Phytophthora megasperma*, the major inductive signal is the *N*-acetylchitooligosaccharide polymer, a major constituent of the cell wall of most fungi. The Elicitor Binding Protein (EBP) from rice has been characterized extensively in terms of the structural specificity required for binding, as well as for its ability to mediate the induction of host-specific defense responses (Shibuya *et al.* 1993; Ito *et al.*, 1996; Nishizawa *et al.*, 1999). Scatchard analysis of ^{125}I-labeled *N*-acetylchitooctaose binding indicated the presence of a single class of binding sites with an apparent K_d of 5.4 nM in microsomal membrane fractions, and a K_d of 30 nM in plasma membrane-enriched fractions. Interestingly, inhibition

analysis showed a size-dependent manner of inhibition, with oligosaccharides of a greater degree of polymerization (e.g., d.p.=8) being the best inhibitors of ligand binding. Recently, the EBP has been solubilized and purified to homogeneity from the plasma membrane of suspension-cultured rice cells (Ito *et al.*, 1999). However, isolation of a cDNA encoding the binding protein has yet to be achieved.

Although the cDNA encoding EBP from rice has not been isolated, the characterization of the cellular responses induced upon perception of chitin oligosaccharides has revealed a vast amount of information about the signal transduction mechanism involved in the establishment of a host-specific defense response. Upon perception of subnanomolar concentrations of chitin elicitors, the plasma membrane undergoes a rapid, transient depolarization, accompanied by an increase in Cl^- efflux (Kuchitsu *et al.*, 1993; Kikuyama *et al.*, 1999). This response, in turn, leads to the acidification of the cytoplasm and a rapid alkalinization of the culture medium (Kuchitsu *et al.*, 1997). Cytoplasmic acidification in plant cells has been postulated to be an intracellular signal (Guern *et al.*, 1991). Recently, He *et al.* (1999) showed that the acidification of the rice cytosol might also serve as a secondary messenger for the induction of a number of early-responsive genes involved in the establishment of a defense response. By monitoring the accumulation of five classes of elicitor-responsive genes; *EL2*, *EL3*, phenylalanine ammonia lyase (*PAL*), chitinase, and β-glucanase, it was shown that activation and expression of these defense-related genes paralleled the structural specificity of elicitor binding to

the rice EBP. Thus, the signal transduction mechanism involved in the perception of chitin elicitors and the subsequent activation and expression of genes involved in mounting a defense response suggests a highly specific response mechanism to invading fungal pathogens.

Tomato

The hypothesis that chitin perception mechanisms in plants are part of a conserved signaling apparatus which allows the plant to sense its environment was further strengthened by the identification of a high-affinity chitin binding site localized in particulate membrane fractions of tomato suspension-cultured cells. Baureithel *et al.* (1994) reported on the identification of a binding site in tomato cells believed to be involved in a defense-response mechanism. Interestingly, this binding site showed not only a high affinity for *N*-acetyl-chitooligosaccharides but also was found to bind modified rhizobial Nod signals.

Using a *N*-acetylchitopentaose coupled to t-butoxycarbonyl-L-^{35}S-methione, competition assays were performed in an effort to characterize the relative affinity of the binding site for both modified (e.g., LCO) and unmodified oligosaccharide elicitors. The radiolabeled ligand exhibited specific, saturable, and reversible binding to both whole cells and membrane particulate fractions with relative affinities (K_d) of 1.4 and 23 nM, respectively. A correlation between elicitor activity (e.g., alkalinization of culture medium) and binding was demonstrated, although chitin

oligosaccharides with a degree of polymerization greater than five were not tested. Interestingly, the binding site also showed a high affinity for the major Nod signal produced by *R. leguminosarum* [(Nod*Rlv*-V(Ac; C18:1), Table 2], with an apparent affinity of 8 nM. This finding brought forth the possibility that chitin perception in non-leguminous species and Nod signal binding in legumes might share a common structural element in terms of oligosaccharide recognition.

Lectins

Carbohydrate-binding proteins (lectins) are found in prokaryotes and eukaryotes, and share a single common property: they possess at least one non-catalytic domain that can bind reversibly to a specific saccharide (Peumans and Van Damme, 1995). The role of lectins in nodulation has had a long and controversial history. Hamblin and Kent (1973) first postulated a role for lectins as determinants of host specificity in the *Rhizobium*-bean (*Phaseolus vulgaris*) symbiosis. Subsequently, Dazzo and Hubbell (1975) postulated the involvement of a lectin as a determinant in the *Rhizobium*-clover symbiosis. Work by Schmidt and Bohlool (1981) further suggested the role of lectins in symbiotic plant-microbe interactions, and thus, established the foundation for the 'legume-lectin recognition hypothesis'. This hypothesis postulated a role for lectins as a 'connector' mediating the specific attachment of rhizobia to the plant root surface.

Within the plant kingdom the carbohydrate binding properties of lectins, as well as their relative distribution within the plant, 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 putative role as Nod signal binding proteins (Etzler *et al.*, 1999). Halverson and Stacey (1985) presented evidence suggesting the role of lectins in host recognition and the *Bradyrhizobium*-soybean symbiosis. This work demonstrated the involvement of a protein factor (i.e., a galactose-specific lectin) present in soybean root extracts in nodulation. By pre-treating a *B. japonicum* nodulation-defective mutant with the root extract, the authors demonstrated the ability of the lectin to restore normal nodulation. These results demonstrated the ability of the lectin to affect the nodulation characteristics of *B. japonicum*, and more specifically, suggested that the lectin may be involved in events prior to root attachment.

Diaz *et al.* (1989) demonstrated the involvement of a seed lectin, PSL, from *Pisum sativum* (garden pea), in determining *Rhizobium* host-specificity. In these experiments, it was shown that transgenic expression of PSL in clover (*Trifolium repens*) roots conferred the ability to be nodulated by *Rhizobium leguminosarum* bv. *viciae*, the symbiont of garden pea. This was the first study that showed definitive evidence that the host range in a *Rhizobium*-legume symbiosis is at least partially determined by symbiont-root-lectin interactions. Moreover, it was the first example of a host-specificity barrier being broken by genetic engineering of the host plant.

More recently, a second study involving the extension of host-specificity based upon the transgenic expression of a legume lectin was published (van Rhijn *et al.*, 1998). In this report, the introduction of the soybean lectin gene, *Le1*, into *Lotus corniculatus* resulted in the ability of *B. japonicum* to nodulate *L. corniculatus*. This study differed slightly from that of the previously published work of Diaz *et al.* (1989) (described above) in that the phylogenetic difference between *B. japonicum* and *S. loti* is greater than that of the two rhizobial biovars that nodulate pea and clover (*R. leguminosarum* bv *viciae* and bv *trifolii*, respectively). Interestingly, the results of van Rhijn *et al.* (1998) also showed that the soybean lectin can change host specificity in a Nod signal-independent manner. These results support the notion that host specificity is dictated by multiple factors. Collectively, the results of Diaz *et al.* (1989) and those of van Rhijn *et al.* (1998) indicate that legume lectins might be involved in mediating host-specificity in the *Rhizobium*-legume symbiosis.

Multiple Recognition Events?

The fact that Nod signals act at very low concentrations (e.g., 10^{-12}-10^{-15} M) and show some degree of chemical specificity suggests that perception is likely mediated by specific protein receptors. Such a receptor should have the ability to recognize the Nod signal at sub-nanomolar concentrations and transduce this binding signal to other cellular components. In addition, the Nod signal receptor should demonstrate some chemical specificity with regard to binding, and this specificity

should correlate with the ability of the Nod signals to induce a biological response in the host plant.

A number of laboratories have sought to define the nature of the Nod signal recognition event(s) by conducting structure/function studies using either rhizobial mutants defective in Nod signal synthesis and/or a collection of natural and synthetic LCO. The first study of this kind was published by Ardourel *et al.* (1994). Working with *S. meliloti* mutants, they demonstrated that different plant responses (e.g., root hair deformation and pre-infection thread formation) had different Nod signal structural requirements. They proposed a model in which a low-stringency signaling receptor mediated such events as root hair deformation, while a second, stringent, high-affinity entry receptor was necessary to trigger sustained, rhizobial invasion. Similarly, Felle *et al.* (1995) examined the ability of various LCO to trigger either cytosolic alkalinization or membrane depolarization in alfalfa root hairs. Sulfated Nod signals, which were morphogenic on alfalfa, were able to induce membrane depolarization, and rapid intracellular alkalinization in root hairs. Non-sulfated Nod signals elicited an intracellular pH change but in the absence of membrane depolarization. Again, this study suggested the presence in alfalfa of two Nod signal recognition events that are independently coupled to cellular events associated with nodulation.

In addition to the 'two-recognition event' model described above, a second hypothesis defining the mechanism of Nod signal perception and specificity has also been proposed. Staehelin *et al.* (1994) proposed a model based on the hypothesis that

structural modifications to the Nod signal backbone influence their stability against hydrolysis by root chitinases. Thus, the ability of a modified Nod signal to avoid hydrolysis and subsequent degradation by legume root chitinases may influence host-specificity. By incubating different concentrations of *R. meliloti* Nod signals in the presence of root chitinases from both *Medicago* (i.e., host) and *Vicia* (i.e., non-host), Nod signal susceptibility to chitinase degradation was assessed. The stability of the Nod signals tested were as follows: Nod*Rm*IV(S)>Nod*Rm*V(S)>Nod*Rm*IV>Nod*Rm*-V. Thus, sulfation of the Nod signal, which had previously been shown to be required for nodulation, "protected" the Nod signal from degradation. Moreover, the stability of the Nod signals correlated with their biological activity on *Medicago*. The finding that Nod signals were cleaved by chitinases at different rates raised the question of whether Nod signal stability could be one of the determinants of host specificity. These data suggested that plant chitinases may be involved in controlling the biological activity of Nod signals by cleaving and inactivating them.

Nod signal structure/function on Soybean

Each host plant has evolved the ability to recognize the specific Nod signal composition produced by its compatible rhizobial symbiont. Purified or chemically synthesized Nod signals with the appropriate structure will elicit, when applied to roots, many of the responses seen upon rhizobial inoculation (e.g., root hair curling, cortical cell division, etc. reviewed in Cohn *et al.*, 1998). Research using various

rhizobial mutants blocked in the decoration of the basic lipo-chitin Nod signal structure led to the general view that Nod signal recognition was highly specific.

Stokkermans *et al.* (1995) and Minami *et al.* (1996a, 1996b) undertook an extensive analysis of the chemical and structural specificity required for Nod signal action in the *B. japonicum*-soybean symbiosis. Initial studies with a *B. japonicum nodZ* mutant suggested that the presence of a 2-*O*-methylfucose residue on the terminal, reducing GlcNAc was critical for biological activity and, by assumption, host recognition (Stacey *et al.*, 1994; Stokkermans *et al.*, 1995). However, using a variety of natural and chemically synthesized LCO, it was found that fucosylation was required only when the LCO was a pentamer (Stokkermans *et al.*, 1995). If the LCO was a tetramer, then fucosylation rendered the molecule inactive at eliciting root hair curling or cortical cell division in soybean. Hence, both the chemical substitutions and the LCO chain length were found to be critical determinants of host specificity.

As previously discussed, analysis of the Nod signal produced by a NodZ mutant suggested that fucosylation was required for Nod signal activity on soybean. Thus, it was surprising to find that a NodZ mutant, incapable of producing fucosylated Nod signals, was able to form nitrogen-fixing nodules on the roots of soybean (Stacey *et al.*, 1994). The results described by Stokkermans *et al.* (1995) offered an explanation for this observation since it was assumed that the NodZ mutant must synthesize an active, non-fucosylated tetrameric Nod signal. Recently, Cohn *et al.* (1999) succeeded in purifying such a Nod signal from the culture supernatant of

the NodZ mutant and showed that it was active on soybean roots. As an extension of earlier studies, the chemical requirements for Nod signal action on an alternative host of *B. japonicum*, rice bean (*Vigna umbellata*) was examined. Rice bean was found to respond only to the fucosylated, pentameric Nod signal but could be nodulated by a *B. japonicum* NodZ mutant (Cohn *et al.*, 1999). Again, the assumption was made that a non-fucosylated, tetrameric Nod signal was likely allowing the NodZ mutant to nodulate rice bean. However, when tested, this molecule was found to be inactive on rice bean roots. Cohn *et al.* (1999) showed that a mixture of non-fucosylated Nod signals was active in inducing cortical cell division and nodule formation on rice bean. Thus, in this case, the chemical requirement for Nod signal fucosylation can be surmounted by adding a mixture of non-fucosylated Nod signals. Such a mixture is naturally produced by the *B. japonicum* NodZ mutant and this appears to explain its ability to nodulate rice bean.

In the case of the *B. japonicum*-soybean symbiosis, Stokkermans *et al.* (1995) demonstrated that only four LCO signals [i.e., Nod*Bj*V(C18:1Δ11, MeFuc), Nod*Bj*V(C18:1Δ9, MeFuc), Nod*Bj*V(C16:0, MeFuc), and Nod*Bj*IV(C16:0)] could elicit a morphogenic response on soybean roots. These studies demonstrated that fucosylation and chitin chain length were the most important determinants for Nod signal specificity. Minami *et al.* (1996a, 1996b) extended these studies by examining the ability of chitin oligomers and LCO to induce the expression of the early nodulins, ENOD40 and ENOD2. Minami *et al.* (1996a) showed that the expression

of ENOD40 mRNA could be induced transiently by a simple, non-acylated chitin pentamer. However, sustained expression of ENOD40 required a soybean-specific Nod signal (i.e., one of the four LCO listed above). In a subsequent study, Minami *et al.* (1996b) showed that the addition of any single LCO to soybean roots failed to elicit the expression of the early nodulin ENOD2. Interestingly, when a mixture of LCO was added, ENOD2 expression was clearly induced in the nodule parenchyma. Analysis of these mixtures demonstrated that addition of a non-acylated chitin pentamer, along with one of the four, morphogenic LCO molecules, was sufficient to induce ENOD2 expression. Taken together, these studies by Minami *et al.* (1996a, 1996b) pointed to the presence of two Nod signal recognition events in soybean that vary in their chemical specificity and are independently coupled to cellular events (Figure 4). Since expression of ENOD40 and ENOD2 mark different stages in nodule ontogeny, these results actually suggest that different Nod signal recognition events are required for the temporal development of the nodule. A low-stringency recognition step is coupled with initiating some of the first steps in nodule ontogeny (e.g., transient ENOD40 expression) but is not sufficient to sustain nodule development. Likewise, activation of a high-specificity recognition step can result in sustained ENOD40 expression and the formation of an incomplete, nodule primordium. However, only when both of these recognition events are simultaneously activated, does nodule development proceed to the formation of vascular tissue and the expression of ENOD2.

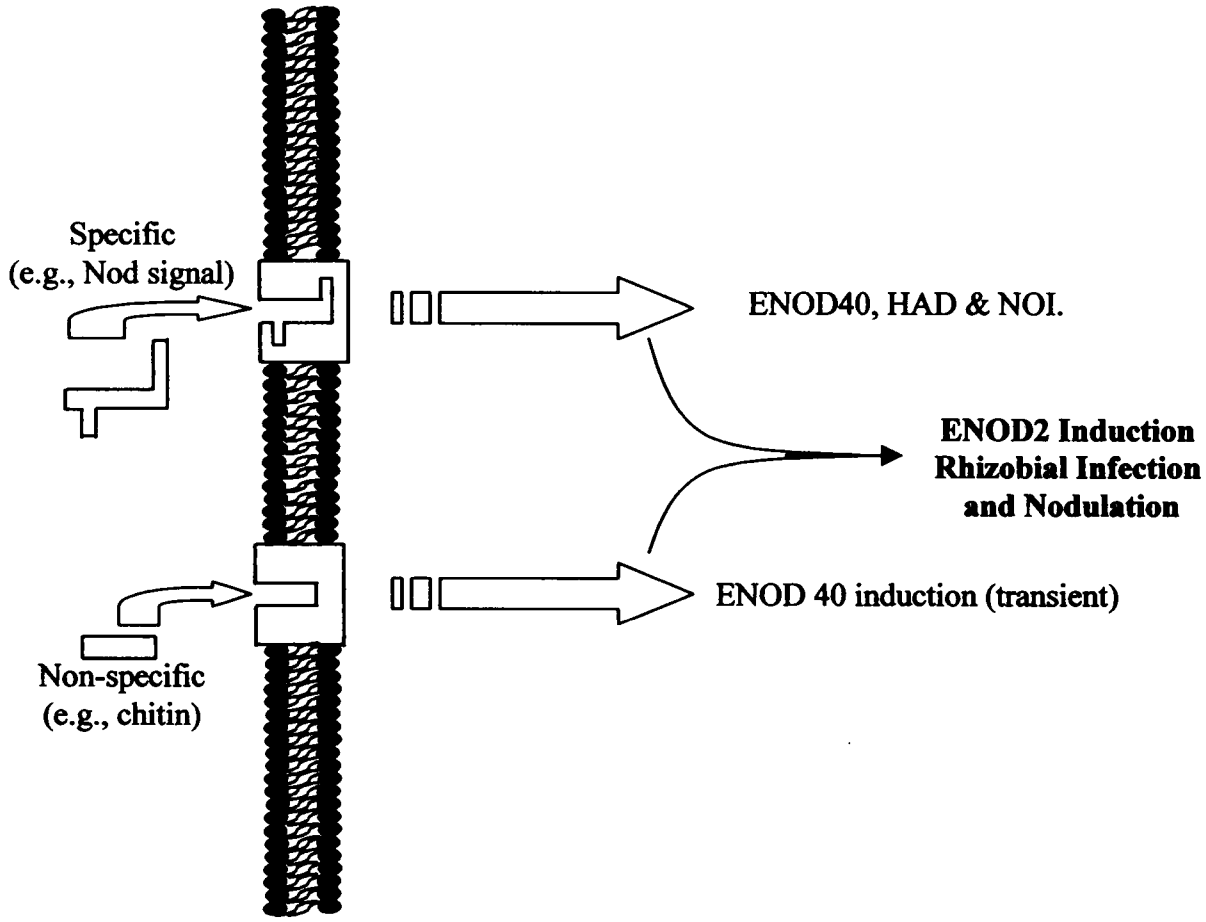


Figure 4. Proposed model for lipo-chitin Nod Signal perception in the *Bradyrhizobium*-soybean symbiosis. Actualization of a host-specific nodulation response involves the concerted effort of two distinct signaling events. ENOD2 induction, and subsequent nodulation, was shown to require the activation of both a non-specific and a specific recognition event (Minami *et al.*, 1996a, 1996b). HAD, root hair deformation; NOI, nodule initiation (e.g., cortical cell divisions).

Nod Signal Binding Proteins

LCO binding proteins have been identified in microsomal membrane preparations from alfalfa (Bono *et al.*, 1995; Gressent *et al.*, 1999) and in the root hairs of the legume *Dolichos biflorus* (Etzler *et al.*, 1999). In alfalfa, the first of two high-affinity binding sites to be identified, termed Nod Factor Binding Site 1 (NFBS1), was identified in root preparations from *M. truncatula* and *M. varia* cell suspensions using tritium-labeled NodRm-1(Ac,S, C16:2), the major Nod signal from *S. meliloti* (Bono *et al.*, 1995). However, NFBS1 was found to have a relatively low affinity for the Nod signal (K_d=86 nM) and low specificity for LCOs. Subsequently, a second binding activity (NFBS2) was found in microsomal membrane preparations from *M. varia* suspension cells which exhibited a high affinity for the *S. meliloti* Nod signal (K_d= 2 nM) (Gressent *et al.*, 1999). Recently, the structural specificity exhibited by NFBS2 was examined in more detail using a ^{35}S-labeled NodRm-1(Ac,C16:2Δ2,9) ligand. Binding studies demonstrated that substitutions on the terminal, non-reducing GlcNAc residue of the Nod signal, including *O*-acetylation, *N*-acylation, and C-4 hydroxylation, were critical determinants of binding specificity. With regard to *N*-acylation, the binding specificity of NFBS2 correlated well with the structure/ function studies of Demont-Caulet *et al.* (1999) discussed previously. The surprising finding of the study by Gressent *et al.* (1999) was that binding to NFBS2 did not require sulfation of the Nod signal, which is thought to be a critical determinant of Nod signal action on alfalfa. Thus, although NFBS2 demonstrated

some characteristics expected of an alfalfa Nod signal receptor (e.g., high affinity and *N*-acylation specificity), it lacked others (e.g., requirement of sulfation). Further work will be required to demonstrate the relevance of both NFBS1 and NFBS2 to nodulation.

Apyrases

Apyrases can be divided into two main classes: ecto-apyrases and endo-apyrases. Ecto-apyrases are characterized as having the catalytic domain outside of the cell, whereas endo-apyrases have the catalytic domain inside the cell (Komoszynski and Wojtczak, 1996). It has been suggested that apyrases function in such diverse roles 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 Golgi lumen (Abeijon *et al.*, 1993; Gao *et al.*, 1999). The functional role of endo-apyrases is best characterized from the work of Abeijon *et al.* (1993) and more recently, from that of Gao *et al.* (1999), who used yeast as a model for studying the role of protein glycosylation in the lumen of Golgi vesicles. These reports described the identification and characterization of two membrane-localized apyrases required for Golgi *N*- and *O*-glycosylation in *Saccharomyces cerevisiae*. In *S. cerevisiae*, mannosylation of *N*- and O-linked oligosaccharides has been shown to be predominantly regulated by a GDPase that converts GDP to GMP (Abeijon *et al.*, 1993). Using a Δ*gda1* null mutation in S.

cerevisiae, this work demonstrated: 1) A block in the elongation of *O*-linked carbohydrate chains in mannoproteins and secreted chitinases. 2) A disruption in the elongation of *N*-linked carbohydrates of carboxypeptidase Y. 3) Under-glycosylation of invertase, and 4) the impaired biosynthesis of mannosylinositolphorylceramides. Thus, it appears that the yeast apyrase (GDA1) is required for the GDP-mannosylation of a number of membrane proteins required for cell wall biosynthesis.

A more recent study (Gao *et al.*, 1999) presented the characterization of a homologue to GDA1, YND1, and provided the first direct evidence for *in vivo* endo-apyrase function in protein glycosylation. Using complementation analysis to overcome the glycosylation defect of invertase (described above) in the Δ*gda1* null mutant, over-expression of the *YND1* gene suggested that YND1 function is partially redundant with GDA1. Thus, phenotypic disruptions due to a loss in apyrase function of either gene can be overcome by over-expression of the complementary homologue. However, when a double mutant was constructed *(Δynd1Δgda1)*, a marked impairment in cell growth and germination was observed. Microscopic analysis further confirmed that the double deletion resulted in a severe defect in cell wall integrity; thereby, suggesting an essential role for the yeast apyrases in cell wall organogenesis.

As described above, lectins have long been implicated in the legume nodulation process but a precise role for these interesting proteins has not been

defined. Recently, Etzler *et al.* (1999) reported on a unique lectin (DB46) isolated from the roots of the legume *Dolichos biflorus*. The *D. biflorus* lectin was found to bind to Nod signals from a variety of rhizobia. However, DB46 showed the highest apparent affinity for the Nod signal produced by *B. japonicum* and *Rhizobium* sp. NGR234, both of which can nodulate *D. biflorus*. Sequence comparisons revealed that the *D. biflorus* lectin was likely an apyrase (i.e., NTPase). Indeed, Etzler *et al.* (1999) demonstrated that DB46 did possess ATPase activity, which was significantly enhanced upon addition of the Nod signal. For this reason, the lectin was termed a lectin-nucleotide phosphohydrolase (LNP). The *D. biflorus* LNP (i.e., DB46) was found on the surface of root hairs using fluorescent antibody labeling. Moreover, pre-treatment of the roots of *D. biflorus* with anti-LNP antibodies inhibited nodulation by its rhizobial symbiont. The properties of this protein suggest that it could play a role in Nod signal recognition and transduce this binding signal by activation of its apyrase enzyme activity.

In contrast to LNP, an apyrase present in pea (*P. sativum*) has been studied for a number of years as a calmodulin-stimulated, nuclei-localized NTPase believed to be involved in mediating phytochrome responses (Chen and Roux, 1986; Chen *et al.*, 1987). Although the ability of the pea apyrase to bind Nod signals has not been demonstrated, mRNA expression data suggests that the pea apyrase may be regulated, in part, by a number of environmental factors which govern the establishment of nodulation. Hsieh *et al.* (1996) examined the differential expression of the pea apyrase in various organs, and found that mRNA was expressed 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. These results suggested that pea apyrase gene expression was regulated by light, a condition which also regulates the onset of nodulation.

Recently, Thomas *et al.* (1999) extended the functional analysis of the pea NTPase/apyrase and demonstrated that transgenic expression of the pea apyrase in *Arabidopsis thaliana* resulted in plants that showed enhanced growth and augmented phosphate transport when additional phosphate was supplied as inorganic phosphate or as ATP. Although these data do not suggest a direct role for the pea apyrase in nodulation, they do provide evidence for a possible role for the pea NTPase as an ecto-apyrase that functions in plant phosphate nutrition.

RATIONALE FOR PRESENT WORK

Oligosaccharide perception in leguminous plants plays a central role in development, defense, as well as in the establishment of a host-specific symbiotic association with members of the *Rhizobiaceae*. Although the mechanism of oligosaccharide signal perception is largely undefined, it is thought to be mediated, in part, by the presence of membrane-localized binding proteins. In soybean, the perception of both chitin oligosaccharides and modified lipo-chitin Nod signals appears to share a number of common features, including the activation of gene expression associated with the onset and development of nodulation. Minami *et al.*

(1996a) first demonstrated the ability of the major lipo-chitin oligosaccharide from *B. japonicum* USDA110 to induce the rapid induction of ENOD40 mRNA expression in the root pericycle of soybean. More importantly, this study also demonstrated the ability of unmodified *N*-acetylated chitin oligosaccharides to induce the rapid induction of ENOD40 expression in the root pericycle. Although the expression of ENOD40 by chitin oligomers was transient, the induction pattern of ENOD40 by both modified lipo-chitin Nod signals, as well as by chitin oligomers, suggested the presence of at least two signal recognition events. In the present study, a two-fold approach was undertaken to identify and characterize a protein factor that might mediate both Nod signal and chitin oligosaccharide perception in the *Bradyrhizobium*-soybean symbiosis. The identification and characterization of a high affinity-binding site for *N*-acetyloligosaccharides present on the plasma membrane of soybean suggests that this site might be involved in the establishment of a defense-related mechanism in response to invading fungal pathogens. Conversely, the identification and characterization of two homologues (GS50 and GS52) to a previously identified Nod signal binding protein present in the root hairs of the legume *Dolichos biflorus* (Etzler *et al.*, 1999) suggests a second, distinct mechanism of modified chitin oligosaccharide perception in soybean. The expression pattern, as well as the cellular localization of GS50 and GS52, suggests that these proteins might be involved in the early events associated with the establishment of a symbiotic association in soybean. In addition, the results presented in this work suggest that

both chitin and Nod signal perception in soybean share a number of similarities in terms of gene activation and structural specificity in the receptor-agonist interaction.

CHAPTER 3

MATERIALS AND METHODS

Chemicals

Chitin and chitosan oligosaccharides were obtained from Seikagaku America, Inc. (Maryland, USA). Chitoheptaose and Chitooctaose were gifts from Dr. Naoto Shibuya (The National Institute of Agrobiological Resources, Tsukuba City, Japan). Na-^{32}Phosphate and Na-^{125}Iodine were purchased from ICN (Costa Mesa, CA). [^{14}C]-Methylated proteins were purchased from Amersham Life Sciences (Arlington Heights, IL). 2-(4-aminophenyl)ethylamine (APEA) was purchased from Aldrich Chemicals (Tokyo, Japan). Protease inhibitors salicylhydroxamic acid (SHAM) and phenylmethylsulfonyl flouride (PMSF) were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were from Fisher Scientific (Pittsburgh, PA).

Bacterial Strains and Plasmids

Bacterial strains and plasmids are listed in Table 4. When necessary, the construction of the strains is described in the text.

Table 4: Bacterial Strains and Plasmids.

Strain or Plasmid	Relevant Characteristics	Source or Reference
B. japonicum USDA110	wild type, Cm^r	USDA, Beltsville, MD
E. coli JM109	*endA1, recA1, gyrA96, thi, hsdR17,* (r_k^-, m_k^+), *relA1, supE44,* Δ(*lac-proAB*),F', *traD36, proAB, lacI*^{q}*ZΔM15*]	Messing, 1983
E. coli INVαF'	F', *endA1, recA1, hsdR17,* (r_k^-, m_k^+), *supE44, thi*-1, *gyrA96, relA1, f80lacZΔDM15D(lacZYA-argF)U169*	Invitrogen, San Diego, CA
pCR2.1	Ap^r, Km^r, T7 promoter	Invitrogen, San Diego, CA
pGEX-4T-3	GST-fusion protein expression vector	Pharmacia, Uppsala, Sweden
E. coli GEX50*	*E. coli* JM109 harboring pGEX46-A	This Study
E. coli GEX52	*E. coli* JM109 harboring pGEX52	This Study
pDAY15	850 bp TD-PCR product of *gs50* cloned into pCR2.1	This Study
pDAY9	351 bp TD-PCR product of gs52 cloned into pCR2.1	This Study
pDAY17	*gs50* 3' RACE product cloned into pCR2.1	This Study
pDAY16	*gs50* 5' RACE product cloned into pCR2.1	This Study
pDAY10	*gs52* 3' RACE product cloned into pCR2.1	This Study
pDAY11	*gs52* 5' RACE product cloned into pCR2.1	This Study
pDAY46-A*	*gs50* cloned into pCR2.1	This Study
pDAY52	*gs52* cloned into pCR2.1	This Study
pDAY52new	*gs52* with modified 5' end for in-frame cloning into pGEX-4T-3	This Study
pGEX46-A*	*gs50* cloned into pGEX-4T-3	This Study
pGEX52	*gs52* cloned into pGEX-4T-3	This Study

Table 4, cont.

* Subsequent sequence analysis revealed that the *gs50* cDNA suffered a 150 bp, in-frame deletion. The deletion occurred between bases 406 and 555.

Bacteria culture media and growth conditions

All strains of *Escherichia coli* were grown and maintained on LB medium (Sambrook *et al.*, 1989) at 37°C. *Bradyrhizobium japonicum* USDA110 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* are: 200μg/ml ampicillin, 100 μg/ml kanamycin, and 25μg/ml tetracycline. Minimal medium (Bergersen, 1961) was used for growth of *B. japonicum* USDA110 for isolation of Nod signals. Minimal medium consists of 0.3 g/L KH_2PO_4, 0.3 g/L K_2HPO_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.

Plant Germination and Growth Conditions

Glycine soja seeds (PI 468397) were surfaced sterilized in 20% hypochlorite for 20 minutes, and washed six times with sterile, distilled water. Seeds were then scarified in concentrated H_2SO_4 for 5 minutes, followed by six washes with sterile, distilled water. Afterwards, seeds were soaked in 0.01N HCl for 10 minutes, followed by six washes with sterile, distilled water. Seeds were germinated in petri dishes on sterile disks of 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), and grown in a model CU-32L plant growth

chamber (Percival Scientific Inc., Boone, Iowa) under a 16 hour day/8 hour night cycle at 28/22°C with 70 to 80% relative humidity.

Enzymes and DNA Primers

Most enzymes in this study were purchased from Promega (Madison, WI), Stratagene (La Jolla, CA), or Gibco BRL (Gaithersburg, MD), and were used according to the manufacturer's specifications. Oligonucleotide primers used in this study were synthesized by either Operon (Alameda, CA) or Integrated DNA Technologies (Coralville, IA), and are listed in Table 5.

Membrane Chitin Binding Assays

Purification of plasma membrane-enriched fractions from suspension-cultured cells and root tissue

Glycine max cv. Essex seeds were surface sterilized in 3% hypochlorite for 20 minutes, followed by shaking in 0.01 N HCl for 15 minutes. After six washes in sterile distilled H_2O, seeds were spread on sterile, moist sheets of Whatman paper. Seeds were germinated in the dark at 28°C for 5 days. After 5 days, roots were

Table 5. DNA Primers used in this study.

Application	Primer sequence	T_m (°C)
gs50 TD-PCR, forward	5′-ATTGATGGAACCCAAGAAGG-3′	64°C
gs50 TD-PCR, reverse	5′-GGYAAAGMTGATATGGCTTC-3′	56°C
gs50 3′ RACE	5′-GGTGAGGCAAGTTGAGTATCAA-3′	64°C
gs50 5′ RACE	5′-GTAATACCCATCAAAGCCAGCCAA-3′	72°C
gs50 5′ RACE, nested	5′-GATTCCACTCCGTCCGGTGC-3′	72°C
gs50 Forward Full	5′-GCCTCTAGAATGAGCAAGGATTATCTAATC-3′	65°C
gs50 walk-1	5′-GCGTCCTGGGGTGAAACTCC-3′	62°C
gs50 Reverse Full	5′-GCCTCTAGACATATGGCTCCTCG-3′	61°C
gs52 TD-PCR, forward	5′-TGGRATWTGGRATGGTGGA-3′	58°C
gs52 TD-PCR, reverse	5′-AGAGGCCATGCDGYTCCACA-3′	65°C
gs52 3′ RACE	5′-GCCAAGTCCACCTATCCAAACC-3′	65°C
gs52 5′ RACE	5′-GCCACTGTAATCTCTTGCC-3′	60°C
gs52 5′ RACE, nested	5′-GCAGATAATAGAAAGACGA-3′	57°C
gs52 Forward Full	5′-GCCTCTAGAGCATGGTACTTGTGC-3′	68°C
gs52 walk-1	5′-GCCATTTGAACTGATCCACC-3′	56°C
gs52 walk-2	5′-GGTCGAAATGGAAGACATGC-3′	56°C
gs52 Reverse Full	5′-GCCTCTAGACCCAGCATAAGC-3′	62°C
gs52 pGEX frame	5’-CCGAATTCCCCCATGGTACTTGTGC-3’	68°C
ENOD40b Forward	5′-AGCTTTGTTGGCTCA-3′	49°C
ENOD40b Reverse	5′-TCAGCTGCAAATTGAGTAA-3′	48°C
Ubiquitin Forward	5′-GGGTTTTAAGCTCGTTGT-3′	48°C
Ubiquitin Reverse	5′-GGACACATTGAGTTCAAC-3′	40°C

TD, touch-down; PCR, Polymerase Chain Reaction; T_m, melting temperature; Y, C/T; M, A/C; W, A/T; D, A/G/T; R, A/G; RACE, Rapid Amplification of cDNA Ends.

excised, frozen in liquid nitrogen, and stored at -80°C. Approximately 12,000 roots were collected to yield 200 grams (fresh weight) of plant material. Suspension-cultured soybean cells of *Glycine max* SB-1, derived from *Glycine max* (L) Merr. cv. Mandarin root tissue, were maintained in a modified B5 medium [1X B5 basal salt mixture (Sigma Chemical, St. Louis, MO) + 2% sucrose 1X B5 vitamins, pH 5.5] at 28°C, according to the method of Ho *et al.* (1988). Suspension cells were a gift from Dr. John Wang (Michigan State University, East Lansing, MI). Suspension cultured soybean cells of *Glycine max* cv. Enrei, also derived from root tissue, were maintained in a similar manner. Cells were harvested 10 days after transfer to fresh medium, frozen in liquid nitrogen, and stored at -80°C until use.

Approximately 200 grams (fresh weight) of either suspension-cultured cells or 5-day-old roots were used as starting material for the isolation of plasma membrane-enriched fractions. Tissue was homogenized in homogenization buffer [0.3M sucrose, 50mM MES-Tris (pH 7.6), 5mM EDTA, 5mM EGTA, 20mM NaF, 1mM DTT, 4mM SHAM, 2mM PMSF, 2.5mM $Na_2S_2O_5$, 0.5% BSA] on ice for 30 minutes. After homogenization, cells were lysed at 1000 p.s.i. using a French pressure cell press (American Instrument Company, Silver Spring, MD). Following lysis, the homogenate was centrifuged at 4000× g for 10 minutes at 4°C. The supernatant was then subjected to an additional centrifugation at 4°C for 10 minutes at 15000×*g*. The resultant supernatant was then centrifuged at 100,000 × *g* for 1 hour at 4°C in a Ti65 fixed angle rotor (Beckman Instruments, Fullerton, CA). After ultra-centrifugation,

the microsomal fraction was re-suspended in microsomal fraction buffer (0.25M sucrose + 10mM sodium phosphate, pH 7.8), and homogenized using a hand-held Potter homogenizer. Plasma membrane-enriched fractions were prepared by aqueous two-phase partitioning using dextran T500 and polyethyleneglycol 3500 according to the method of Shibuya *et al.* (1996). Purified plasma membrane was re-suspended in 1ml plasma membrane buffer (0.25m sucrose + 5mM HEPES-bistrispropane, pH 7.0 + 0.1M DTT). Typically, 3-5 mgs of plasma membrane-enriched fractions were obtained from 200g of cells (fresh weight). Protein concentrations were determined using the BCA Protein Assay (Pierce Chemical, Rockford, IL), using bovine serum albumin standards. In all assays, the quantity of plasma membrane is based on protein determination.

Synthesis of ^{125}I-labeled aminophenyl derivatives of N-acetylchitopentaose and N-acetylchitooctaose

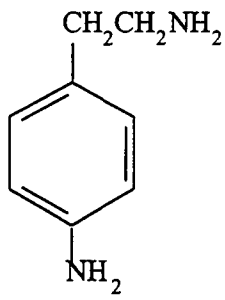
(GN_8-APEA and GN_5-APEA were synthesized by Yuki Ito in the laboratory of Dr. Naoto Shibuya at the National Institute of Agrobiological Resources, Tsukuba City, Japan).

GN_5-APEA and GN_8-APEA, the aminophenyl conjugates used as a ligands in the binding assays, were prepared by a similar reductive amination reaction, as previously described (Ito *et al.*, 1996). Briefly, five hundred microliters of 2-(4-

Aminophenyl)ethylamine (APEA) (Figure 5), 225 μl of acetic acid and 200 mg of $NaCNBH_3$ were dissolved in a final volume of 7 ml of dH_2O. Ten milligrams of either *N*-acetylchitopentaose or *N*-acetylchitooctaose were added to the mixture and dissolved on ice. The mixture was then heated at 80°C for 1 hour. Purification of the conjugates was performed as previously described (Ito *et al.*, 1997) using an Amberlite CG-120 cation exchange column, followed by reverse-phase HPLC using an Inertsil PREP-ODS column (20×250 mm, GL Sciences Inc., Tokyo, Japan). The major peak detected at 285 nm was desalted using a Sephadex G-25 column (Pharmacia Biotech, Uppsala, Sweden), lyophilized, and stored at -20°C.

Iodination of the GN_5 and GN_8 ligands was performed using Iodogen (Figure 5) (Pierce Chemical Company, Rockford, IL) as the oxidizing agent. Two microliters of either GN_5-APEA or GN_8-APEA (1 mM stock) and 10 μl of 0.5M sodium phosphate buffer (pH 7.5) were mixed in a glass test tube, pre-coated with 50 μg of Iodogen. Five microliters of Na-[^{125}I] (16.3×10^5 Bq) (ICN, Costa Mesa, CA) were added to the mixture and incubated at room temperature for 1 hour. After incubation, 5 μl of 0.1M cold NaI were added to the reaction tube and incubated for 5 minutes. The mixture was applied to a Poly Prep AG 1 × 8 column (BioRad Laboratories, Hercules, CA) and eluted with distilled water. The radioactive, unbound fractions were stored at -20°C until use for affinity cross-linking and binding assays. GN_5-APEA and GN_8-APEA were typically radio-iodinated to a specific activity of 1.8×10^9 Bq μmol^{-1}, which was sufficient for all binding assays, inhibition analysis,

A.



B.

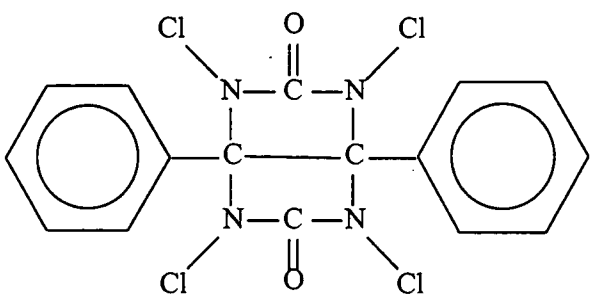


Figure 5: Chemical structure of A) 2-(4-Aminophenyl)ethylamine and B) Iodogen.

and affinity cross-linking of the plasma membrane enriched fraction.

Isolation of lipo-chitin Nod signals produced by Bradyrhizobium japonicum USDA110

LCOs were purified according to the method of Sanjuan *et al.* (1992), with slight modifications. Early log phase (OD_{600nm}=0.3) cultures of *B. japonicum* USDA110 were induced with 1% soybean seed extract (Barbour *et al.*, 1991) for 48 hours at 28°C. After induction, cell cultures were extracted with ½-volume of distilled, HPLC-grade *N*-butanol (Fischer Scientific, Pittsburgh, PA), followed by concentration of the extract using a vacuum-driven Büchi Rotavapor evaporator (Brinkmann Instruments, Westbury, NY), and re-suspended in acetonitrile/water (82/18, vol/vol). The crude butanol extract was chromatographed on a silica gel 60 (EM Science, Gibbstown, NJ) column (Pharmacia LKB, 1.6 × 100cm, Uppsala, Sweden). Fractions were eluted with a 60:40 acetonitrile/water gradient, and further purified using reverse phase high performance liquid chromatography (HPLC) using a C18 column (5μ phase, 300Ǎ, Phenomonex, Torrance, CA). HPLC analysis was performed using a Waters 996 photodiode array detector coupled with Millennium chromatography software (Waters, Milford, MA). Structural analysis of purified lipo-chitin Nod signals was performed by mass spectrometry by Dr. Russell Carlson

(Complex Carbohydrate Research Center, The University of Georgia, Athens) as previously described (Sanjuan *et al.*, 1992).

Binding assays and inhibition analysis

Binding assays were performed according to Shibuya *et al.* (1996), with slight modifications. In most cases, 20 μg of plasma membrane (i.e., 20μg protein) were mixed with 10pmol of either [^{125}I]-GN_5-APEA or [^{125}I]-GN_8-APEA, and the final volume was adjusted to 300 μl with binding buffer [25mM Tris-HCl (pH 7.0) +1mM $MgCl_2$, 1M NaCl, 2mM DTT]. For inhibition analysis, an appropriate amount of unlabeled chitin oligosaccharide, chitosan oligosaccharide, or lipo-chitin Nod signal was added to the reaction mixture 1 hour prior to the addition of the labeled ligand. After incubation on ice for 1 hour, the reaction mixture was directly applied to a Durapore membrane filter (Multiscreen-GV 96-well filtration plate, 0.22 μm, Millipore Corporation, Bedford MA, USA). A vacuum was applied to the membrane, and the samples were washed twice with 200 μl of wash buffer [25mM Tris-HCl (pH 7.0) + $MgCl_2$, 2mM DTT]. Radioactivity retained on the membranes, which corresponds to bound ligand, was counted on a gamma counter (RiaGamma 1274 system, LKB Wallac Oy, Finland).

Affinity cross-linking

Affinity cross-linking of the [^{125}I]-GN_5 and [^{125}I]-GN_8 ligands to the plasma membrane or the microsomal fraction was performed in manner similar to the binding assays. After 1 hour incubation on ice, the reaction mixture was centrifuged at 41,000 rpm for 30 minutes (TLA 100.2 Fixed-Angle Rotor, Beckman Instruments, Fullerton, CA). The pellet was washed once with 1 ml of binding buffer, followed by 1 ml of distilled water. The pellet was then re-suspended in 50μl of phosphate-buffered saline (PBS, pH 7.4). Two hundred microliters of 2.5% glutaraldehyde and 50μl of Na-$CNBH_3$ (1mg/ml) were added to the reaction tube. The reaction was incubated at room temperature (25°C) for 1 hour. After incubation, the mixture was centrifuged at 41,000 rpm for 30 min. The pellet was washed twice with 1 ml of binding buffer, followed by two washes each with 1 ml of PBS. The pellet was re-suspended in 50μl of SDS sample buffer and analyzed by SDS-PAGE according to the method of Laemmli (1970) using 12% acrylamide. Dried gels were analyzed by autoradiography using a phosphor screen processed on a Storm 840 Optical Scanner (Molecular Dynamics, Sunnyvale, CA). Images were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Reactive oxygen detection and quantitation

(The following experiments were performed by Mitsuo Okada in the Laboratory of Dr. Naoto Shibuya, The National Institute of Agrobiological Resources, Tsukuba City, Japan).

The generation of reactive oxygen species induced by chitin oligomers was monitored as follows. Chitin oligosaccharides of various degrees of polymerization (d.p.=1-8) were added to suspension-cultured SB-1 soybean cells (Ho *et al.*, 1988). Fifty milligrams of suspension-cultured soybean cells were transferred to fresh modified B5 medium (Ho *et al.*, 1988), and grown at 25°C. After 1 hour, various chitin oligosaccharides were individually added to a final concentration of 1μM. An equal amount of sterile dH_2O was added to a control sample, instead of the chitin oligomers. The cells were incubated at 25°C for an additional 15 minutes, after which time they were transferred to an ice bath to quench the reaction. The cells were removed by low speed centrifugation, and the supernatant was used to determine the presence of reactive oxygen species by the luminol-dependent chemiluminsecence assay (Anderson *et al.*, 1991). Briefly, 50 μl of the supernatant was added to 50μl of 1.1mM luminol, 100 μl of 14mM potassium ferricyanide, and 300 μl 50mM potassium phosphate buffer (pH 7.9). Samples were immediately analyzed for

chemiluminescence using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Alkalinization of soybean suspension cell medium in response to perception of chitin oligosaccharides

(The following experiments were performed by Dr. Koji Tsukada in the laboratory of Dr. Naoto Shibuya, The National Institute of Agrobiological Resources, Tsukuba City, Japan).

To avoid wounding suspension-cultured soybean cells by mechanical stirring, and thus preventing a slow, but continuous alkalinization of the culture medium, 700 mg of cells were sequestered in a 15mm×50mm polyester mesh bag (Kureha Chemical Co., Tokyo). Mesh bags containing the suspension cells were placed into Erlenmeyer flasks containing 30 ml of fresh, sterile, B5 medium (Ho *et al.*, 1988), pH 5.5. After stirring the cells 300 rpm for 30 minutes at 25°C, chitin oligosaccharides of various degrees of polymerization (d.p.=1-8) were added individually to a final concentration of 5μM. A HM-5S pH meter (TOA Electronics, Tokyo) equipped with a glass pH electrode (GS-5015C, TOA Electronics, Tokyo) was used to monitor the pH change (ΔpH) of the medium. After each replicate experiment, the cells were

harvested from the mesh bags, and their fresh weight determined. Data were expressed as the magnitude of ΔpH normalized for cell mass.

ENOD40 induction by chitin oligomers

ENOD40 induction by chitin oligomers was monitored as previously described (Minami *et al.*, 1996). Briefly, 3-day-old seedlings of *G. soja* were spot-inoculated with 100μg of chitin oligosaccharides of various degrees of polymerization (d.p=1-8) in 1% (w/v) carboxymethylcellulose. Forty hours post-inoculation, root tissue corresponding to 1 cm above and 1 cm below the inoculated region of the root was harvested in liquid nitrogen and stored at -80°C until further use.

Total RNA was isolated from root tissue using the Trizol reagent (GibcoBRL Laboratories, Rockville, MD), and was treated with RNAse-free DNAse I (Promega, Madison, WI). RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI) using oligo-$(dT)_{12-18}$ as a primer, according to the manufacturer's protocol. Products were recovered by ethanol precipitation. Five microliters of reverse-transcribed cDNA was used in a polymerase chain reaction in the presence of 10mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 5 units Taq DNA Polymerase (Promega, Madison, WI). PCR reaction conditions were as

follows: 35 cycles (94°C for 1 min, 55°C for 3 min, 72°C for 1 min), with 10 minutes of extension time at 72°C. Primers, ENOD40b Forward and ENOD40b Reverse, are listed in Table 5, and were added to a final concentration of 2.5μM (Kouchi and Hata, 1993). An internal control was performed using primers identical to the soybean Ubiquitin gene (Table 5, Ubiquitin forward and Ubiquitin reverse). PCR products were separated by electrophoresis in a 0.8% agarose gel followed by blotting to ZetaProbe nylon membrane (BioRad Laboratories, Hercules, CA). The ENOD40 or Ubiquitin products were detected by hybridization with a ^{32}P-labeled full-length cDNA product for ENOD40b or Ubiquitin. Control reactions treated as above in the absence of reverse transcriptase gave no signal after hybridization (data not shown).

Thin-layer chromatography of $(GN)_5$-APEA and $(GN)_8$-APEA

To ascertain the structural integrity of the APEA ligands following the binding assays, the ligands were extracted from the binding reactions after 1-hour incubation on ice, and analyzed by thin-layer chromatography (TLC). Binding assays were performed as described above, using either $(GN)_5$-APEA and $(GN)_8$-APEA as ligands. After 1 hour incubation on ice, the reaction mixture was centrifuged at 41,000 rpm for 30 minutes (TLA 100.2 Fixed-Angle Rotor, Beckman Instruments, Fullerton, CA). Following centrifugation, the supernatant was removed to a sterile Eppendorf tube and stored at room temperature until TLC analysis. The pellet was

washed once with 500μl binding buffer, then re-suspended in 200μl N-butanol. The tube was vortexed vigorously for 20 seconds, then centrifuged at 41,000 rpm for 30 additional minutes. After centrifugation, the supernatant was transferred to a sterile Eppendorf tube and dried under filtered N_2 gas. The dried extract was re-suspended into 50μl of dH_2O. Ten microliters of each extract was spot inoculated onto a normal-phase silica TLC plate (Sigma Chemical Co., St. Louis, MO). The dried plate was developed in 100% acetonitrile. After chromatography, the plate was air-dried and exposed to X-OMAT AR X-ray film (Kodak, Rochester, NY) for seven days.

Nucleic Acid Isolation and Manipulation

Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* 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

Nine-day-old *G. soja* plants were homogenized in liquid nitrogen using a mortar and pestle, and genomic DNA was isolated using Promega's Wizard® Genomic DNA Kit (Promega, Madison, WI), according to the manufacturer's instructions.

Isolation of *gs50* and *gs52* cDNA clones

Isolation of total RNA and reverse transcription

Total RNA was isolated from 3-day and 5-day-old roots of *G. soja* using the Trizol® Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. RNA quantity and integrity was determined by monitoring the optical density (OD) at 260nm and by agarose gel electrophoresis, respectively. Reverse transcription of total RNA was performed as described above. An aliquot (5μl) was

utilized for touchdown polymerase chain reactions (Don *et al.*, 1991) in the presence of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 1 unit Taq DNA polymerase (Promega, Madison, WI). PCR cycle conditions were as follows: 94°C, 1 min.; 52°C, 1 min.; 74°C, 3 min. The annealing temperature was decreased 2°C every fourth cycle from 55°C to a 'touchdown' at 37°C. Degenerate primers designed to a conserved, internal, apyrase motif found in pea (Hseih *et al.*, 1996), potato (Handa and Guidotti, 1996), and LNP (Etzler *et al.*, 1999) were used at a 5μM final concentration. The sequences of the primers specific for *gs50* (*gs50* TD-PCR forward/reverse) and *gs52* (*gs52* TD-PCR forward/reverse) are listed in Table 5. PCR products were separated by electrophoresis in a 0.8% (w/v) agarose gel, and visualized by staining with ethidium bromide.

TA cloning and DNA sequencing of PCR-amplified products

PCR products corresponding to DNA fragments of expected sizes (*gs50*, 850bp; *gs52*, 357bp) were cloned into the TA-cloning vector pCR2.1 using Invitrogen's TA Cloning® Kit (Invitrogen, Carlsbad, CA). Ligated products were transformed into *INVαF´* One Shot™ competent cells (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Plasmid DNA was isolated according to the method of Sambrook *et al.* (1989).

All 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). The initial sequence data text files were edited following comparison with the same data displayed in four-color electropherograms before they were analyzed further. In both cases, both strands of two independent clones were sequenced to ensure the fidelity of the sequences.

Extension of cDNA ends

Extension of the cDNA ends of the initial gs50 and gs52 partial clones was achieved using the 5' and 3' Rapid Amplification of cDNA Ends (RACE) kit (Gibco BRL, Gaithersburg, MD), according to the manufacturer's specifications. Cloning, transformation, and DNA sequencing was performed as described above.

Isolation of full-length cDNAs encoding gs50 and gs52

After obtaining sequence data from the initial PCR reaction, as well as the 3' and 5' RACE products, primers were designed to regions flanking the coding sequence (ATG start) of both *gs50* and *gs52*. RT-PCR was performed as described

above. Five microliters of cDNA was used in a PCR reaction, with 5μM/each of the full-length primers flanking the coding sequence of *gs50* and *gs52*. PCR reaction conditions were as described above, except 1 unit of *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was substituted for *Taq* polymerase. The primer sequences and T_m of the full-length cDNA primers are listed in Table 5.

Southern blot analysis

Twenty micrograms of genomic DNA was digested with *Eco*RI, *Hin*dIII, or *Pst*I overnight at 37°C, according to the supplier's specifications. Digests were loaded onto a 0.8% agarose gel and separated for 1 hour at 100 volts, 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. Gene-specific probes for *gs50* and gs52 were labeled to a specific activity of 10^8 cpm/μg DNA using random primer labeling (Promega, Madison, WI).

Northern blot analysis

Total RNA 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).

gs50 and *gs52* mRNA Expression Analysis

Developmental expression

Root tissue was harvested at time points ranging from 2 days to 9 days post-imbibition of *G. soja* seeds. In all *gs50* and *gs52* expression-analysis experiments (e.g., development, nitrate, bacterial inoculation assays) described in this dissertation, the zone of the first emergent root hairs on each plant was marked when plants were transferred to growth pouches. To standardize 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 described above. Primers corresponding to the 5' and 3' full-length ends of *gs50* (*gs50* forward/reverse full) and *gs52* (*gs52* forward/reverse full) (Table 5) were used in PCR reactions as follows: 5μl cDNA + 10 mM Tris-HCL (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 5 units Taq DNA Polymerase (Promega, Madison, WI). DNA primers were added to a final concentration of 2.5μM. PCR conditions were as follows: 25 cycles (94°C for 1 min, 55°C for 3 min, 72°C for 1 min), with 10

minutes of extension time at 72°C. As an internal control, primers specific for Ubiquitin were also included (Ubiquitin forward/reverse, Table 5).

Tissue-specific expression of gs50 and gs52

Total RNA was isolated from roots, hypocotyls, cotyledons, stems, leaves, flowers, and nodules using the Trizol reagent (Gibco BRL, Gaithersburg, MD). Total RNA was reverse-transcribed and processed as described above. As an internal control, primers specific for Ubiquitin were also included (Ubiquitin forward/reverse, Table 5).

Bacterial inoculation assays

For rhizobial inoculation assays, plants were grown in pouches for 2 days, then inoculated with 10^6 cells of *B. japonicum* USDA110 re-suspended in ½-strength plant nutrient solution (PNS) (Wacek and Brill, 1976). At various time points (3hr-120 hr post-inoculation), 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. PCR reactions were performed exactly as described above. As an internal control, primers specific for Ubiquitin were also included (Ubiquitin forward/reverse, Table 5).

Nitrate expression assays

To determine the effect of nitrate on the expression pattern of *gs50* and *gs52*, plants were germinated and grown in the presence of a sterile 20 mM ammonium nitrate solution (pH 7.0). Control plants, receiving only treatment with distilled H_2O, were also included. Root tissue was harvested at time points ranging from 2 days to 9 days post-imbibition of *G. soja* seeds. Total RNA was isolated and reverse-transcribed as described above. PCR reactions were performed exactly as described above. As an internal control, primers specific for Ubiquitin were also included (Ubiquitin forward/reverse, Table 5).

Generation of Antibodies to GS50 and GS52

Construction of plasmids expressing GST-GS50 and GST-GS52 fusion proteins

GS50 and GS52 were expressed in *E. coli* cells from the IPTG-inducible Tac promoter of the expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ). The plasmid used in expressing GS50 was constructed as follows: A *Bam*HI-*Not*I fragment containing *gs50* (1423 bp) was released from pDAY46A (Table 4) and purified using Qiagen's gene clean kit (Qiagen, Valencia, CA). The 1.4 kb fragment was directionally cloned into pGEX-4T-3, which was *Bam*HI/*Not*I digested and subjected to alkaline phosphatase (Promega, Madison, WI) according to

the supplier's specifications. In-frame ligation of *gs50* in pGEX-4T-3 was confirmed by automated DNA sequencing using the pGEX forward primer (Pharmacia Biotech, Uppsala, Sweden).

The plasmid used in expressing GS52 was constructed as follows: A EcoRI fragment containing *gs52* (1467 bp) was released from pDAY52new (Table 4) and purified using Qiagen's gene clean kit (Qiagen, Valencia, CA). Orientation of the insert was determined by restriction analysis using *Hin*dIII 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 strains GEX50 and GEX52 were grown overnight in 50 ml of LB containing 200μg/ml ampicillin at 37°C and 200 rpm. This culture was then diluted into 8 liters of LB containing the same concentration of ampicillin and incubated under the same conditions. When an A_{600nm} 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 37°C 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 4°C 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 -80°C, 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 4°C to remove excess urea, and stored in 5 mL aliquots at -80°C until further use.

GST-sepharose 4B affinity purification of GS50 and GS52

Five milliliters of TN-dialyzed protein extracts 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 4°C 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 OD_{280nm} of zero. The bound fusion protein was eluted with 12 mM glutathione (pH 8.2), and protein content of eluted fractions was determined by monitoring the OD at 280nm.

Glycerol was added to the collected fractions to a final concentration of 15%, and fractions were stored at -80°C until further use.

Immunization and antibody purification

For the first immunization, a solution containing 1.5 mg purified GST-GS50 or 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 a two-week interval 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 -20°C in 500μl 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-GS50 and GST-GS52 antigen-affinity columns

Antigen purification of anti-GS50 and 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. One and a half milligrams of fusion protein (GST-GS50 or GST-GS52) dissolved in coupling buffer was added to the activated Sepharose 4B matrix and mixed end-over-end overnight at 4°C. Following overnight incubation, the mixture was transferred to blocking buffer (0.2M glycine, pH 8.0), and again incubated overnight at 4°C. The mixture was then transferred to a disposable, 10 ml chromatography column (BioRad Laboratories, Hercules, CA). Excess absorbed protein was removed by washing with 10 ml of coupling buffer, followed by acetate buffer (0.1 M sodium acetate + 0.5M NaCl, pH 4), followed by coupling buffer. Antigen-affinity purification of anti-GS50 or anti-GS52 antibodies was performed as follows: 1.5 mgs of Protein A purified IgG (see above) was dialyzed against TN

buffer overnight at 4°C. 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 5 ml of elution buffer (100mM Glycine, pH 2.5), and immediately mixed with 400 μl of 1M Tris (pH 8.0) to neutralize the sample. Affinity-purified antibodies were dialyzed overnight at 4°C in TBS (100 mM NaCl + 50 mM Tris, pH 7.0), and stored in 100μl aliquots at -80°C.

Western Blot Analysis

Tissue-specific expression of GS50 and GS52

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. 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 8.1) + 10 mM EDTA + 10 mM KCl + 1mM 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 -80°C 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 14,0000 rpm. The supernatant was transferred to a sterile Eppendorf tube and stored at -80°C until further use. Total proteins were separated by 12% preparative SDS-PAGE. Proteins were visualized by staining with 0.05% Coomassie brilliant blue R-250 (BioRad Laboratories, Hercules, CA). Detection of GS50 and GS52 was performed by immunoblotting using anti-GS50 or anti-GS52 antibodies.

Fractionation of soybean root membranes on sucrose density gradients

Membrane preparation was as above except for the following modifications. After isolation of the microsomal membrane fraction, the membranes were re-suspended into 40 ml of gradient medium [50 mM Mops (pH 7.5) + 2 mM EDTA + 5 mM DTT]. An aliquot (10 ml) of each fraction was layered onto four, 20-ml linear 20 to 45% (w/w) sucrose density gradients in gradient medium for isopycnic centrifugation at 100,000g at 4°C for 3 hours (Beckman SW-28 rotor, Beckman Instruments, Fullerton, CA). Following centrifugation, 1.5-mL fractions were collected (bottom to top) using a Versitaltic Pump Plus peristaltic pump (Manostat Corporation, New York, NY) connected to a Model 2110 fraction collector (BioRad Laboratories, Hercules, CA). The refractive index was determined using a Bausch and Lomb Abbe-3L refractometer (Bausch and Lomb, Rochester, NY), and sucrose concentrations were determined from a standard curve prepared using known sucrose concentrations. Protein concentrations of membrane fractions were determined using

the BCA protein assay (Pierce Chemical, Rockford, IL), using BSA as standard curve.

Membrane marker enzyme assays

Endoplasmic reticulum. Localization of ER membrane fractions were confirmed using a monoclonal antibody specific for calnexin (Li *et al.*, 1998). Calnexin monoclonal antibody was a gift from Dr. Heven Sze (University of Maryland, College Park, MD).

Golgi. Latent UDPase activity was determined as follows: Fifty micrograms of membrane was added to 50 μl assay buffer (3mM UDP + 3 mM $MnSO_4$ + 30 mM MES-Tris, pH 6.5), and incubated at 37°C for 20 minutes. Released phosphate was determined using the malachite green method (described below).

Plasma membrane. Vanadate-sensitive ATPase assays were conducted immediately after membrane fractionation. Twenty-five micrograms of membrane was added to 250 μl of ATPase assay buffer [40 mM Tris-MES (pH 6.75) + 3 mM $MgSO_4$ + 1 mM Na_2MoO_4 + 10 mM EGTA + 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, 3mM final concentration) was added to the reaction mixture, and the samples were incubated for an additional 30 minutes at 30°C. The reaction was stopped by the addition of 50 μl 5N H_2SO_4. Released phosphate was determined by

the malachite green method (described below). Vanadate- and nitrate-sensitive ATPase activities were calculated as the difference between the activities with and without the respective inhibitor.

Malachite green method of phosphate determination

Orthophosphate release was determined using a modified method of Baykov *et al.* (1988). To prepare the color reagent, 3 parts 0.045% (w/v in water) malachite green/HCl, are mixed with 1 part 4.2% (w/v in 4N HCl) NH_4-molybdate. The mixtures was covered with foil, stirred for 30 minutes, then filtered through two No. 1 Whatman filters into a polyethylene container. To 10 ml of this mixture, 200μl 2% (w/v) tergitol NP (Pierce Chemical, Rockford, IL) was added. This mixture could be stored at 4°C for up to 1 week. Released phosphate was determined as follows: 800μl of the above mixture was added to 50-100μl ATPase assay. The mixture was incubated at room temperature for 1 minute. After incubation, 100μl of 34% (w/v) sodium citrate-$2H_2O$ was added to the reaction, and the mixture was incubated for 1 hour at room temperature. Following incubation, the absorbance was read at 630 nm. Phosphate standards were prepared containing 0 to 30 nmol P_I from potassium phosphate (monobasic).

Restriction Fragment-Linked Polymorphism (RFLP)-based mapping of GS52

(These experiments were performed by Dr. Roxanne Denny in the laboratory of Dr. Nevin Young, The University of Minnesota, St. Paul, MN).

GS52 was mapped in a *G. max* cv. Evans x PI209332 RIL population. Unfortunately, a polymorphism for GS50 was not found in this mapping population and, therefore, GS50 was not mapped. Leaf tissue from 90 lines of a RIL (Recombinant Inbred Line) population generated from the Evans x PI209332 F2 population to the F4 generation by single seed descent were pooled for bulk DNA extraction and analysis. Plant DNA was extracted and analyzed by the method of Concibido *et al.* (1996). Parental survey blots containing parental genomic DNA was digested with *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, and *Taq*I, and blotted according to Concibido *et al.* (1996). DNA probes corresponding to *gs50* and *gs52* were end-labeled using γ-[^{32}P]-dATP to a specific activity of 10^8 cpm/μg DNA. GS52 was analyzed by two-way contingency table analysis using Statview II (Abacus Concepts, Berkley, CA). Linkage between RFLP markers or microsatellite markers was inferred if the probability of observing a chi-square value was 0.0001.

CHAPTER 4

RESULTS

Section I: A high-affinity chitin binding site on the soybean plasma membrane.

Preparation of radiolabeled chitin oligosaccharide ligands

An aminophenyl group was conjugated to both *N*-acetylchitopentaose and *N*-acetylchitooctaose by reductive amination with 2-(4-aminophenyl) ethylamine (APEA) according to the method of Ito *et al.* (1997). The structure of the synthesized ligands is shown in Figure 6. The ligands, 1(2-(4-aminophenyl) ethyl) amino-1-(tetra-N-acetylchitotetraosyl)-*N*-acetyldeoxyglucosaminitol(GN_5-APEA), and 1(2-(4-aminophenyl)ethyl)amino-1-(hepta-*N*-acetylchitoheptaosyl)-*N*-acetyldeoxyglucosaminitol (GN_8-APEA), were purified by gel filtration cation exchange chromatography, and reverse-phase HPLC as previously described (Ito *et al.*, 1997, data not shown).

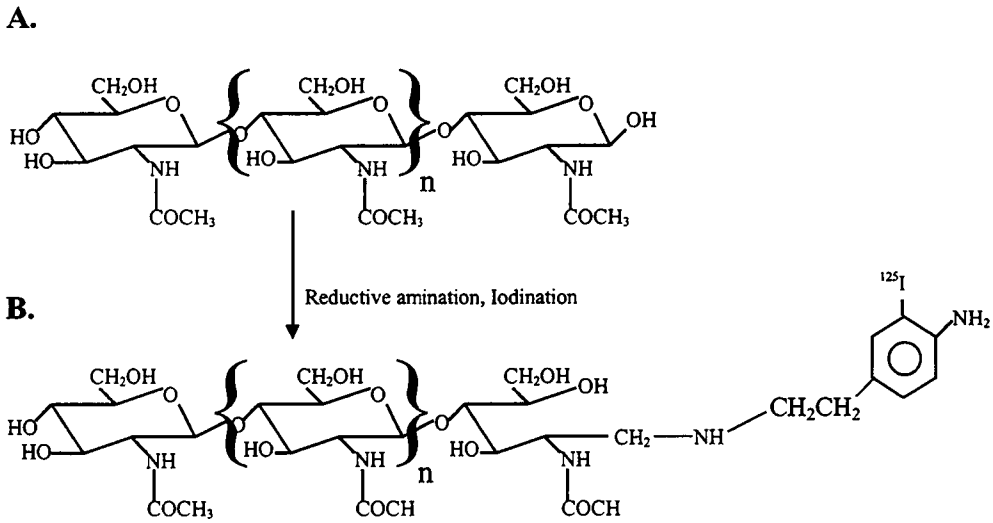


Figure 6: Structure of ligands used in saturation analysis, inhibition analysis, and affinity cross-linking studies. A) β-1,4-linked *N*-acetylchitooligosaccharide. B) Chitin-APEA ligand.

Identification of a high-affinity binding site for chitin in the soybean plasma membrane

To further define the structural requirements of chitin recognition in soybean, we investigated the binding affinities of a number of chitin oligosaccharides for a high-affinity binding site localized in the soybean plasma membrane. We employed two different modified chitin oligosaccharides, GN_5-APEA and GN_8-APEA. The binding of the APEA-ligands was determined using plasma membrane enriched fractions from both suspension-cultured cells, as well as 5-day root tissue. As can be seen in Figure 7, binding of GN_8-APEA to plasma membrane-enriched fractions isolated from suspension-cultured soybean cells was saturable at a ligand concentration of approximately 80nM. Ligand binding to plasma membrane purified from 5-day root tissue showed a similar pattern of saturation (data not shown). Saturation of ligand binding was determined in the presence and absence of a "cold" competitive inhibitor, $(GlcNAc)_7$, to determine non-specific and total binding. Specific binding was calculated as the difference in the two values. The saturation of GN_5-APEA binding to plasma membrane-enriched fractions from both suspension-cultured cells and root tissue showed a similar pattern of binding to that of the GN_8-APEA ligand (data not shown).

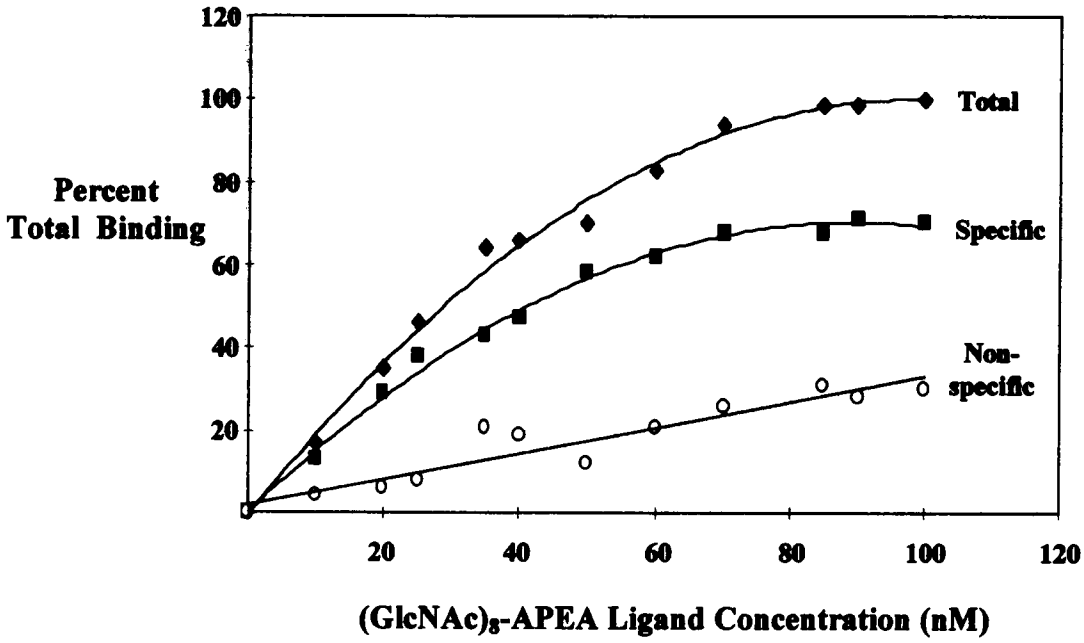


Figure 7: Binding of ^{125}I-(GlcNAc)$_8$-APEA ligand to purified plasma membrane is saturable. Total binding represents binding in the absence of cold, competitive inhibitor ([GlcNAc]$_7$). Specific binding, the difference in binding in the presence and absence of (GlcNAc)$_7$. Non-specific binding, binding in the presence of saturating concentrations of (GlcNAc)$_7$.

Scatchard and Hill plot analysis

Scatchard analysis (Scatchard, 1949) of ^{125}I-GN$_5$-APEA and ^{125}I-GN$_8$-APEA binding to the plasma membrane preparations from both suspension-cultured cells, as well as root tissue, indicated the presence of a highly specific binding site for chitin oligosaccharides. Data obtained using ^{125}I-(GN)$_8$-APEA yielded a K_d of 35nM and a B_{max} of 12.2 fmol/μg protein for suspension-cultured cells. Binding of GN$_8$-APEA to plasma membrane preparations from root tissue indicated a K_d of 47nM and a B_{max} of 7.6 fmol/μg protein. (Figure 8). In both cases, the data identify a high-affinity binding site for chitin at the plasma membrane surface. The slight difference in the K_d value most likely represents a difference in homogeneity of the preparations and not the presence of two distinct sites. The Hill coefficient for all Scatchard data, with an average value of ~0.93 (Figure 9), indicated a single class of binding sites that does not show cooperativity (Hill, 1913).

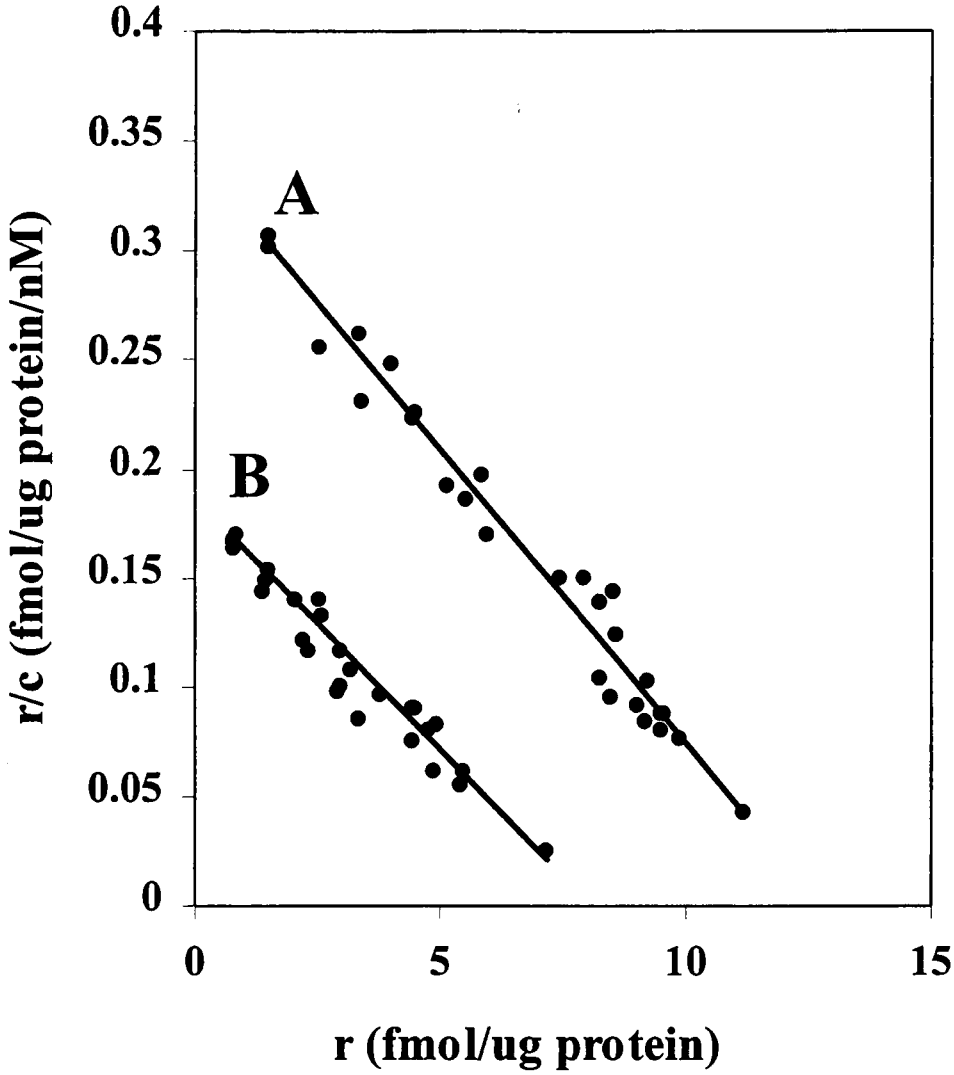


Figure 8. Scatchard analysis of ^{125}I-(GN)$_8$-APEA binding to soybean plasma membrane. A) Suspension-cultured soybean cells, K_d=35 nM. B) Five-day-old root cells, K_d=47 nM.

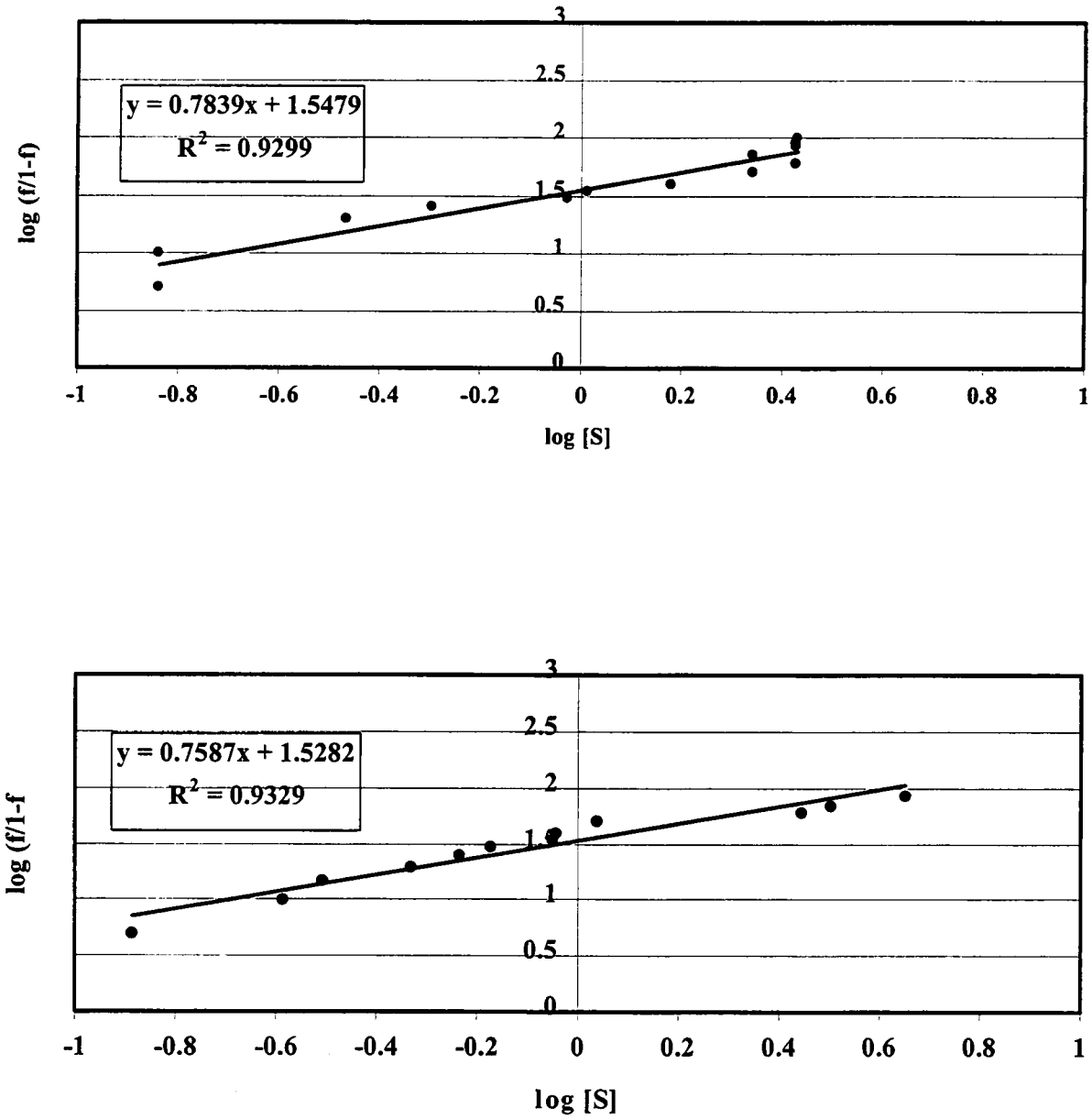


Figure 9. Hill plot analysis. Data obtained using plasma membrane from A) Suspension-cultured soybean cells or B) 5-day-old root tissue. [S]=^{125}I-(GlcNAc)-APEA ligand concentration. f = percent specific bound ligand.

Structural specificity and inhibition analysis of chitin binding

The biological action of the lipo-chitin Nod signals in the legume-*Rhizobium* symbiosis exhibits a strict specificity with regard to the Nod signal chemical structure. To determine if chitin perception at the plasma membrane surface demonstrates the same specificity, we performed a detailed analysis of chitin binding in the presence of competing chitin oligomers (d.p.=1-8). As can be seen in Figure 10, inhibition of chitin binding proceeded in a size-dependent fashion. The larger chitin oligosaccharide compounds were the better inhibitors of ligand binding to plasma membrane isolated from suspension-cultured soybean cells. In all cases, the *N*-acetylchitooctaose showed complete inhibition of ligand binding at a concentration of 25μM, indicating a high degree of specificity for the chitooctaose. Figure 10B shows the result of ^{125}I-GN_8-APEA-inhibition analysis using root plasma membrane. Again, inhibition of chitin binding was size-dependent, with chitooctaose being the best inhibitor of ligand binding. Similar results were obtained using ^{125}I-GN_5-APEA as the ligand (data not shown). Additionally, *N*-acetylglucosamine and *N*-acetylchitobiose were shown to have no inhibitory effect on ligand binding at concentrations as high as 250μM; thereby, suggesting that there is a size minimum for binding inhibition. In addition to assessing the inhibitory effect of various chitin oligosaccharides, de-acetylated chitin oligosaccharides (chitosan) at concentrations as high as 250μM were also tested for their ability to inhibit ligand binding. None of the

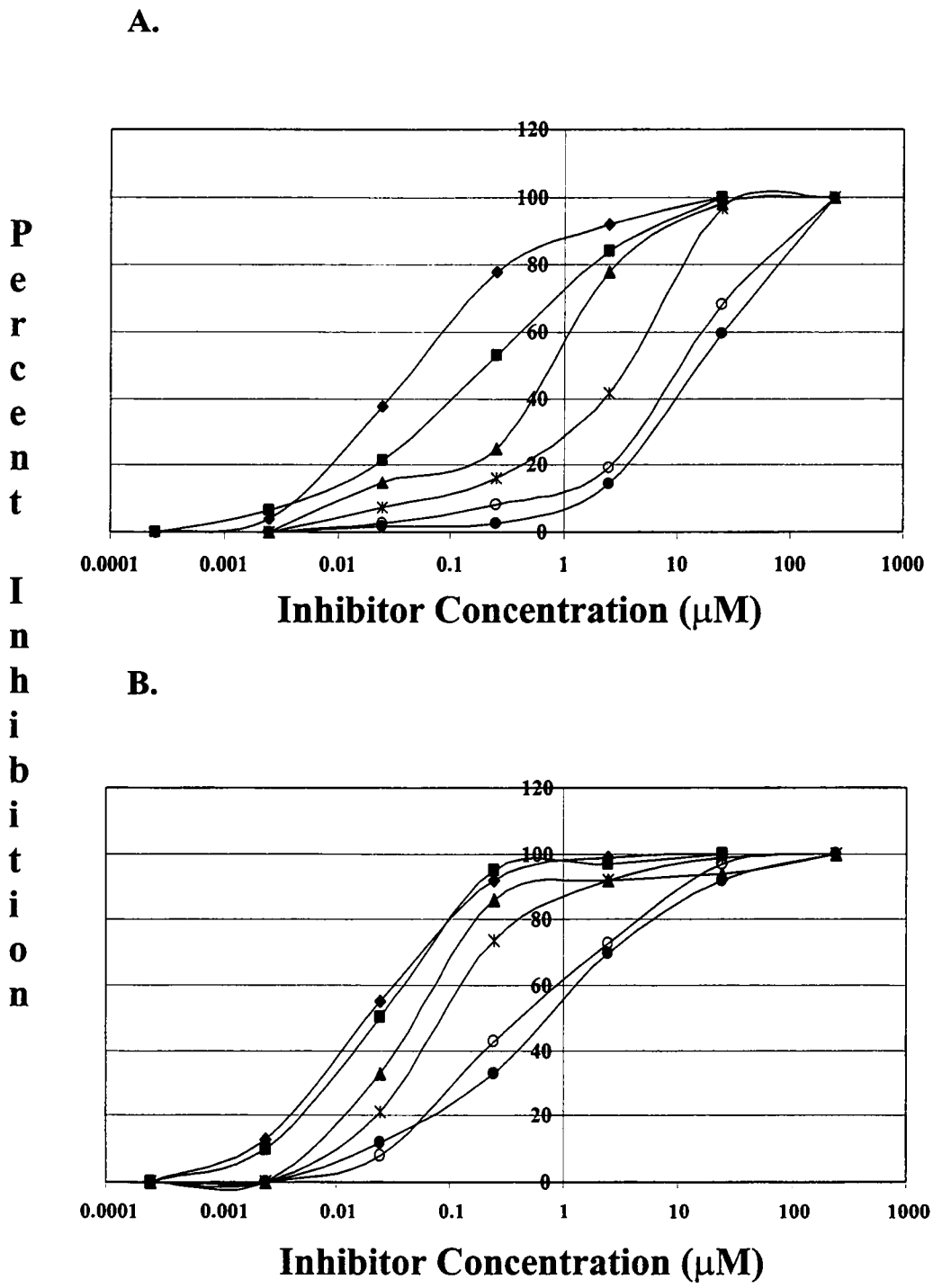


Figure 10. Inhibition of chitin oligosaccharide binding to a plasma membrane-enriched fraction from soybean proceeds in a size-dependent manner. A Inhibition of binding to plasma membrane isolated from suspension-cultured soybean cells. B) Inhibition of binding to plasma membrane isolated from 5-day-old roots tissue (*Glycine max*). ♦, Octamer; ■, Heptamer; ▲, Hexamer; *, Pentamer; o, Tetramer; ·, Trimer.

chitosan oligomers (d.p.=2-8) had an inhibitory effect on ligand binding (data not shown). Taken together, the data from the suspension-cultured cells, as well as the root tissue plasma membrane, suggest that GN_5-APEA and GN_8-APEA are interacting with a single class of binding sites which prefers larger *N*-acetylated chitin oligosaccharides.

Purification of lipo-chitin Nod signals produced by B. japonicum USDA110

Figure 11 shows a high-performance liquid chromatography (HPLC) elution profile of the LCO of *B. japonicum* USDA110. The various Nod signals were distributed among four HPLC peaks (Figure 11, asterisks). The major peak, which eluted at 44.3 minutes (48% acetonitrile/water (v/v)), was identified using mass spectrometry (data not shown, analysis performed by Dr. Russell Carlson, The Complex Carbohydrate Research Center, The University of Georgia, Athens, GA) as the *B. japonicum* USDA110 Nod signal [Nod*Bj*V(C18:1Δ11, MeFuc)] (Sanjuan *et al.*, 1992).

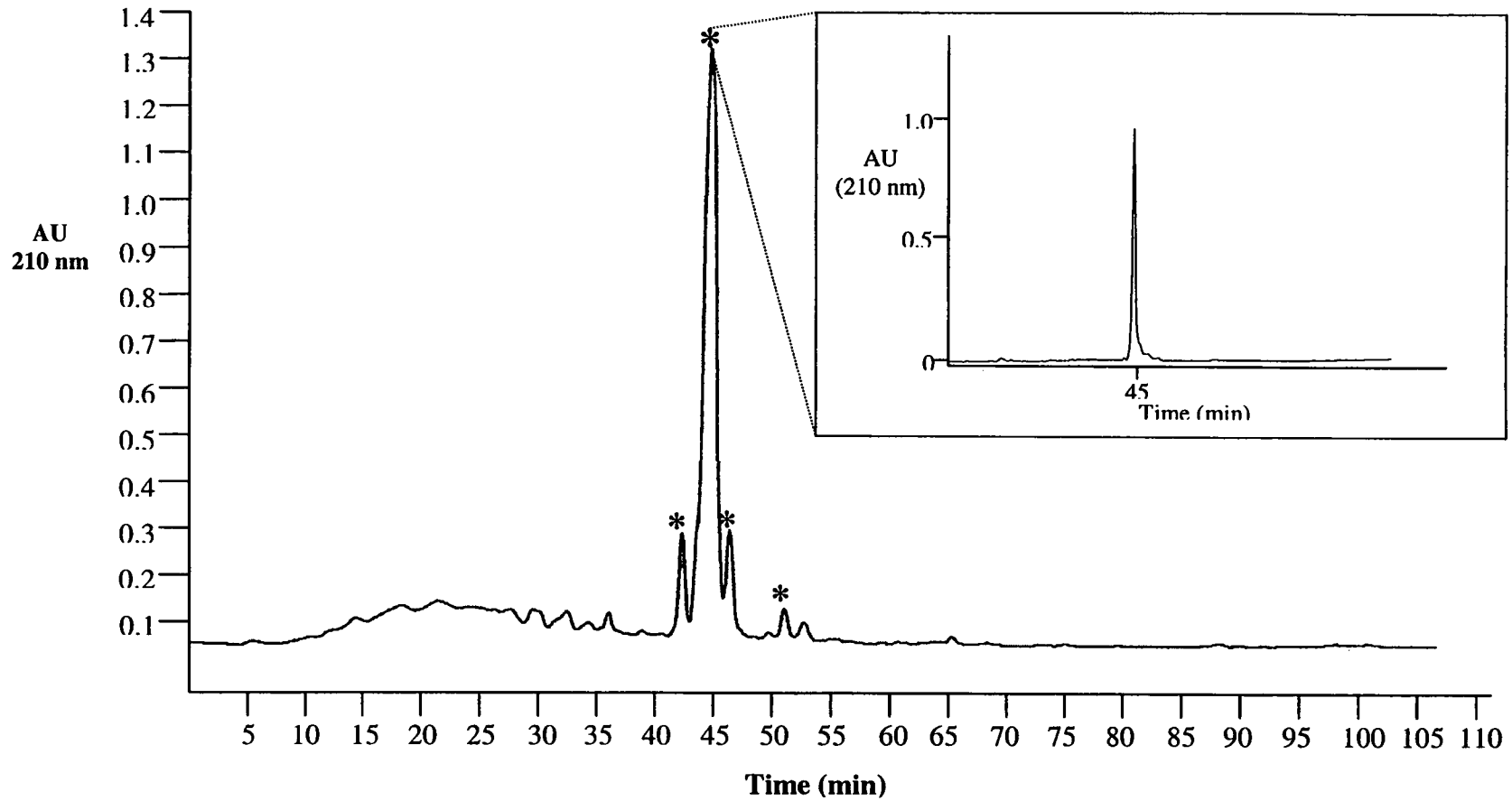


Figure 11. HPLC elution profile of the Nod signals produced by *B. japonicum* USDA110. Inset chromatogram shows the elution profile of the major Nod signal, Nod*Bj*V(C18:1Δ11, MeFuc).

The major lipo-chitin Nod signal from B. japonicum USDA110 inhibits ^{125}I-(GlcNAc)$_5$-APEA binding to the plasma membrane of soybean

The structural requirements for inhibition of chitin binding to the soybean plasma membrane suggests that Nod signals may also inhibit binding. Therefore, we tested the ability of the major Nod signal produced by *B. japonicum* USDA110 to inhibit chitin binding to soybean plasma membrane. As shown in Figure 12, inhibition of ^{125}I-(GlcNAc)$_5$-APEA binding to soybean root plasma membrane was observed. However, the level of inhibition was less than that of the structurally similar *N*-acetylchitopentaose. With an IC_{50} value of approximately 10 μM, the major Nod signal produced by *B. japonicum* USDA110 is similar to the chitotriose in its ability to inhibit chitin binding to the plasma membrane of soybean. Surprisingly, the same range of Nod signal concentrations added as a "cold" competitor did not inhibit ^{125}I-(GlcNAc)$_8$-APEA binding to the plasma membrane (data not shown).

Affinity cross-linking of GN$_8$-APEA to soybean plasma membrane

To detect the presence of the high affinity chitin-binding site, an affinity cross-linking method was employed, as previously described for the detection of a chitin binding site in rice plasma membrane (Ito *et al.*, 1997). SDS-PAGE analysis identified the presence of a major band of approximately 85 kDa, with minor bands at

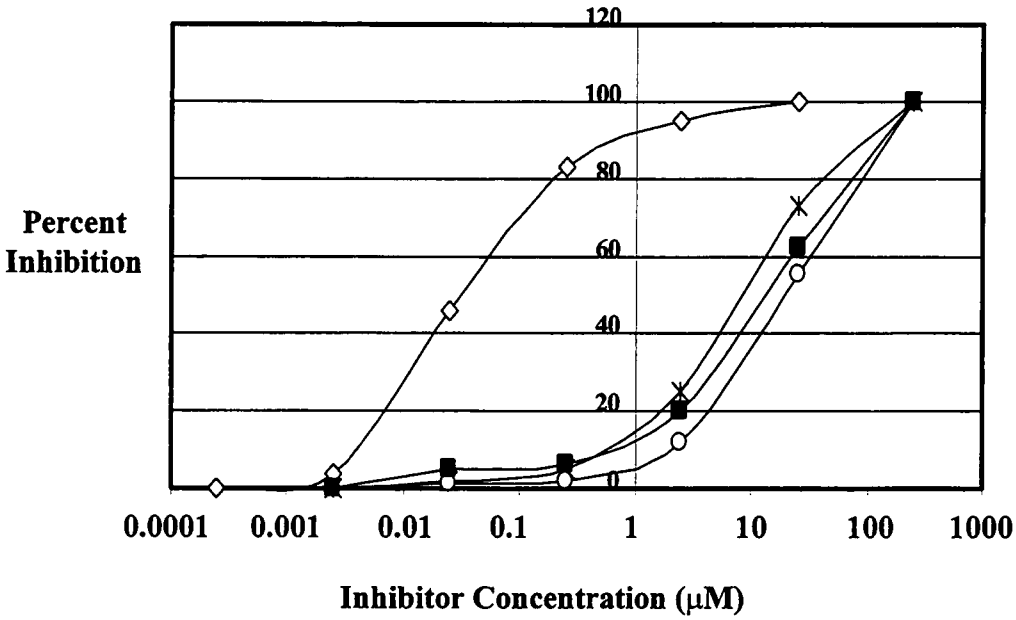


Figure 12. Inhibition analysis of ^{125}I-(GlcNAc)$_5$-APEA binding to soybean root plasma membrane by: , (GlcNAc)$_8$; *, (GlcNAc)$_5$; ■, Nod*Bj*V(C18:1Δ11, MeFuc); and o, (GlcNAc)$_3$.

~14 and ~150 kDa (Figure 13). The major band at 85 kDa is similar in size to the Elicitor Binding Protein (EBP) previously described in rice (Ito *et al.*, 1997). The largest band, 150 kDa, may represent a dimer of the 85 kDa protein. The smaller band is likely a proteolytic fragment of the major band. In the presence of a 250-fold molar excess (25μM) of competitive $(GlcNAc)_7$ inhibitor, no signal was detected, indicating saturation of the binding site.

In addition to using chitin oligosaccharides as "cold" competitive inhibitors of ligand binding in the cross-linking of the plasma membrane, we also employed a variety of concentrations of the major lipo-chitin Nod signal from *B. japonicum*. Affinity cross-linking of soybean plasma membranes to the ^{125}I-(GlcNAc)-APEA ligands showed results similar to those obtained from the inhibition analyses described above (Figure 12). Purified Nod*Bj*V(C18:1Δ11, MeFuc) was added to the binding reaction at final concentrations ranging from 0.0025 μM to 500 μM. Again, when ^{125}I-$(GlcNAc)_8$-APEA was used as a ligand, no inhibition of binding was detected at concentrations in excess of 500 μM, again suggesting that the major lipo-chitin Nod signal produced by *B. japonicum* USDA110 is a relatively weak inhibitor of octamer binding to soybean plasma membrane (Figure 14A). When ^{125}I-$(GlcNAc)_5$-APEA was used as a ligand, a slight inhibition of binding was detectable (Figure 14B). However, when compared to Figure 14A, in which $(GlcNAc)_8$ was used as a "cold" competitor (100% inhibition), the major Nod signal is again a relatively weak competitor.

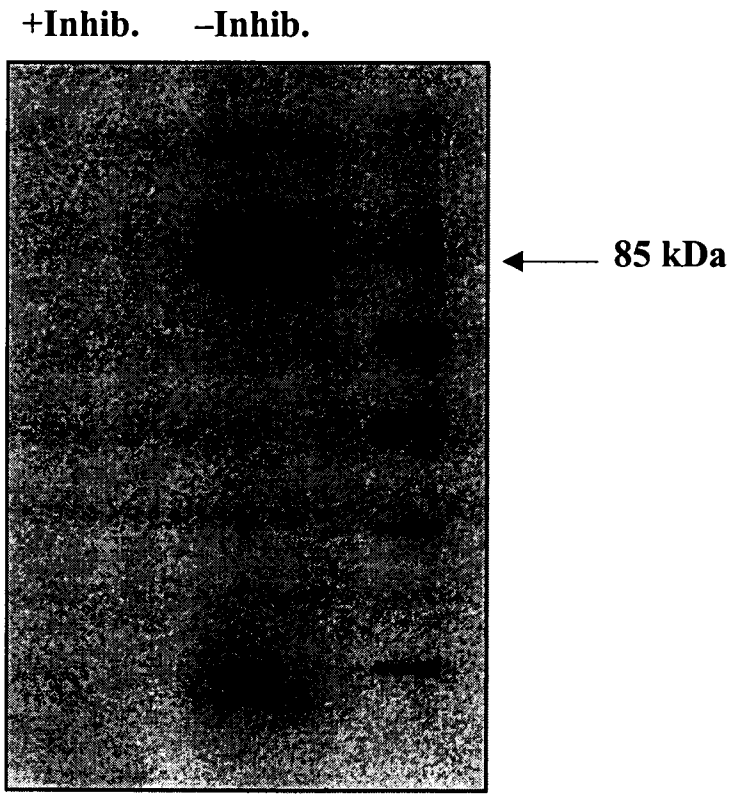


Figure 13: Affinity cross-linking of ^{125}I-(GlcNAc)$_8$-APEA ligand to the plasma membrane-localized 85 kDa binding protein. Plasma membrane was incubated in the presence of 10 pmol of ^{125}I-ligand in the presence (+Inh.) or absence (-Inh.) of chitin oligosaccharide inhibitor [(GlcNAc)$_7$,(250 molar excess)].

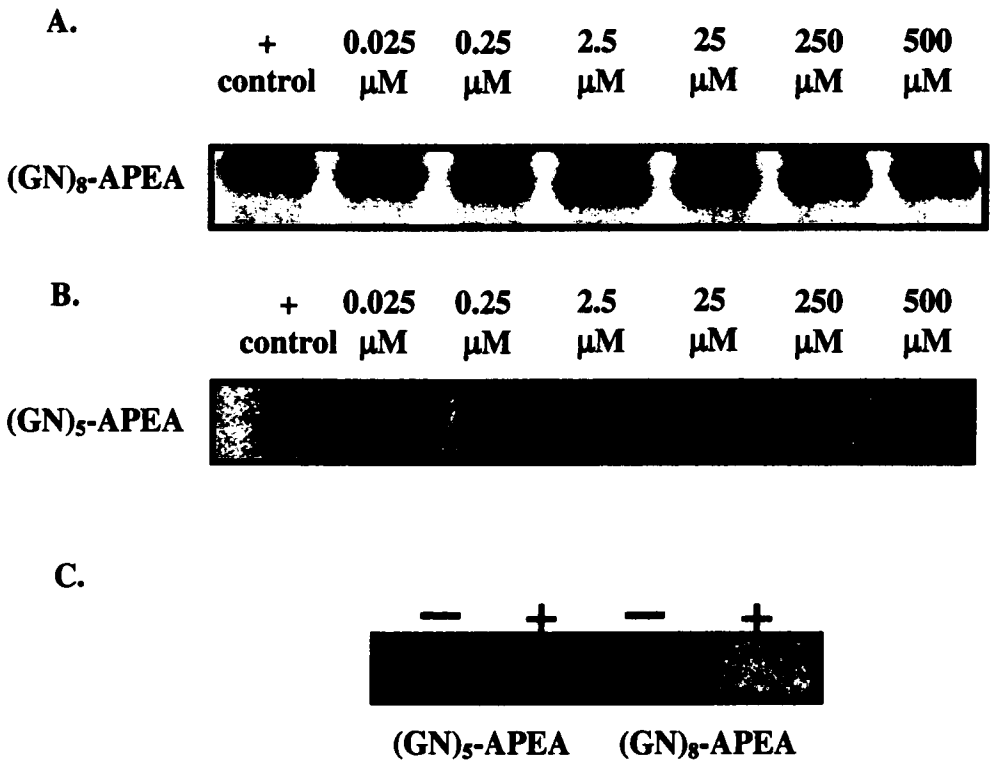


Figure 14: Affinity cross-linking of soybean plasma membrane. Nod signal inhibition of: A) ^{125}I-(GlcNAc)$_8$-APEA binding by *Bj*NodV (C18:1Δ11, MeFuc), or B) ^{125}I-(GlcNAc)$_5$-APEA binding by *Bj*NodV (C18:1Δ11, MeFuc). C) Cross-linking the presence (+), or absence (-), of (GlcNAc)$_5$ or (GlcNAc)$_8$.

Generation of reactive oxygen in suspension-cultured soybean cells by N-acetylchitooligosaccharides

To correlate the presence of a high-affinity chitin binding site in the plasma membrane with a biological response, the generation of reactive oxygen (ROX) in response to chitin elicitation was monitored by the luminol-chemiluminescence assay (Anderson *et al.*, 1991). As can be seen in Figure 15, ROX was generated in suspension-cultured soybean cells upon treatment with purified chitin oligosaccharides. The generation of ROX was directly dependent on the degree of polymerization of the chitin oligomers. The pattern of the response to chitin oligomers shown in Figure 15 is very similar to the specificity of the chitin binding site in the plasma membrane, shown in Figure 10. In both cases, the larger chitin oligosaccharides (i.e., $[GlcNAc]_8$) were preferred. Furthermore, (GlcNAc) or $(GlcNAc)_2$, which lacked the ability to inhibit ^{125}I-$(GlcNAc)_8$-APEA binding to plasma membrane preparations, were also incapable of eliciting a significant generation of reactive oxygen (ROX) (Figure 15). The levels of ROX production shown in Figure 15 are similar to the levels previously reported using rice suspension-cultured cells in response to chitin elicitation (Kuchitsu *et al.*, 1993; Minami *et al.*, 1996a; Shibuya *et al.*, 1996).

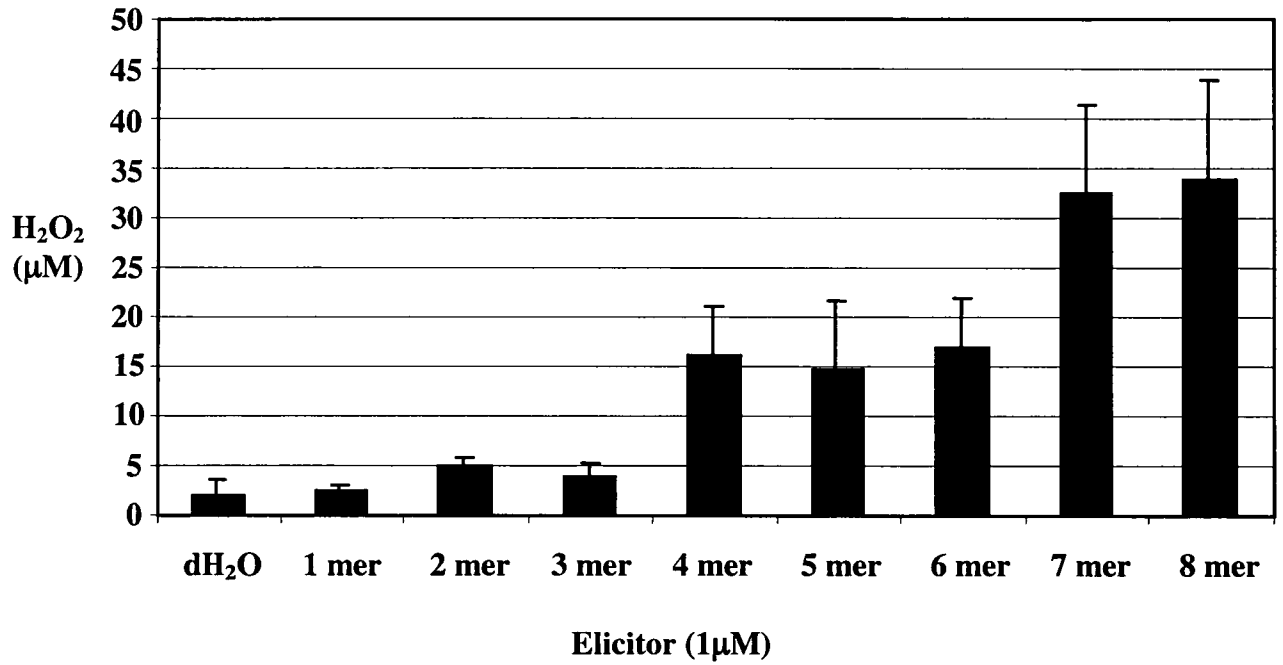


Figure 15: Generation of reactive oxygen in suspension-cultured soybean cells by *N*-acetylchitooligosaccharides. Suspension-cultured soybean cells were incubated in the presence or absence (dH_2O) of 1 μM chitooligosaccharides of varied degrees of polymerization, and assayed for the generation of reactive oxygen species. Data represents the average of 3-independent trials in triplicate.

N-acetylchitooligosaccharides induce alkalinization of culture medium of soybean suspension cells in a size-dependent manner

Alkalinization of the culture medium of suspension-cultured plant cells has been reported as an early response to microbial infection (He *et al.*, 1998), as well as to the perception of purified chitin-oligosaccharide elicitors compounds (Kuchitsu *et al.*, 1993; Baureithel *et al.*, 1994; Minami *et al.*, 1996c). To determine if perception of chitin oligosaccharides by soybean suspension cells induces a rapid alkalinization of the culture medium, chitin oligosaccharides of varied degrees of polymerization were tested for their ability to induce a change in pH (ΔpH) of the medium upon elicitor treatment. As seen in Figure 16, *N*-acetylchitooligosaccharides induced the alkalinization of the culture medium of soybean suspension cells in a size dependent manner. That is, treatment of soybean cells with chitin oligosaccharides of a greater degree of polymerization (i.e., $[GlcNAc]_8$) induced a greater ΔpH, as compared to *N*-acetylchitooligosaccharides of a lesser degree of polymerization (i.e., $[GlcNAc]_1$). Further supporting the binding specificity of the plasma membrane-localized protein, treatment of suspension cells with equal molar amounts of chitosan, the de-acetylated chitin oligosaccharide, resulted in no measurable ΔpH of the culture medium ($[GlcNH_2]_7$, Figure 16).

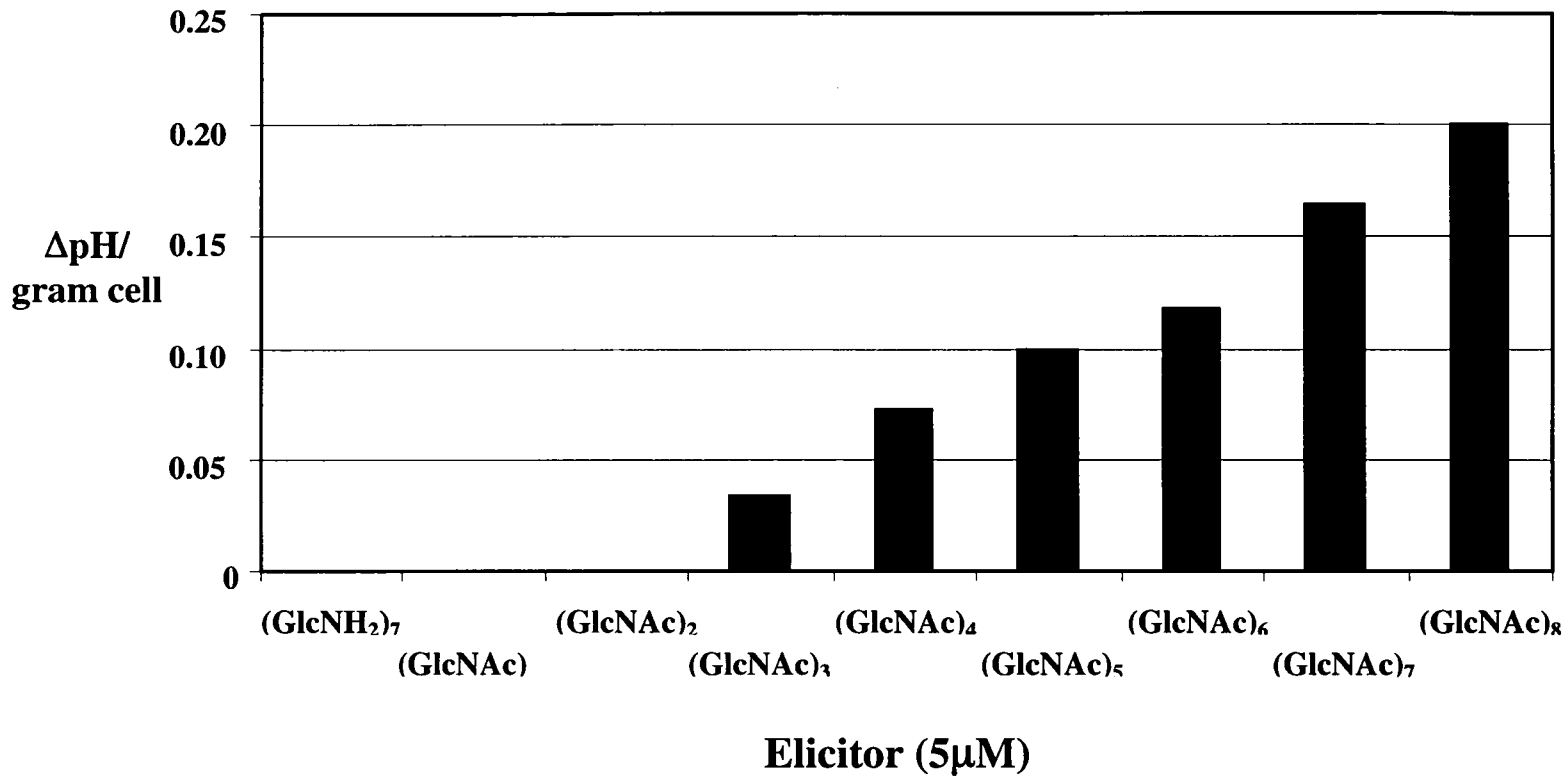


Figure 16: Alkalinization of soybean suspension cell culture medium in response to chitin oligomers. Suspension-cultured cells were incubated in the presence of 5 μM chitooligosaccharides of various degrees of polymerization, and the culture medium was monitored for a change in pH (ΔpH). All data was normalized for cell mass. Standard deviation of samples was +/- 5%. Data provided by Dr. Koji Tsukada, National Institute of Agrobiological Resources, Tsukuba City, Japan.

Accumulation of ENOD40 mRNA by N-Acetylchitooligosaccharides is dependent upon the degree of polymerization of the chitin backbone

Minami *et al.* (1996a) showed rapid mRNA accumulation of the soybean early nodulin ENOD40 in soybean roots treated with a variety of natural and synthetic LCO molecules. Interestingly, ENOD40 induction was observed using LCO molecules defined as both active and inactive in terms of inducing root hair deformation and nodule initiation in *G. soja* roots. Indeed, treatment with an unsubstituted chitin pentamer induced ENOD40 expression in soybean roots. In lieu of our identification of a high affinity chitin-binding site on the soybean plasma membrane, we decided to re-examine the induction of ENOD40 in response to chitin oligomers of different lengths. As shown in Figure 17, the accumulation of ENOD40 mRNA proceeded in a size-dependent fashion, with the chitin pentamer being the most active inducer of ENOD40 expression. Quantitation of raw data is shown in Figure 18. Interestingly, chitin oligosaccharides of a greater degree of polymerization (e.g., heptamer and octamer) were also capable of inducing the accumulation of ENOD40 mRNA to a significant level above the control sample (Figure 17, cmc only). In addition, the modified chitin oligomers used as ligands in the binding assays were also able to induce the rapid accumulation of ENOD40 mRNA; therefore, demonstrating that modification to the reducing end of the backbone does not eliminate activity.

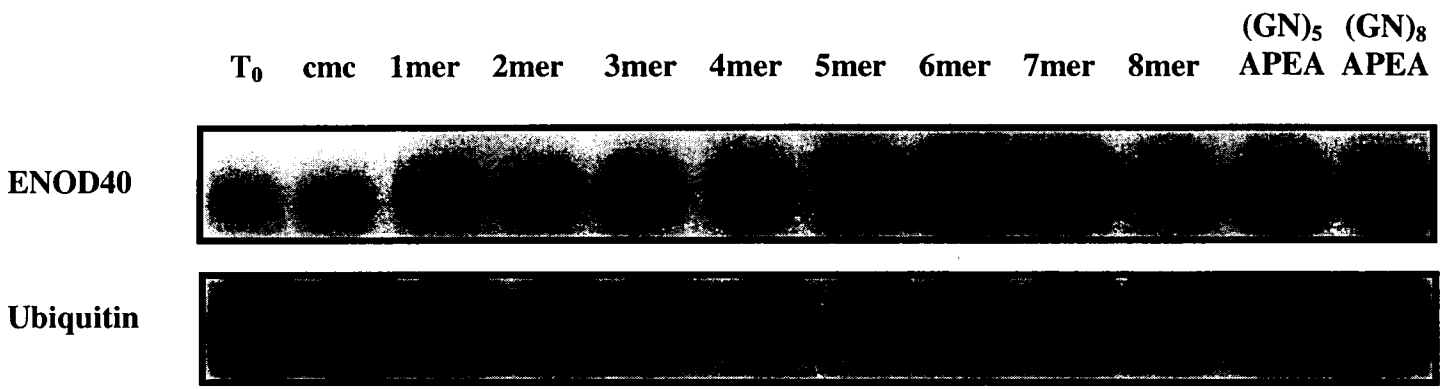


Figure 17. Size-dependent induction of ENOD40 mRNA by *N*-Acetylchitooligosaccharides. T_0, time-point at which plants were spot-inoculated with oligosaccharides (dissolved in 1% cmc), or with cmc, carboxymethylcellulose, control.

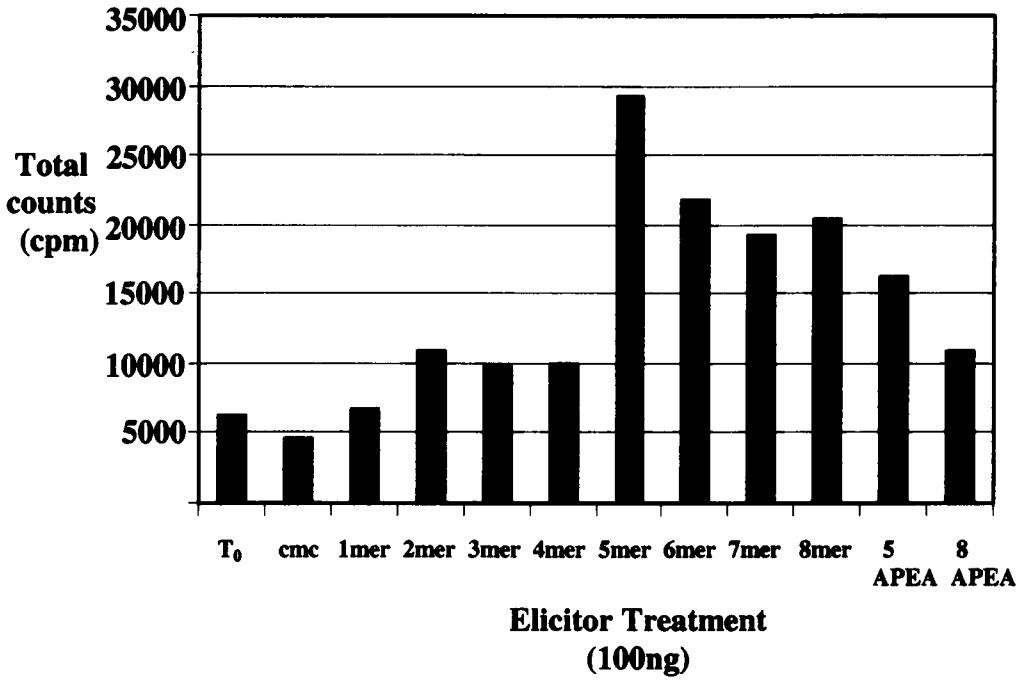


Figure 18. Induction of ENOD40 expression by chitin oligomers 40 hours post-inoculation. cmc, carboxymethylcellulose; (GlcNAc), *N*-acetylglucosamine; T_0, expression of ENOD40 at 3 days; 5 APEA, $(GlcNAc)_5$-APEA; 8 APEA, $(GlcNAc)_8$-APEA; APEA, amino-phenylethylamine.

Section II: Identification of Two Soybean Orthologs, *gs50* and *gs52*, to a Nod Signal Binding Protein From *Dolichos biflorus*.

Etzler *et al.* (1999) demonstrated the ability of an apyrase (LNP) present in the roots of *D. biflorus* to bind the major Nod signal produced by *B. japonicum* USDA110. Based on these results, we sought to identify a putative ortholog to LNP present in the roots of soybean. Using primers designed to a conserved catalytic domain present in apyrases from a variety of plant species, two soybean orthologs were identified using a TD-PCR approach. The 850 bp (*gs50*) (pDAY15, Table 4) and 351 bp (*gs52*) (pDAY9, Table 4) partial cDNAs isolated using TD-PCR were extended using RACE PCR (Frohman *et al.*, 1988) to yield two full-length cDNA sequences. To guard against the possibility of errors introduced during the PCR amplification, two clones were independently generated from each end, and both strands were sequenced in entirety. Isolation of the full-length cDNA clones (e.g., DAY46-A, *gs50* and DAY46-B, *gs52*; Table 4) was performed using PCR with *Pfu* DNA polymerase and primers complementary to the 3' and 5' ends of *gs50* and *gs52* (*gs50* full forward/reverse and *gs52* full forward/reverse, Table 5). As shown in Figure 19, the sequence of *gs50* predicts has an open reading of 463 amino acids, encoding a protein with an apparent molecular weight of 50,600. The sequence of the gs52 cDNA (Figure 20) predicts an open reading frame of 468 amino acids encoding a protein with an apparent molecular weight of 51,817. Using NCBI Blast analysis (Altschul *et al.*, 1997), the sequences of *gs50* and *gs52* were compared to known DNA and protein sequences in the NCBI database.

Figure 19. Nucleotide and translated protein sequence of *gs50*. Conserved catalytic apyrase motif is indicated by **bold** font.

```
        10                  30                  50
gcaactATGAGCAAGGATTATCTAATCATACTTTCTCTCCTTCTTTCCACGGTGCTTGCA
---------+---------+---------+---------+---------+---------+
      M  S  K  D  Y  L  I  I  L  S  L  L  L  S  T  V  L  A
        70                  90                 110
GCGGCAAGTTCTTCTTCTTCTTCTTCGTCCCTCTATCTTGGGAAGGGCTTCTCCCATCGC
---------+---------+---------+---------+---------+---------+
A  A  S  S  S  S  S  S  S  S  L  Y  L  G  K  G  F  S  H  R
       130                 150                 170
AAATTGTCTCCCAGTTATCACATTCACAAAACCATTGACGAATCCTATGCAGTTATCTTC
---------+---------+---------+---------+---------+---------+
K  L  S  P  S  Y  H  I  H  K  T  I  D  E  S  Y  A  V  I  F
       190                 210                 230
GATGCTGGTAGCACGGGAAGCCGTGTACATGTCTATCGTTTTAACCAGCAATTAGATCTC
---------+---------+---------+---------+---------+---------+
D  A  G  S  T  G  S  R  V  H  V  Y  R  F  N  Q  Q  L  D  L
       250                 270                 290
CTTCGCATTGGCCAAGACCTTGAGTTGTTCGTCAAGACTATGCCAGGTTTAAGTGCTTAC
---------+---------+---------+---------+---------+---------+
L  R  I  G  Q  D  L  E  L  F  V  K  T  M  P  G  L  S  A  Y
       310                 330                 350
GCTGAGAATCCACAAGATGCTGCAGAATCTCTTATTCCACTTTTGGAGGAAGCAGAAGCT
---------+---------+---------+---------+---------+---------+
A  E  N  P  Q  D  A  A  E  S  L  I  P  L  L  E  E  A  E  A
       370                 390                 410
GCTGTTCCTCAGGAGTTTCACCCCAGGACGCCAGTTAAACTTGGAGCAACTGCAGGTTTA
---------+---------+---------+---------+---------+---------+
A  V  P  Q  E  F  H  P  R  T  P  V  K  L  G  A  T  A  G  L
       430                 450                 470
AGGCAATTGGAAGGGGATGCTTCTGATAGGATATTGCAGGCGGTCAGTGACATGCTCAAG
---------+---------+---------+---------+---------+---------+
R  Q  L  E  G  D  A  S  D  R  I  L  Q  A  V  S  D  M  L  K
       490                 510                 530
AACAGAAGCACACTGAACGTTGGGGCCGATGCAGTTTCAGTGTTGAGTGGAAACCAAGAA
---------+---------+---------+---------+---------+---------+
N  R  S  T  L  N  V  G  A  D  A  V  S  V  L  S  G  N  Q  E
       550                 570                 590
GGAGCTTATCAATGGGTAACAATTAACTATCTACTGGGAAACTTGGGAAAGCATTATTCA
---------+---------+---------+---------+---------+---------+
G  A  Y  Q  W  V  T  I  N  Y  L  L  G  N  L  G  K  H  Y  S
       610                 630                 650
GAAACTGTTGCAGTAGTTGATCTTGGAGGTGGATCTGTTCAAATGGCTTATGCAGTCTCA
---------+---------+---------+---------+---------+---------+
E  T  V  A  V  V  D  L  G  G  G  S  V  Q  M  A  Y  A  V  S
       670                 690                 710
GAGACAGATGCTGCGAAGGCTCCACGAGCACCGGACGGAGTGGAATCATACATAACGGAG
---------+---------+---------+---------+---------+---------+
E  T  D  A  A  K  A  P  R  A  P  D  G  V  E  S  Y  I  T  E
       730                 750                 770
ATGTTCCTTAGGGGAAAGAAATATTACCTCTATGTACACAGTTACTTGCGATACGGTATG
---------+---------+---------+---------+---------+---------+
M  F  L  R  G  K  K  Y  Y  L  Y  V  H  S  Y  L  R  Y  G  M
       790                 810                 830
CTAGCAGCTCGCGCAGAGGCTTTAAAGGTTCGTCATTCTGAAAATCCTTGTATTTTGGCT
---------+---------+---------+---------+---------+---------+
L  A  A  R  A  E  A  L  K  V  R  H  S  E  N  P  C  I  L  A
       850                 870                 890
GGCTTTGATGGGTATTACGTATATGGAGGAGTGCAGTATAAGGCCAAAGCTCCCCCTTCT
---------+---------+---------+---------+---------+---------+
G  F  D  G  Y  Y  V  Y  G  G  V  Q  Y  K  A  K  A  P  P  S
       910                 930                 950
GGCTCAAGCTTCAGCCAATGCCAAAATGTTGTTGTTGAAGCACTCCATGTTAATGCAACA
---------+---------+---------+---------+---------+---------+
G  S  S  F  S  Q  C  Q  N  V  V  V  E  A  L  H  V  N  A  T
```

Figure 19, cont.

```
   970                  990                 1010
TGCTCTTACAAGGATTGCACTTTCGGAGGGATATGGAATGGTGGTGGGGGAGCTGGAGAA
---------+---------+---------+---------+---------+---------+
C  S  Y  K  D  C  T  F  G  G  I  W  N  G  G  G  G  A  G  E
      1030                1050                1070
AACAACTTTTTTATTGCCTCATTTTTCTTTGAAGTTGCCGATGAGGCTGGTTTTGTTGAT
---------+---------+---------+---------+---------+---------+

N  N  F  F  I  A  S  F  F  F  E  V  A  D  E  A  G  F  V  D
      1090                1110                1130
CCAAACGCCCCCAATGCCAAAGTTCGTCCTGTGGATTTTGAGAATGCAGCAAAGGTTGCC
---------+---------+---------+---------+---------+---------+
P  N  A  P  N  A  K  V  R  P  V  D  F  E  N  A  A  K  V  A
      1150                1170                1190
TGTAACACAGAATTAAAGGATCTCAAATCCATTTTCCCTCGTGTTAAGGATGGAGACGTT
---------+---------+---------+---------+---------+---------+
C  N  T  E  L  K  D  L  K  S  I  F  P  R  V  K  D  G  D  V
      1210                1230                1250
CCTTATATATGCTTGGATCTTGTATACGAATATACATTGCTCGTTGATGGATTTGGCATA
---------+---------+---------+---------+---------+---------+
P  Y  I  C  L  D  L  V  Y  E  Y  T  L  L  V  D  G  F  G  I
      1270                1290                1310
GATCCGCAGCAAGAGATTACGTTGGTGAGGCAAGTTGAGTATCAAGATTCTCTCGTGGAA
---------+---------+---------+---------+---------+---------+
D  P  Q  Q  E  I  T  L  V  R  Q  V  E  Y  Q  D  S  L  V  E
      1330                1350                1370
GCAGCACGGCCACTAGGCAGTGCCATAGAAGCCATATCATCATTACCTAAATTTGAGAAA
---------+---------+---------+---------+---------+---------+
A  A  R  P  L  G  S  A  I  E  A  I  S  S  L  P  K  F  E  K
      1390                1410                1430
TTAATGTATTTCATTTAAgctttaaaacgaggagccatatgtatgtgtggaatgaactcc
---------+---------+---------+---------+---------+---------+
L  M  Y  F  I
      1450                1470
atcactcttaaataaaaatcgttttgttccttttttct
---------+---------+---------+---------
```

Figure 20. Nucleotide and translated protein sequence of *gs52*. Conserved catalytic apyrase motif is indicated by **bold** font.

```
        10                  30                  50
cggagagtttgaaaATGGTACTTGTGCTATGGTCAAGAGGGACTAAGAACATGGACTTCC
---------+---------+---------+---------+---------+---------+
             M  V  L  V  L  W  S  R  G  T  K  N  M  D  F  L
        70                  90                 110
TAACCCTCTTCACTCTTCTACTCTTGTTATTCATTCATACAGCACTCTCTTCTACCCAAT
---------+---------+---------+---------+---------+---------+
  T  L  F  T  L  L  L  L  L  F  I  H  T  A  L  S  S  T  Q  Y
       130                 150                 170
ATCATGATGGGAACATCTTACTCACTCATCGTAAGATCTTCCCCAAACAGGAAGCTATCA
---------+---------+---------+---------+---------+---------+
  H  D  G  N  I  L  L  T  H  R  K  I  F  P  K  Q  E  A  I  T
       190                 210                 230
CCTCGTACGCTGTCATCTTTGATGCTGGTAGCACTGGTAGCCGTGTGCATGTCTTCCATT
---------+---------+---------+---------+---------+---------+
  S  Y  A  V  I  F  D  A  G  S  T  G  S  R  V  H  V  F  H  F
       250                 270                 290
TCGACCAGAACTTGGATCTCCTTCGTATTGGCAACGAACTCGAGTTTTATGATAAGGTAA
---------+---------+---------+---------+---------+---------+
  D  Q  N  L  D  L  L  R  I  G  N  E  L  E  F  Y  D  K  V  T
       310                 330                 350
CACCCGGTTTGAGTGCATACGCAGATAATCCTCAACAAGCTGCAGAATCTCTTATCCCAC
---------+---------+---------+---------+---------+---------+
  P  G  L  S  A  Y  A  D  N  P  Q  Q  A  A  E  S  L  I  P  L
       370                 390                 410
TTTTAGAGGAAGCTGAAAGTGTTGTCCCTGAGGACCTGTACCCTACTACACCCGTTAAGC
---------+---------+---------+---------+---------+---------+
  L  E  E  A  E  S  V  V  P  E  D  L  Y  P  T  T  P  V  K  L
       430                 450                 470
TTGGGGCGACTGCAGGTTTAAGGCTTTTGGAAGGGGATGCTTCTGAAAGGATATTGCAAG
---------+---------+---------+---------+---------+---------+
  G  A  T  A  G  L  R  L  L  E  G  D  A  S  E  R  I  L  Q  A
       490                 510                 530
CGGTTAGGGATTTGCTCAAGAACAGAAGTACCCTGAACGCTCAACCAGATGCAGTAGCTA
---------+---------+---------+---------+---------+---------+
  V  R  D  L  L  K  N  R  S  T  L  N  A  Q  P  D  A  V  A  I
       550                 570                 590
TTATTGATGGAACCCAAGAAGGTTCTTATCTATGGGTGACAATTAATTATTTATTGGGGA
---------+---------+---------+---------+---------+---------+
  I  D  G  T  Q  E  G  S  Y  L  W  V  T  I  N  Y  L  L  G  K
       610                 630                 650
AGTTGGGAAAAAGGTTTTCAAAGACAGTAGGAGTAGTTGATCTTGGAGGTGGATCAGTTC
---------+---------+---------+---------+---------+---------+
  L  G  K  R  F  S  K  T  V  G  V  V  D  L  G  G  G  S  V  Q
       670                 690                 710
AAATGGCATATGCCATCTCAAGGAACACAGCAAAAAATGCTCCAAAACCACCAGATGGAG
---------+---------+---------+---------+---------+---------+
  M  A  Y  A  I  S  R  N  T  A  K  N  A  P  K  P  P  D  G  E
       730                 750                 770
AGGAACCTTACATAAAGAAGCTTGTACTCAAGGGAAAGGAATATGACCTCTATGTTCACA
---------+---------+---------+---------+---------+---------+
  E  P  Y  I  K  K  L  V  L  K  G  K  E  Y  D  L  Y  V  H  S
       790                 810                 830
GTTATTTGCATTACGGTAGAGAAGCATCTCGTGCCGAGATTTTGAAGGTCACTGATGGCT
---------+---------+---------+---------+---------+---------+
  Y  L  H  Y  G  R  E  A  S  R  A  E  I  L  K  V  T  D  G  S
       850                 870                 890
CCGCTAACCCTTGTATTTTAGCTGGCTACGATGGGACCTACACATATTCAGGAGCAGACT
---------+---------+---------+---------+---------+---------+
  A  N  P  C  I  L  A  G  Y  D  G  T  Y  T  Y  S  G  A  D  Y
```

Figure 20, cont.

```
       910                 930                 950
ATAAGGCCTTCGCTCCCATTTCTGGCTCCAGTTACGATGAATGTAGAGAGGTTGTTCTTC
---------+---------+---------+---------+---------+---------+
  K  A  F  A  P  I  S  G  S  S  Y  D  E  C  R  E  V  V  L  Q

       970                 990                1010
AAGCTCTCAAATTGAATGAGTCATGCCCCCATCAAAATTGCACATTTGGTGGTATATGGG
---------+---------+---------+---------+---------+---------+
  A  L  K  L  N  E  S  C  P  H  Q  N  C  T  F  G  G  I  W  D
      1030                1050                1070
ATGGTGGAAGGGGAAGTGGACAGAAAATTCTTTTCGGCACTTCGTCTTTCTATTATCTAC
---------+---------+---------+---------+---------+---------+
  G  G  R  G  S  G  Q  K  I  L  F  G  T  S  S  F  Y  Y  L  P
      1090                1110                1130
CTACAGAGATTGGTATCATTGATCTAAATAAACCCAACTCCAAAATTCATCCTGTTGATC
---------+---------+---------+---------+---------+---------+
  T  E  I  G  I  I  D  L  N  K  P  N  S  K  I  H  P  V  D  L
      1150                1170                1190
TTGAGATTGAAGCTAAACGAGCTTGTGAAACAAAATTGGAGGATGCCAAGTCCACCTATC
---------+---------+---------+---------+---------+---------+
  E  I  E  A  K  R  A  C  E  T  K  L  E  D  A  K  S  T  Y  P
      1210                1230                1250
CAAACCCTGCTGAGGATAGACTTCCATATGTATGCTTGGATATTGCATACCAGTATGCAT
---------+---------+---------+---------+---------+---------+
  N  P  A  E  D  R  L  P  Y  V  C  L  D  I  A  Y  Q  Y  A  L
      1270                1290                1310
TGTACACCGATGGATTTAGCCTTGATCCATGGCAAGAGATTACAGTGGCAAATGAAATAG
---------+---------+---------+---------+---------+---------+
  Y  T  D  G  F  S  L  D  P  W  Q  E  I  T  V  A  N  E  I  E
      1330                1350                1370
AATATCAGGATGCTCTTGTGGAAGCTGCATGGCCTTTAGGCACTGCCATAGAAGCCATAT
---------+---------+---------+---------+---------+---------+
  Y  Q  D  A  L  V  E  A  A  W  P  L  G  T  A  I  E  A  I  S
      1390                1410                1430
CGTCATTGCCTAAATTTGATCGATTAATGTATTTTATTtaaagcttatgctgggatattt
---------+---------+---------+---------+---------+---------+
  S  L  P  K  F  D  R  L  M  Y  F  I  *
      1450
cagttgtttaaaggccactttac
---------+---------+---
```

Using the NCBI *BlastP* program (Altschul *et al.*, 1997), more than 15 similar proteins were identified from both plants and animals. Analysis of all the sequences revealed four well-conserved regions (Figure 21, selected sequences only). None of the putative conserved sites showed any striking similarity to the consensus Walker ATP binding motifs (Walker *et al.*, 1982). A phylogenetic tree generated using an alignment of 16 published sequences from a diverse group of apyrases, including GS50 and GS52, is shown in Figure 22. It is noteworthy that the grouping of the legume apyrases fall into discrete sub-categories, which might indicate specialized function which distinguish them from non-legume orthologs. References and GenBank accession numbers for the apyrase sequences discussed in the text and figures are as follows: *gs50* and *gs52* (This Study, BankIt305028 and BankIt305031, respectively), LNP (Etzler *et al.*, 1999; AF139870), *L. japonicus* (Roberts *et al.*, 1999; AF156780), *M. sativa* (Roberts *et al.*, 1999; AF156782), *P. sativum* NTPase (Hsieh *et al.*, 1996; Z32743), *A. thaliana* (Roberts *et al.*, 1999; AF156783), *S. tuberosum* (potato) (Handa and Guidotti, 1996; U58597), CD39 (Chadwick and Frischauf, 1998; NM_001249), Human ecto-apyrase (Matsumoto *et al.*, 1999; AJ133133), and mouse apyrase (Wang and Guidotti; U81295).

Hydropathic plot analysis was performed using MegAlign™ sequence analysis software (DNASTAR Inc, Madison, WI) (Figure 23). The complete deduced amino acid sequence of GS50 shows only one predicted transmembrane segment at residues 1 to 22. Hydropathy analysis of GS52 also suggests the presence of

```
GS50              52   ESYAVIF [redacted] VHVYRFN  73
GS52              52   TSYAVIF [redacted] VHVFHFD  77
LNP               52   TSYAVIF [redacted] VHVFNFD  73
PNTPASE           42   SSYAVVF [redacted] IHVYHFN  63
POTATO APYRASE    44   EHYAVIF [redacted] VHVFRFD  65
GDA1              91   HKYVIMI [redacted] VHIYKFD  112
HUMAN CD39        47   VHYGIVL [redacted] LYIYKWP  68

GS50             127   RT [redacted] KLG [redacted] QLEGDAS  146
GS52             131   TT [redacted] KLG [redacted] LLEGDAS  150
LNP              117   KT [redacted] KLG [redacted] LLDGDAS  136
PNTPASE          118   KT [redacted] RLG [redacted] LLNGDAS  135
POTATO APYRASE   120   KT [redacted] ELG [redacted] MLKGDAS  140
GDA1             165   KT [redacted] AVK [redacted] LL.GDAS  184
HUMAN CD39       124   KT [redacted] AVK [redacted] LL.GDAS  143

GS50             172   VLS [redacted] NQ [redacted] AYQWVTI [redacted] YLLGNLGK 195
GS52             176   IID [redacted] TQ [redacted] SYLWVTI [redacted] YLLGKLGK 199
LNP              172   VID [redacted] TQ [redacted] SYLWVTV [redacted] YLLGKLGK 196
PNTASE           163   IID [redacted] TQ [redacted] SYLWVTV [redacted] YALGNLGK 184
POTATO APYRASE   165   ILD [redacted] TQ [redacted] SYMWAAI [redacted] YLLGNLGK 186
GDA1             211   .MG [redacted] DE [redacted] VFAWITT [redacted] YLLGNIGA 232
HUMAN CD39       169   .IT [redacted] QE [redacted] AYGWITI [redacted] YLLGKFSQ 183

GS50             199   ETVAVV [redacted] L [redacted] MAYAVSET     224
GS52             203   KTVGVV [redacted] L [redacted] MAYAISRN     228
LNP              179   KTVGVI [redacted] L [redacted] MAYAVSKK     210
PNTPASE          190   KTVGVI [redacted] L [redacted] MAYAVSKK     210
POTATO APYRASE   192   KTTATI [redacted] L [redacted] MAYAISNE     212
GDA1             239   PTAAVF [redacted] L [redacted] IVFAVSEP     257
HUMAN CD39       207   ETFGAL [redacted] L [redacted] MAYAVSET     220
```

Figure 21. Multiple protein sequence alignment of apyrase proteins from a variety of eukaryotic species. Highlighted text indicates conserved catalytic motif.

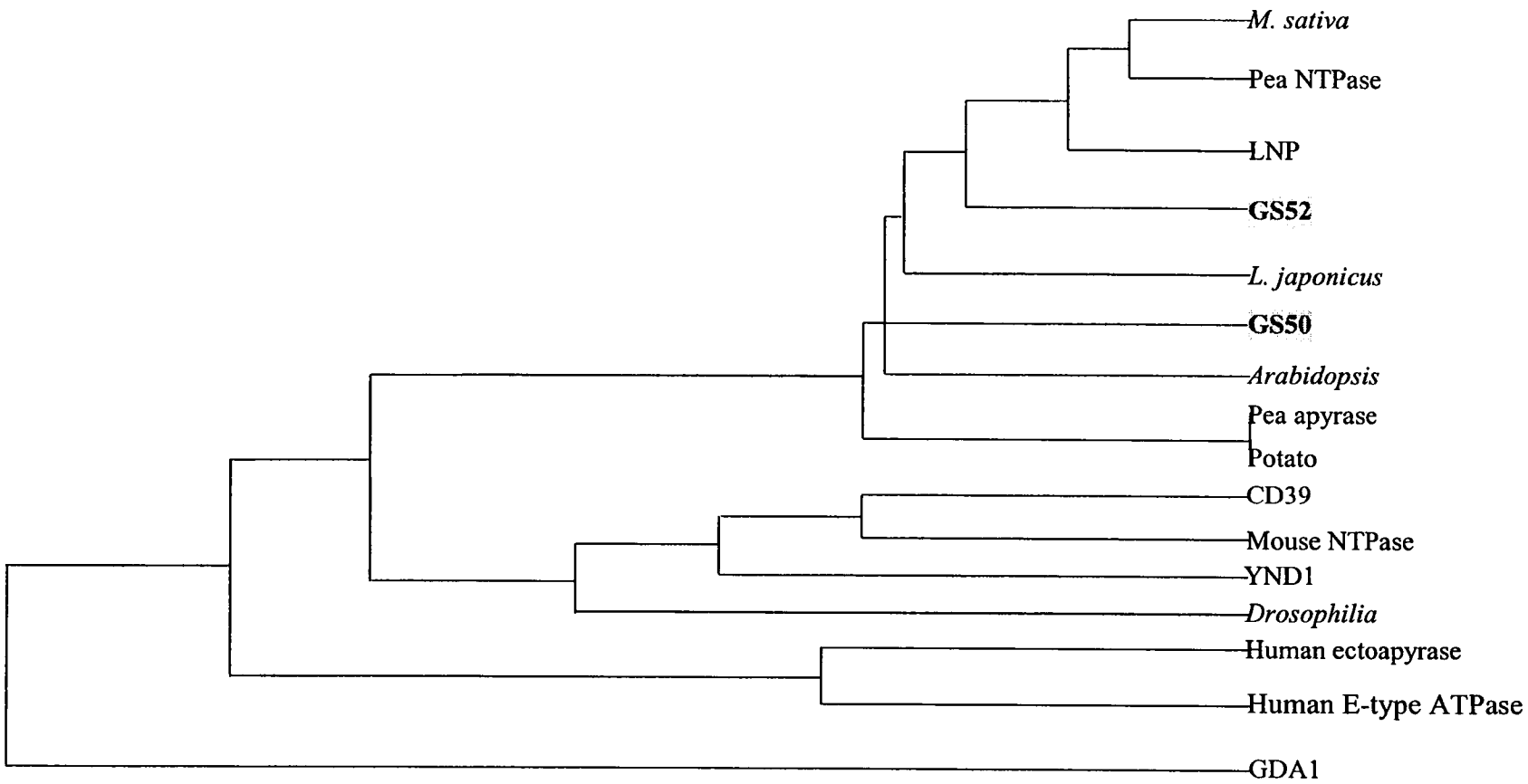


Figure 22. Phylogenetic analysis of apyrase proteins from plants and animals. References and accession numbers are provided in the text.

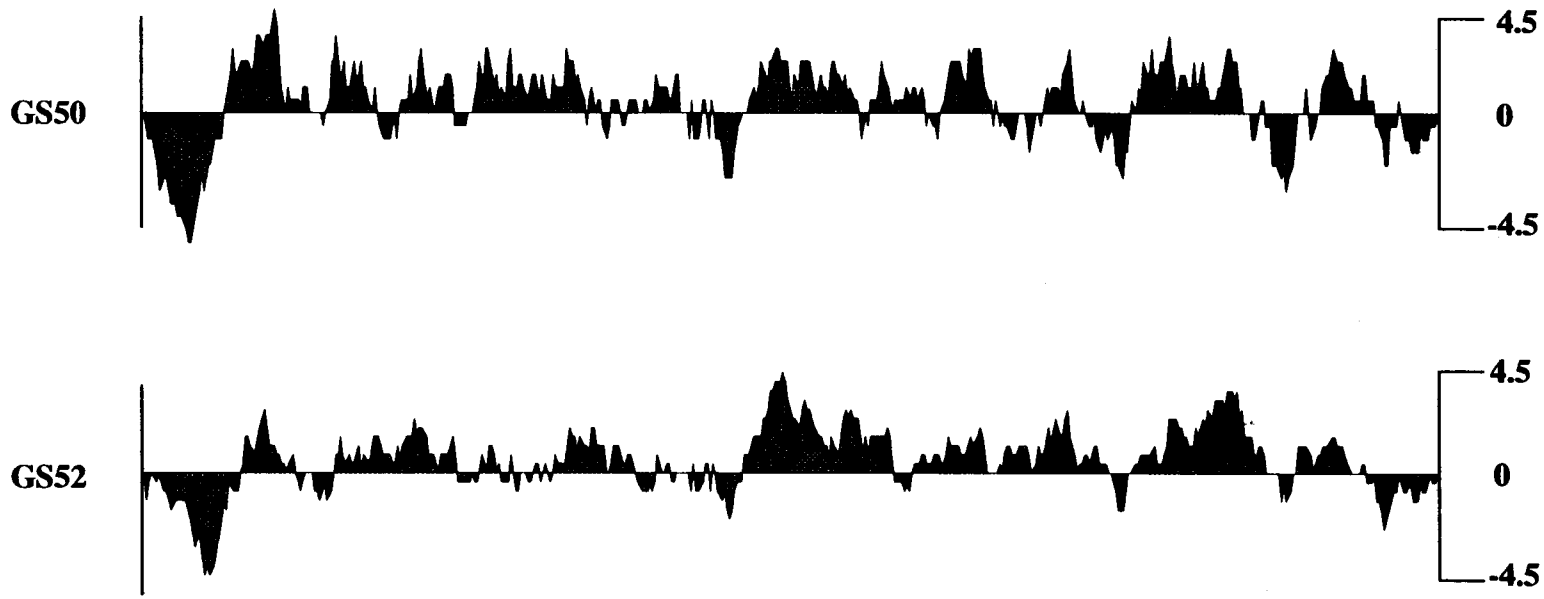


Figure 23. Hydropathy analysis of GS50 and GS52. Predicted transmembrane domain for each protein is located at the NH_2-terminus, and is characterized as having a TM domain of at least 19 amino acid residues. Negative/Positive on the COOH axis reflect the hydrophobicity scale. The hydropathy index is the mean value of the hydrophobicity of the amino acids with a 19 residue "window" around each position (Kyte and Doolittle, 1982).

single transmembrane segment in a similar location. However, PSORT analysis (Nakai and Kanehis, 1991) predicts the likelihood that GS52 is a peripheral protein, with a cleavable N-terminal signal sequence at amino acid residue 33. In the case of GS50, the predicted transmembrane segment is 22 amino acids, beginning with methionine. This is characteristic of a hydrophobic signal sequence necessary for translocation across the endoplasmic membrane (von Heijne, 1989).

PCR products generated using DNA primers to GS50 and GS52 hybridize to genomic DNA from G. soja

To ensure that the *gs50* and *gs52* cDNA clones isolated using a TD-PCR approach are in fact representative of true DNA sequences derived from *G. soja*, Southern blot analysis was performed using ^{32}P-labeled DNA probes. Using endonuclease-restricted genomic DNA isolated from *G. soja,* DNA probes corresponding to regions of *gs50* and *gs52* derived from the extreme 3' and 5' ends of each cDNA, respectively, were used as probes to enhance the differences in nucleotide sequences. This was done to ensure that cross-hybridization of the 2 cDNAs with the reciprocal gene would be avoided. As shown in Figure 24, labeled probes hybridize with genomic DNA isolated from soybean under stringent conditions, thus indicating that the clones were derived from *G. soja* sequences and were not the result of contamination during PCR. These results suggest that the two

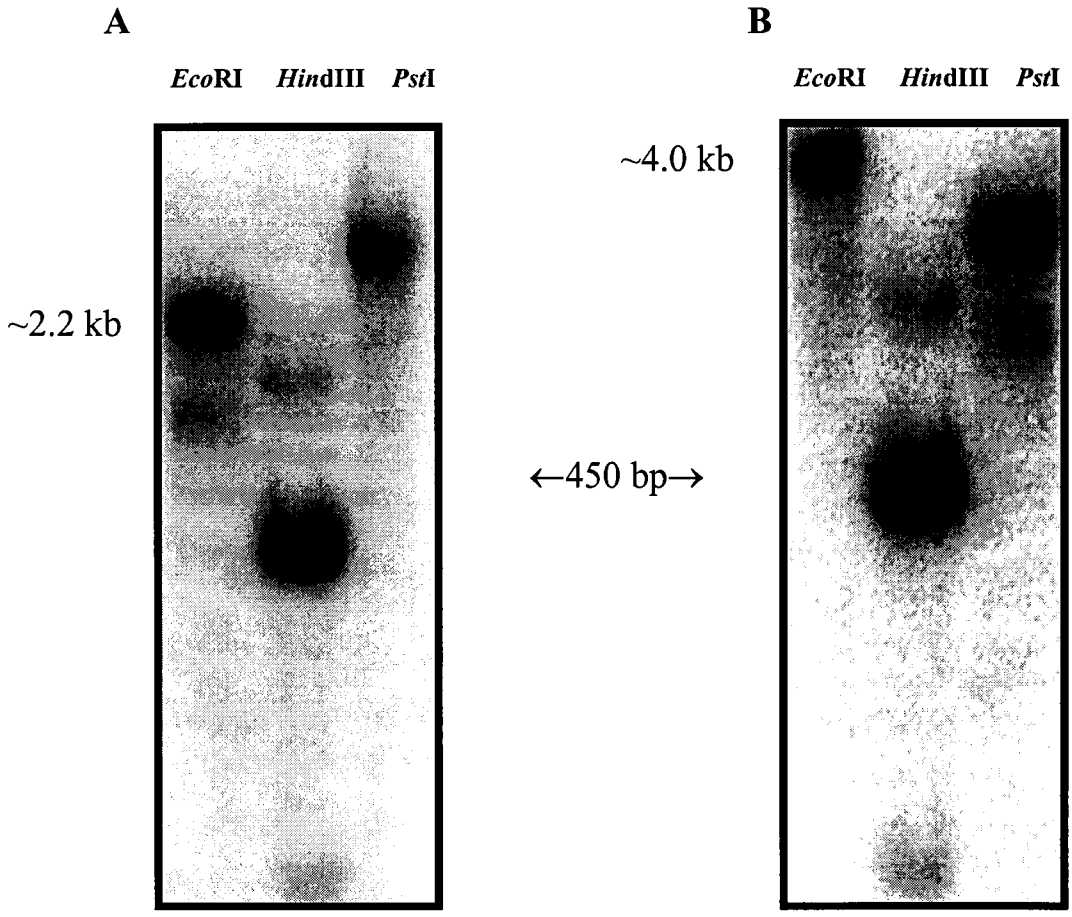


Figure 24. Southern blot analysis of *G. soja* genomic DNA digested with either *Eco*RI, *Hin*dIII, or *Pst*I. Blots were hybridized with either a *gs50* probe (A), or a *gs52* probe (B).

clones isolated using PCR are representative of two independent genes present in *G. soja*. For example, while the *gs50* probe hybridized to a 2.2 kb *Eco*RI fragment, the *gs52* probe hybridized to a 4.1 kb *Eco*RI fragment. Similarly, the hybridization of both *gs50* and *gs52* to an internal *Hin*dIII fragment of approximately 450 bp, demonstrates the high degree of nucleotide sequence similarity between GS50 and GS52 within the cDNA coding sequences.

gs50 and gs52 mRNA are differentially expressed in soybean tissues

The initial identification and isolation of *gs50* and *gs52* was achieved using total RNA from root tissue of *G. soja*. Northern blot analysis was initially used to determine tissue and developmental expression, as well the induction of *gs50* and *gs52* upon rhizobial inoculation (data not shown). However, because of the high degree of sequence similarity between the two cDNAs, an RT-PCR approach was undertaken to differentiate the expression pattern of GS50 and GS52 mRNA. To further characterize *gs50* and *gs52* expression in terms of tissue localization, RT-PCR was performed using total RNA isolated from a variety of tissues from *G. soja*. In this, and all subsequent experiments describing results generated using RT-PCR, the DNA primers used for the amplification of *gs50* and *gs52* are listed in Table 5 (*gs50* full forward/reverse; *gs52* full forward/reverse). As shown in Figure 25, *gs50* and *gs52* are in fact expressed in root tissue from soybean. However, their levels of

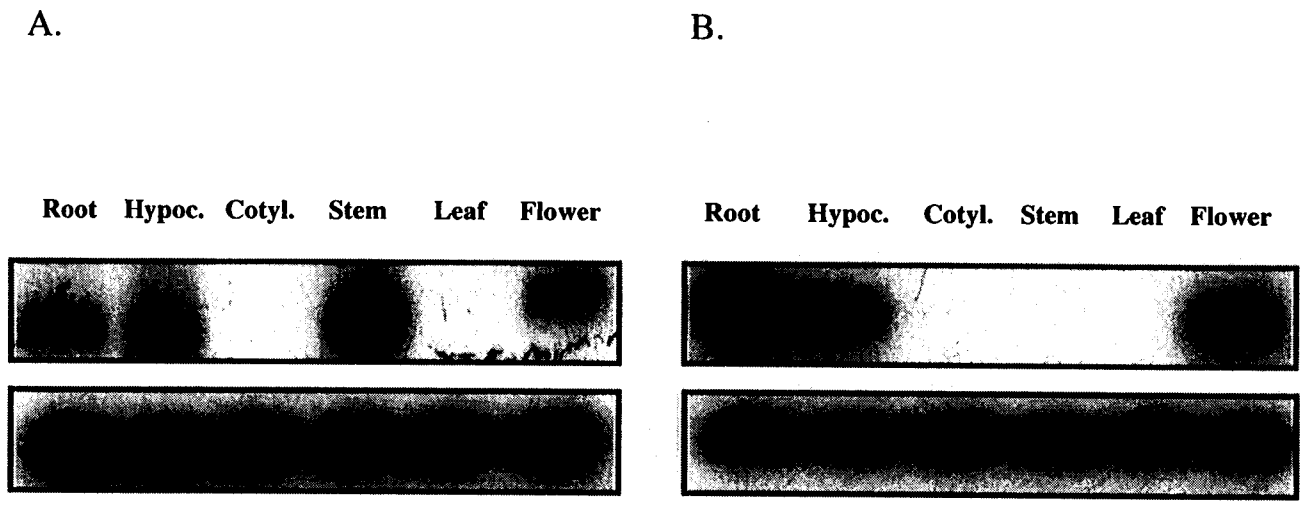


Figure 25. Reverse-transcription PCR analysis of *gs50* and *gs52* expression in various tissues from *G. soja*. A) *gs50*. B) *gs52*. Hypoc., hypocotyl; Cotyl., cotyledon.

expression, as well as their relative distribution in soybean differ significantly. *gs52* mRNA was expressed at a much higher level in the roots of soybean than *gs50.* Interestingly, both *gs50* and *gs52* mRNA was highly expressed in flowers, yet neither mRNA was detected in the leaf or in the cotyledon. Conversely, *gs50* mRNA expression was maximally localized in the stem of 5-day-old seedlings, whereas gs52 mRNA was expressed at a significantly lower level. Thus, the differential expression pattern of *gs50* and *gs52* mRNA might suggest that these two proteins function in distinct cellular processes in soybean. Interestingly, the mRNA expression pattern of *gs52* in the developing roots and flowers of soybean coincides with that of the previously described LNP from *D. biflorus* (Etzler *et al.*, 1999).

gs52 mRNA is induced in the presence of B. japonicum USDA110

Upon inoculation and perception of compatible rhizobia by the host legume, the expression of a number of plant genes involved in the establishment of nodulation is up-regulated (e.g., *rip1*, Cook *et al.*, 1995; *ENOD40*, Minami *et al.*, 1996a; *ENOD2*, Minami *et al.*, 1996b). In order to determine if *gs50* and/or *gs52* mRNA levels are also significantly enhanced upon inoculation with *B. japonicum*, RT-PCR was performed using total RNA from root tissue corresponding to the zone of the developing root which is most susceptible to rhizobial infection. As shown in Figure 26 (-USDA110), the developmental expression pattern of *gs50* reached maximal expression at 3 days, and by 9 days post-imbibition, expression of *gs50* had

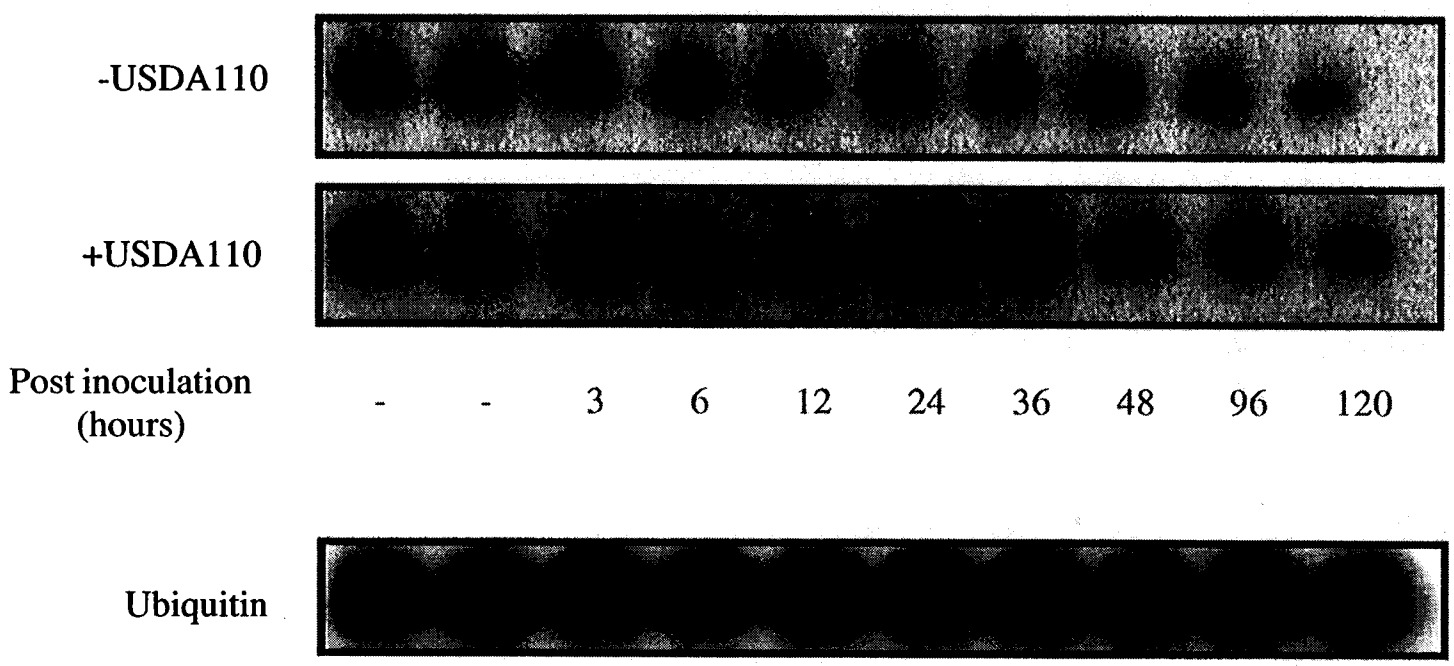


Figure 26. RT-PCR analysis of GS50 mRNA developmental expression (-USDA110) and expression under conditions of rhizobial inoculation (+USDA110). Internal control for PCR (Ubiquitin) is shown in the lower panel.

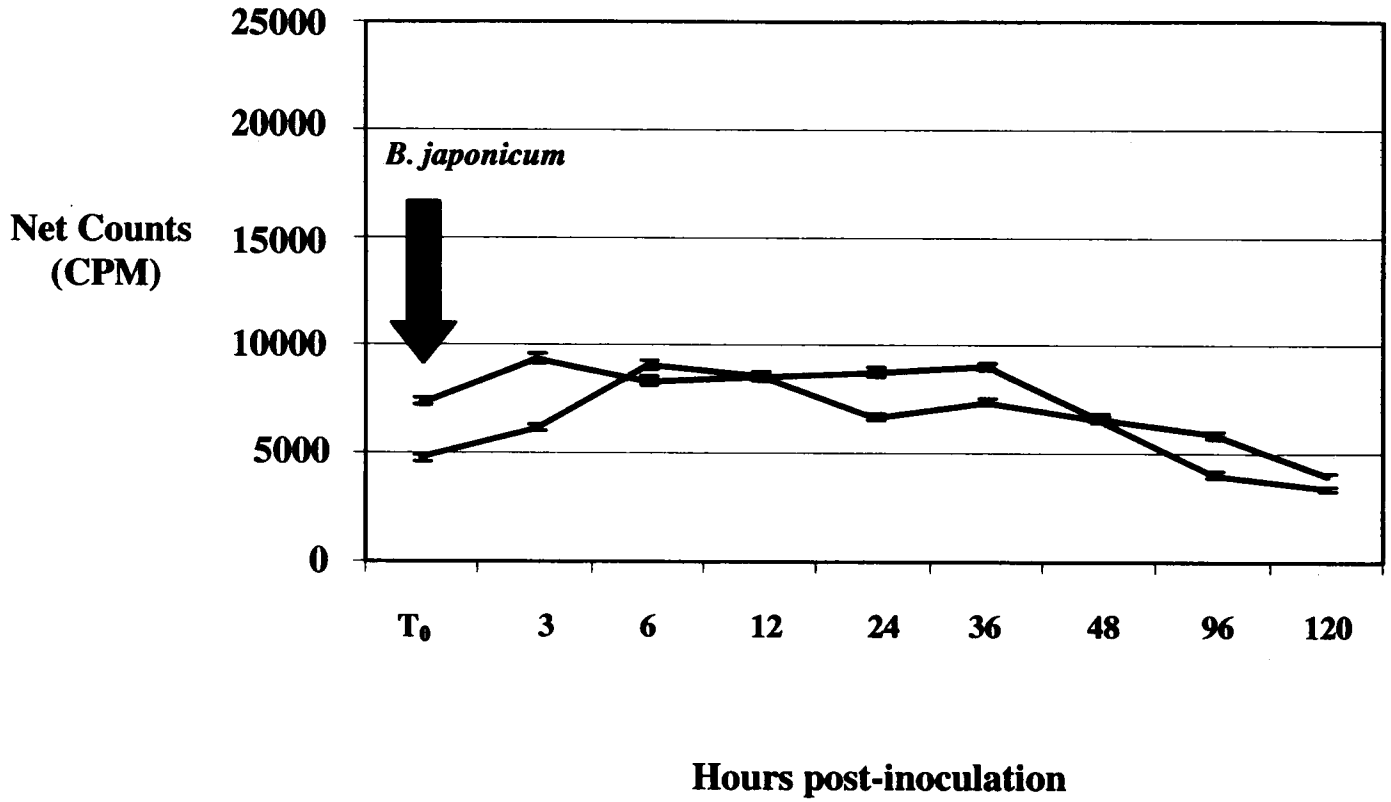


Figure 27. Graphical analysis of total counts from developmental and *B. japonicum* inoculation expression of GS50 mRNA. Corresponding Southern blot is shown in Figure 26. Net counts represent dosimeter readings generated fromm corresponding blot. T_0= time point of inoculation.

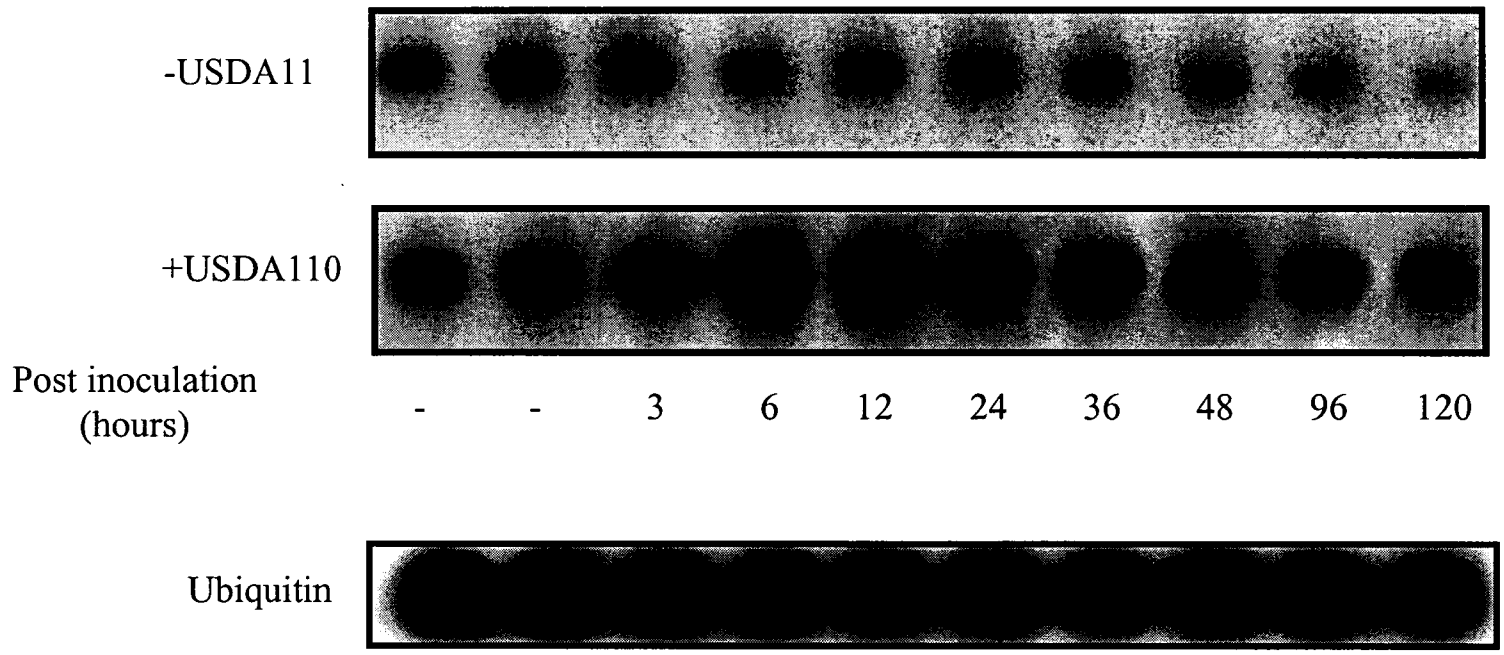


Figure 28. RT-PCR analysis of GS52 mRNA developmental expression (-USDA110) and expression under conditions of rhizobial inoculation (+USDA110). Internal control for PCR (Ubiquitin) is shown in the lower panel.

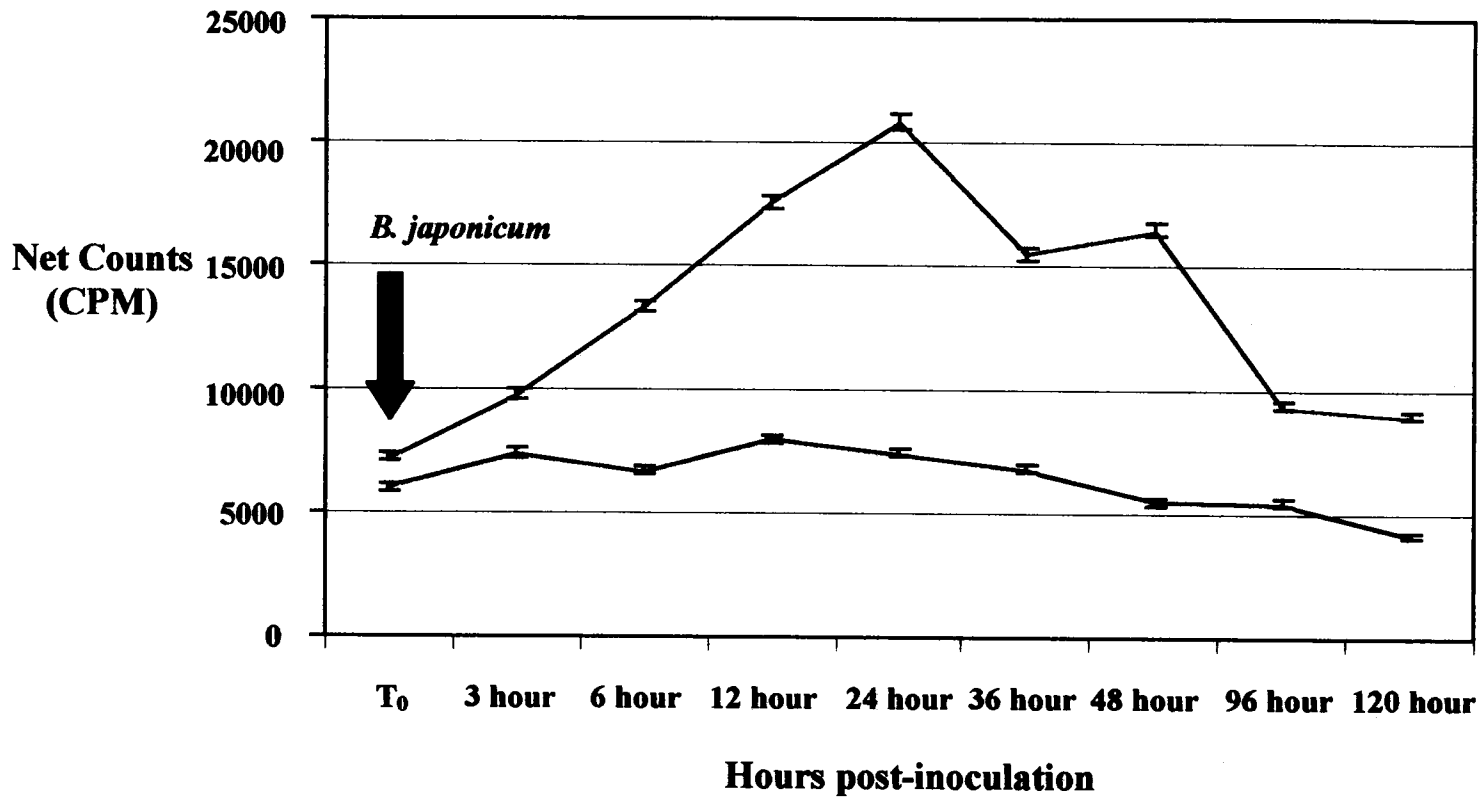


Figure 29. Graphical analysis of total counts from developmental and *B. japonicum* inoculation expression of GS52 mRNA. Corresponding Southern blot is shown in Figure 28. T_0= time point of inoculation.

significantly decreased. After inoculation of 3-day-old roots of *G. soja* with 10^6 cells of *B. japonicum* USDA110 (+USDA110, Figure 26), mRNA expression was unchanged when compared to roots that were inoculated with water. The developmental progression of *gs52* mRNA was similar to that of *gs50* (Figures 28 & 29). However, when roots were inoculated with *B. japonicum* cells 3 days post-imbibition, there was a marked increase in *gs52* mRNA levels within 6 hours post-inoculation.

gs52 mRNA expression is repressed in the presence of nitrate

Nodulation is regulated, in part, by the availability of nitrogen resources. Hseih *et al.* (1996) demonstrated the down-regulation of mRNA expression of the *P. sativum* NTPase in the presence of nitrate. Additionally, previous work by Heidstra *et al.* (1997) demonstrated that expression of the late nodulin leghemoglobin was down regulated in the presence of 20 mM ammonium. Based on these observations, we sought to determine if the expression of *gs50* and *gs52* mRNA is also regulated by the presence of nitrate. *G. soja* seeds were germinated in the presence of 20 mM ammonium nitrate for two days, then transferred to sterile plant growth pouches. At various time points ranging from 2 days to 9 days post-imbibition, root tissue was harvested, and stored at -80°C. As shown in Figure 30, the expression of *gs50* in the presence of nitrate was unaffected when compared to the developmental expression

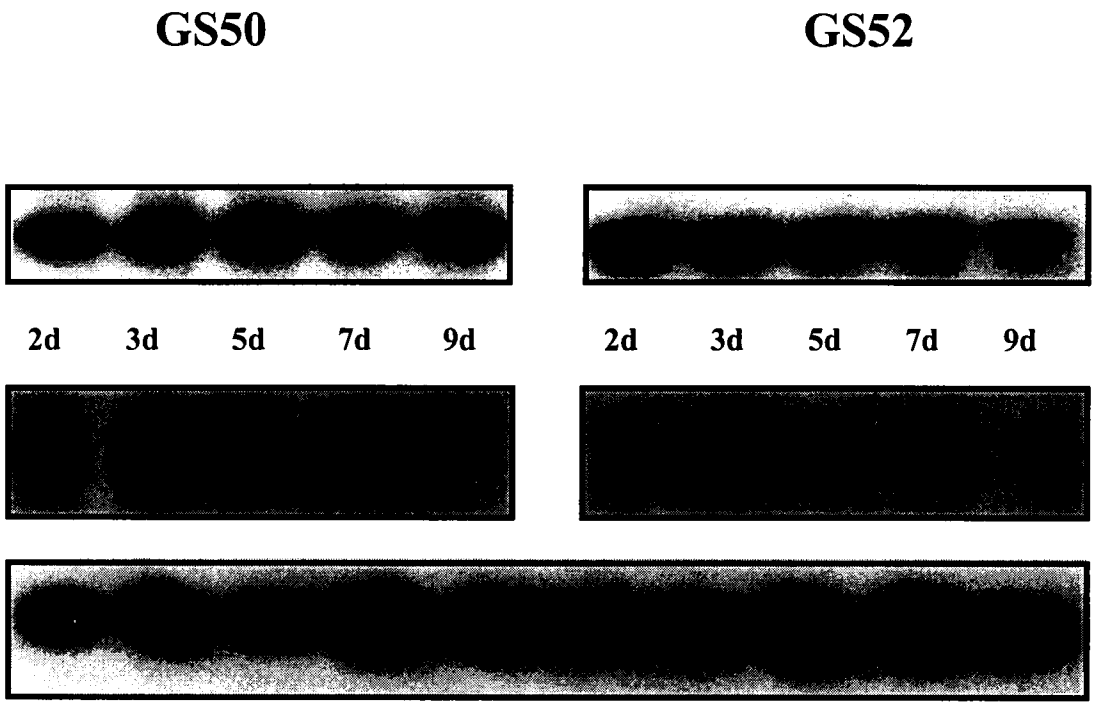


Figure 30. RT-PCR time course expression of *gs50* and *gs52* in the presence of 20 mM ammonium nitrate. Tissue was isolated at time points ranging from 2 days to 9 days post-imbibition. As a developmental control, plants receiving only H_2O (no nitrate) were included in parallel (top panels). RT-PCR analysis of RNA isolated at time points ranging from 2d to 9d post-imbibition of plant grown in the presence of 20 mM ammonium nitrate (pH 7.0) (center panels). As an internal control for RNA loading during reverse transcription and PCR, primers specific for soybean Ubiquitin were included (lower panel).

of *gs50* (Figure 30). Alternatively, the expression of *gs52* in the presence of nitrate is significantly diminished, thus indicating that GS52 is in part regulated by the availability of nitrogen resources. These data further strengthen the hypothesis that GS52 may play a role in the cascade of events leading to the onset of nodulation in soybean.

Production and purification of GS50 and GS52 fusion proteins

In order to express GS50 and GS52 in *E. coli*, *gs50* and *gs52* were cloned into the expression vector pGEX-4T-3 to generate pGEX46-4 and pGEX52 (Table 4). Cell extracts of *E. coli* harboring pGEX46-A and pGEX52 were analyzed by SDS-PAGE. As shown in Figure 31, dominant bands of 76 and 78 kDa were observed in the lanes corresponding to the induced fractions (Figure 31, I). These polypeptides had a molecular weight consistent with that predicted for the GST-GS50(e.g., GST=26 kDa, GS50=50 kDa) and GST-GS52 (e.g., GST=26 kDa, GS52=52 kDa). In the absence of IPTG inducer, no fusion protein was detected (Figure 31, U). Immunoblot analysis using anti-GST antibodies confirmed SDS-PAGE results (data not shown). The majority of the GS50 fusion protein was present in insoluble inclusion bodies. Therefore the protein was solubilized in a series of urea extractions (1M to 7M), and further analyzed using SDS-PAGE (Figure 32). As shown in Figure 32, optimal extraction of GS50-GST fusion protein was achieved at 4M Urea. The

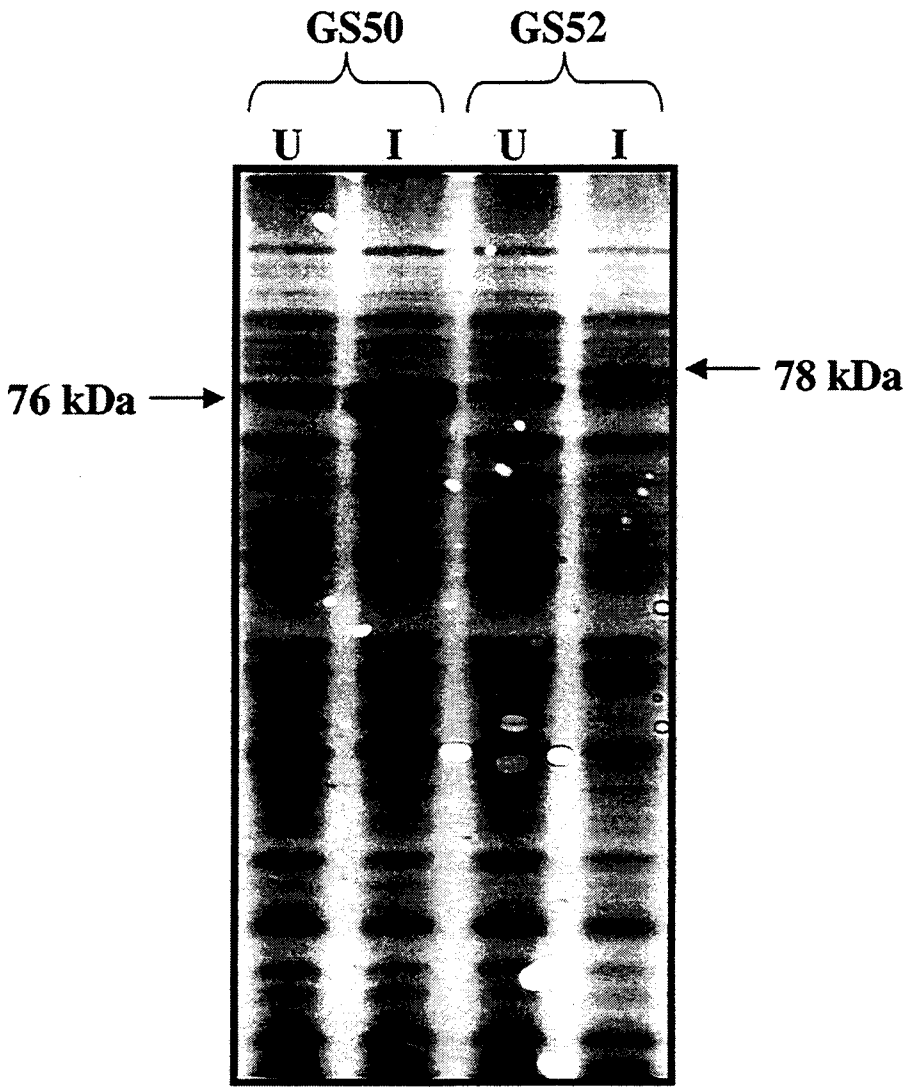


Figure 31. Silver stain SDS-PAGE analysis of GS50 and GS52 GST-fusion proteins expressed in *E. coli*. Log phase cultures of *E. coli* were induced with 500 μM IPTG for 1 hour. Total protein was separated on a 12% SDS-PAGE gel. U, Un-induced culture; I, Induced culture (e.g., 500 μM IPTG).

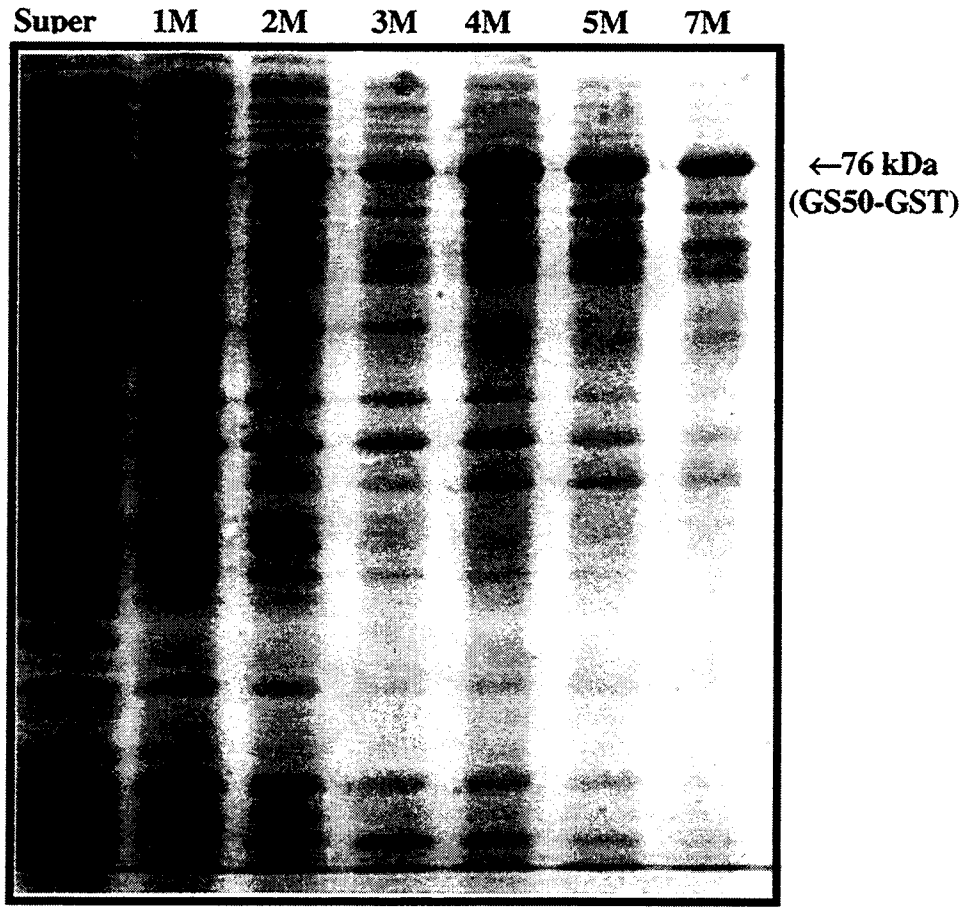


Figure 32. SDS-PAGE and silver stain analysis of urea extraction of GST-GS50 fusion protein from *E. coli* GEX50 inclusion bodies. Super, supernatant of total cell lysate. 1M-7M indicates the final concentration of urea added to solubilize inclusion bodies.

GS52 fusion protein was also solubilized with urea and purified in a similar manner (data not shown). Figure 33A and 33B show the presence of a single band corresponding to the 76 and 78 kDa GS50 and GS52 fusion proteins, respectively, following purification of the proteins on GST-Sepharose 4B. Immunoblot analysis using anti-GST antibodies to the eluted proteins confirmed both the presence and purity of the eluate. The purified fusion proteins were used to immunize rabbits for the generation of anti-GS50 and anti-GS52 antibodies (Figure 33).

GS50 and GS52 proteins are differentially expressed in soybean tissues

The mRNA expression analysis described above suggested that GS50 and GS52 have distinct patterns of tissue localization in *G. soja*. To determine if the RT-PCR data was representative of the protein localization of these two apyrases, total protein extracts were prepared from various tissues of *G. soja* and used in western blot analysis to determine protein localization. As shown in Figure 34B, the tissue localization of GS50 strongly supported the RT- PCR data described above (Figure 34b). Maximal expression of GS50 was detected in 5-day-old stem tissue from *G. soja*, with minimal expression of GS50 localized in 5-day-old roots. Again, similar to the RT-PCR data, GS50 was also expressed at a relatively high level in the flowers of *G. soja*.

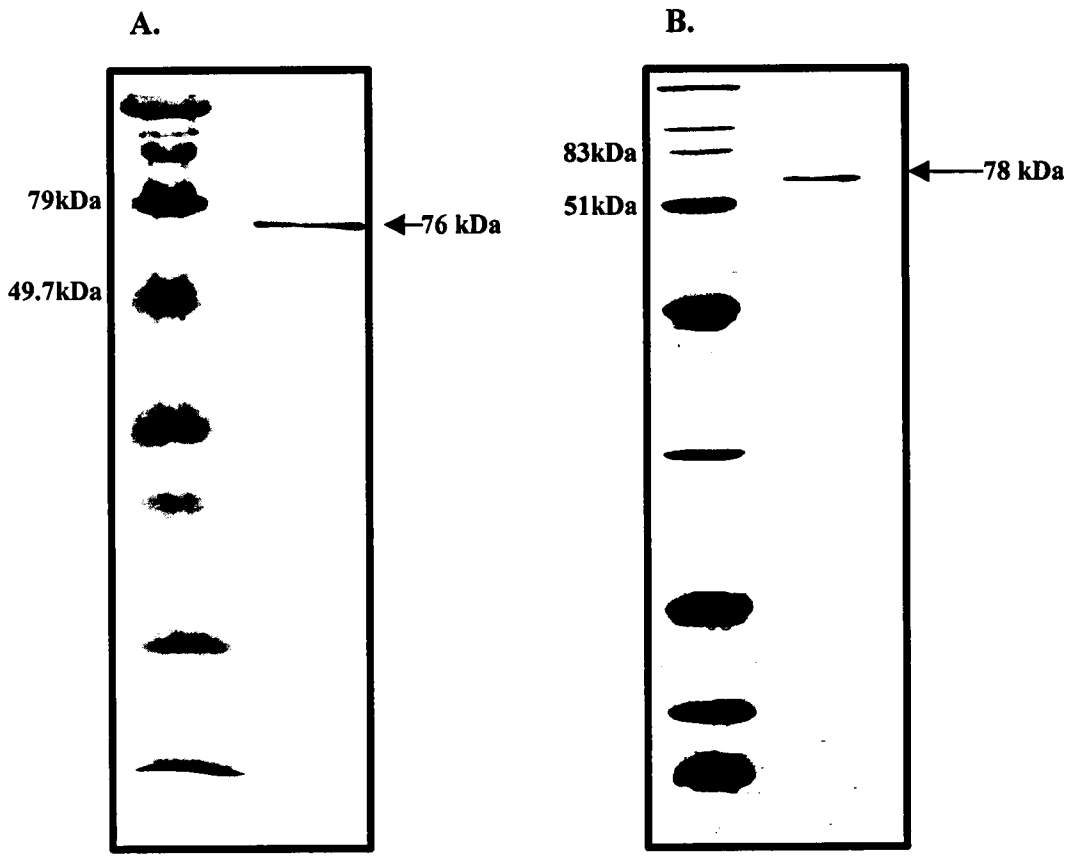


Figure 33. Purified GST-fusion proteins. **A**. GST-GS50. **B**. GST-GS52. Molecular weights of proteins reflect the apyrase protein plus the 26 kDa GST fusion tag. In both cases, a broad-range molecular weight marker is shown in the left lane; only significant molecular weights are shown.

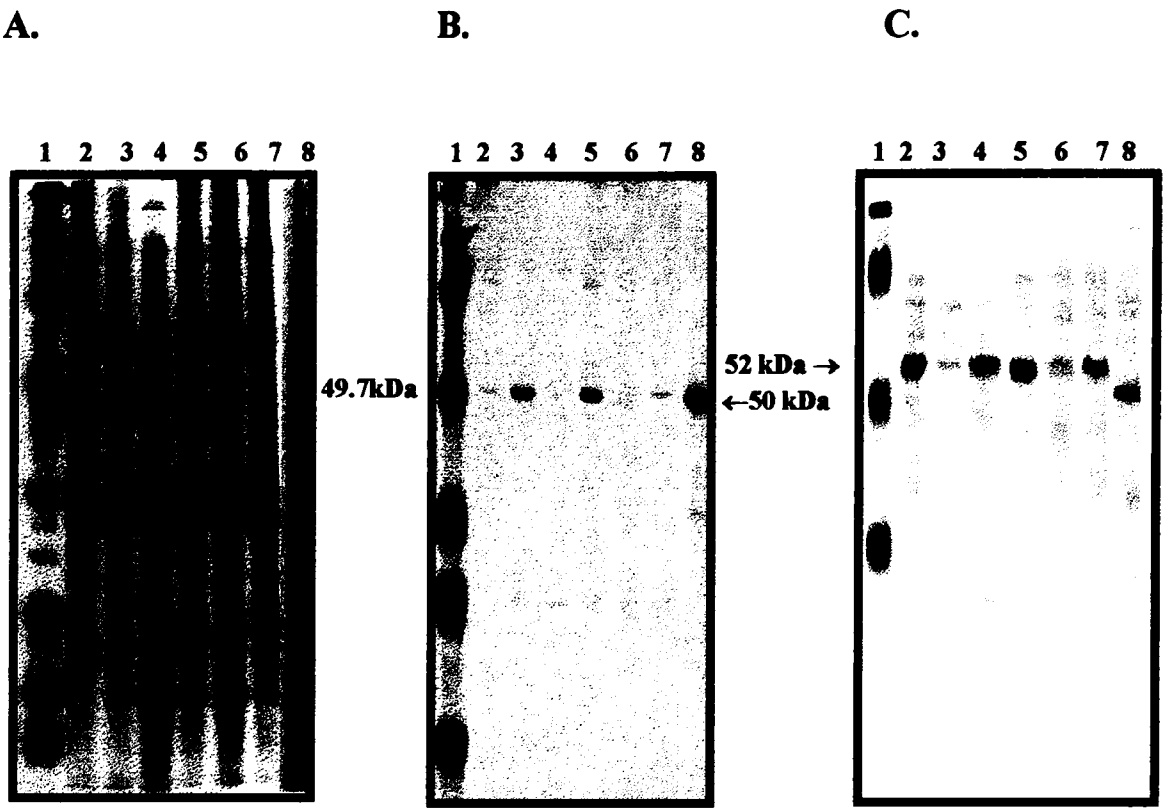


Figure 34. Western blot analysis of total protein extracts from various tissues and nodules from *G. soja*. A) SDS-PAGE silver stain of total protein extracts. B) Western blot using antigen-purified anti-GS50 IgG antibodies. C) Western blot using antigen-purified anti-GS52 IgG antibodies. Lanes: 1, Broad range MW marker; 2, Root; 3, Hypocotyl; 4, Cotyledon; 5, Stem; 6, Leaf; 7, Flower; 8, Nodule.

Western blot analysis of GS52 expression showed a similar pattern of tissue localization (Figure 34C) to that detected using RT-PCR, with one minor exception. GS52 signal was most abundant in the developing roots of soybean. However, in sharp contrast to the RT-PCR results, GS52 was also detected in the cotyledon and stem at significant levels. The failure of RT-PCR to detect *gs52* in the cotyledon may reflect the possibility that the *gs52* mRNA is rapidly turned-over in this tissue, thereby limiting the ability of PCR to detect the messenger RNA. However, we cannot rule out the presence of a cross-reacting GS52 ortholog in the cotyledon and stem. Again, similar to GS50, GS52 was also found at high levels in the developing flower. Interestingly, expression of GS52 in the infected nodules of soybean suggests that GS52 may be post-transitionally modified within nodule tissues. As shown in Figure 34C, anti-GS52 antibodies detected a signal corresponding to a protein with an apparent mass of 50 kDa. The absence of this band in the other samples (e.g., root, stem, and flower) excludes the possibility that antibodies to GS52 cross-react with GS50. SDS-PAGE analysis and subsequent western blotting showed no cross-reactivity of anti-GS50 with GS52, or anti-GS52 antibodies with GS50. Thus, the detected protein is likely a post-transitionally-modified GS52.

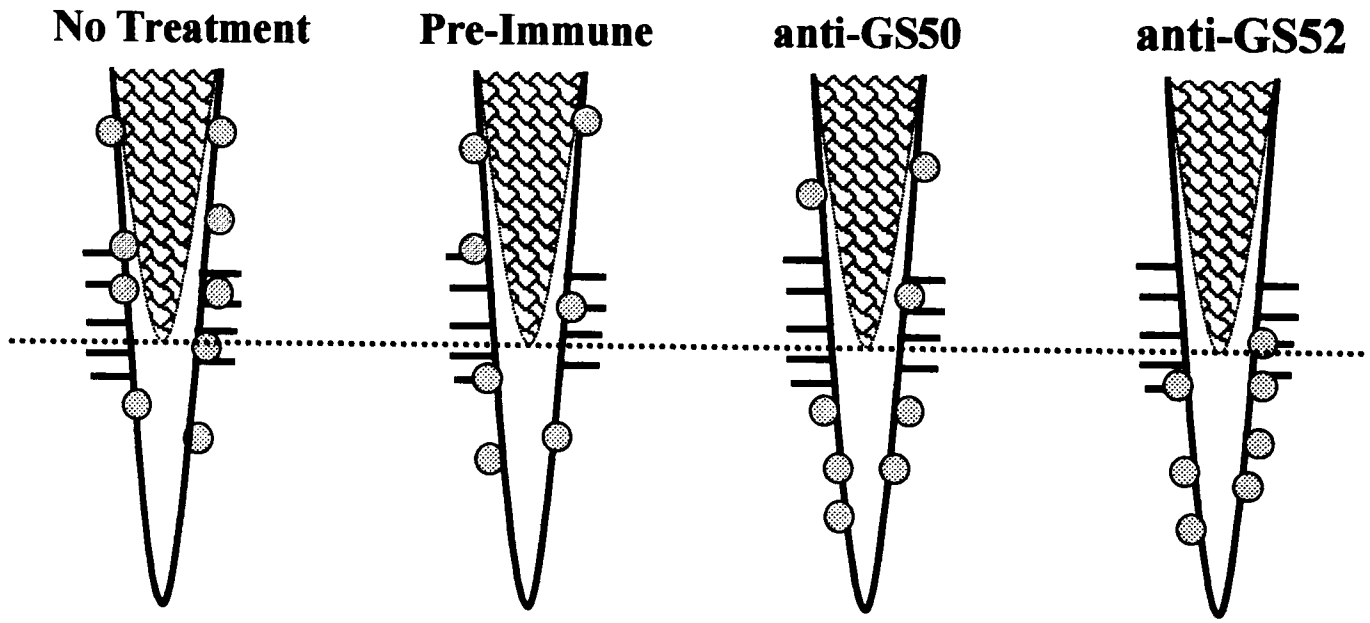
Anti-GS52 antibodies block nodulation of soybean by B. japonicum USDA110

Etzler *et al.* (1999) demonstrated the ability of anti-LNP anti-serum to block nodulation of *D. biflorus* by *B. japonicum* 24A10, thereby suggesting a critical role

for LNP in the *D. biflorus* nodulation process. To determine if anti-GS50 and anti-GS52 serum affected the ability of *G. soja* to be nodulated by its rhizobial host *B. japonicum* USDA110, 2-day-old seedlings were incubated for 1 hour in the presence of antiserum against each protein. As controls, plants that were also incubated in the presence of pre-immune serum or left untreated. As shown in Figure 35, treatment with antisera to GS52 inhibited nodulation by *B. japonicum*. This inhibition occurred only in the region of the root that had been exposed to the antiserum (i.e., the region of the root above the root tip at the time of nodulation = upper zone). Interestingly, antiserum against GS50 had no effect on the ability of *G. soja* to be nodulated by rhizobia. Control plants (no treatment and pre-immune) showed normal nodulation phenotypes.

Subcellular localization of GS50 and GS52 using membrane separation in sucrose-density gradients

Previous reports on the localization of apyrase proteins suggested that some of these proteins may be membrane-localized (Chen and Roux, 1986; Abeijon *et al.*, 1993; Hseih *et al.*, 1996; Etzler *et al.*, 1999). Hydropathy analysis suggested that GS50 and GS52 may also possess putative membrane-spanning domains and, therefore, may be expressed in soybean tissues as an integral membrane protein. The membrane localization of GS50 and GS52 was determined after separating soybean



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

Figure 35. Effect of anti-GS50 and anti-GS52-serum on nodulation of *G. soja* by *B. japonicum* USDA110. Pink (hatched) inset represents root at time of antibody treatment and inoculation. Black-bounded (un-hatched) drawing represents root at time of analysis. Therefore, upper zone represents part of root exposed to antibody and lower zone represents new root growth untreated with antibody. n= number of plants per treatment.

root membranes by ultracentrifugation on linear 20 to 45% (w/w) sucrose density gradients. The distribution of three membrane marker enzymes separated by sucrose gradients is shown in Figure 36. The location of the enzyme activities coincided with sucrose density gradients previously published for these plant membrane markers (Leonard and Vanderwoude, 1976; Widell and Larsson, 1990; Giannini *et al.*, 1991). The relative distribution of GS50 and GS52 after centrifugation of soybean root cell homogenate is shown in Figure 37. Interestingly, GS50 and GS52 appeared to differ in their subcellular localization. By comparing Figure 36 and 37, GS50 localized to membrane fractions corresponding to the membrane marker activity (latent UDPase) for the Golgi. That is, UDPase activity peaked at fraction 10, while maximum GS50 localization peaked in fraction 9. Conversely, GS52 localized to membrane fractions corresponding to the peak of H^+-ATPase activity. ATPase activity (a marker enzyme for the plant plasma membrane) peaked in fraction 6 (Figure 36), and this same fraction number contained the most GS52 by western blot analysis. Composite localization data for both GS50 and GS52 is shown in Figure 38.

Genetic mapping of GS52 on an integrated genetic linkage map of the soybean genome

(This data was generated by Dr. Roxanne Denny and Dr. Nevin Young at the University of Minnesota, St. Paul).

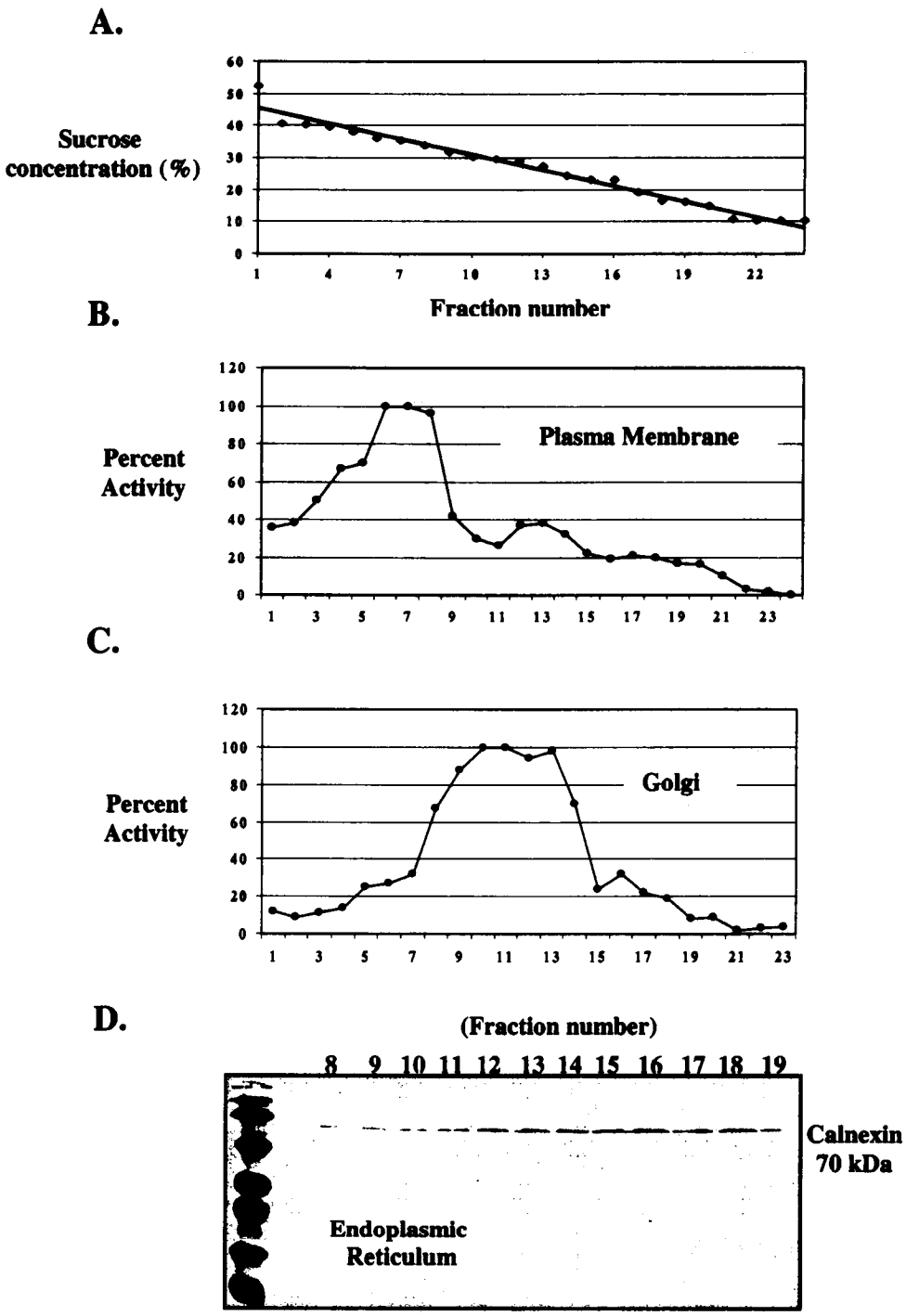


Figure 36. Sucrose membrane marker standards. **A**. Sucrose concentrations of the fractionated membranes based upon refractive indices. **B**. Vanadate-sensitive ATPase activity, plasma membrane. **C**. Latent UDPase activity, Golgi. **D**. Western blot analysis of ER localization using monoclonal antibodies to oat calnexin.

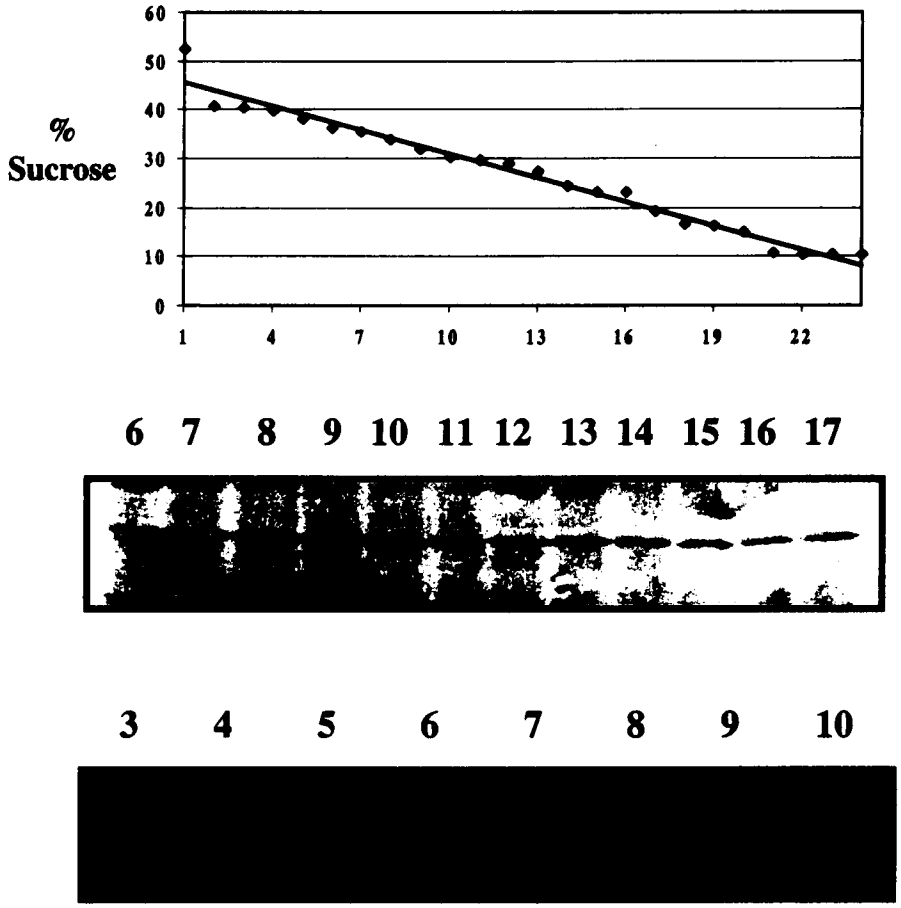


Figure 37. Western blot analysis of sucrose-fractionated membranes isolated from 5-day-old soybean roots. Upper panel, sucrose concentrations of membrane fractions. Sucrose fractions were immuno-blotted using antigen-purified anti-GS50 and anti-GS52 antibodies, center and lower panels, respectively. Numbers above panels represent sucrose gradient fraction numbers.

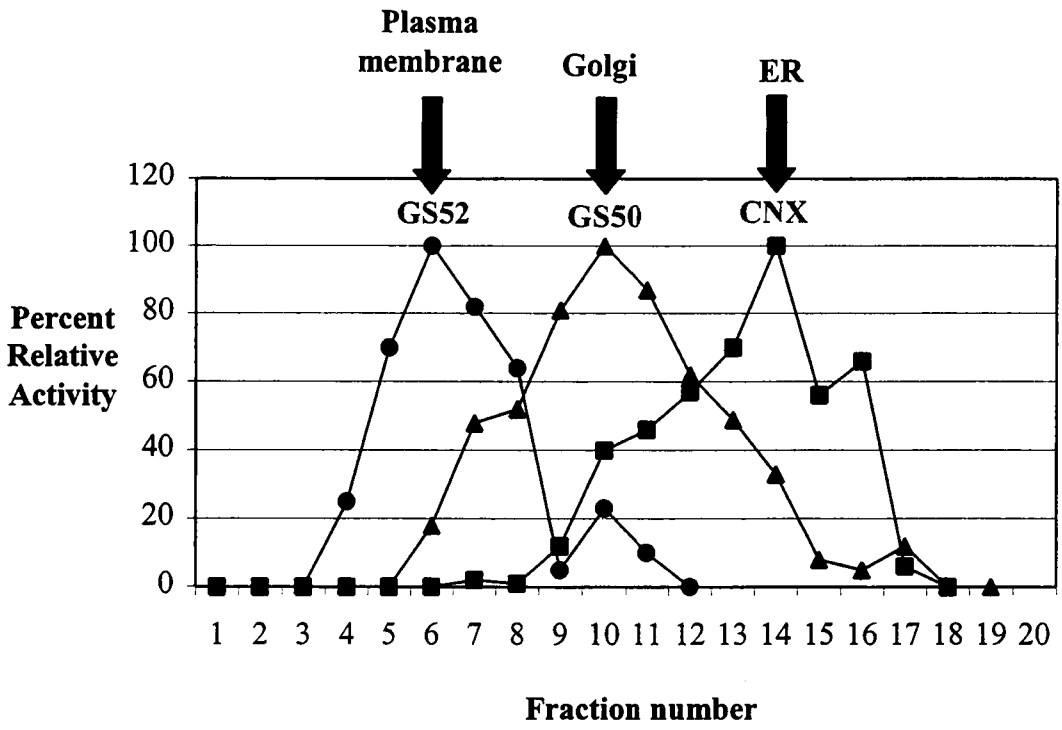


Figure 38: Membrane localization of GS50 and GS52 based on sucrose density centrifugation of microsomal membrane fractions from *G. soja* roots. Percent relative activity was determined based on densitometry analysis of western blots of sucrose-fractionated membranes using anti-GS50 and anti-GS52 antibodies. Localization of GS50 and GS52 based on western blot analysis of root membrane fractions coincided with peak activity of membrane marker assays for the Golgi (latent UDPase) and the plasma membrane (Vanadate-sensitive ATPase), respectively. Calnexin (CNX), a ER membrane-associated chaperone protein is shown as a marker for the ER membrane.

The availability of molecular genetic maps saturated with single-locus, multiple allele markers provide a foundation for identifying protein function based on functional genomics. In an effort to determine if GS50 and GS52 map to regions of known protein function within the soybean genome, or whether RFLP-based mapping might identify regions/clusters of other apyrase homologues, full-length cDNAs encoding GS50 and GS52 were hybridized with a parental survey blot containing genomic DNA from 90 lines of a soybean RIL population. Southern blot analysis showed that GS52 was polymorphic within the mapping population used (*G. max* cv. Evans × PI209332 RIL). However, Southern blot analysis failed to detect a polymorphism using *gs50* as a probe (data not shown). Based on hybridization analysis, as well as two-way contingency table analysis, GS52 mapped 18 cM below marker Satt249 and 10 cM below markerA060_1 (Figure 39). Interestingly, *gs52* mapped onto Molecular Linkage Group (MLG) J, which carries quantitative trait loci (QTLs) for cyst nematode resistance genes, as well as the restricted nodulation gene *Rj2* (Cregan *et al.*, 1999).

Marker	cM
A363_2	0.0
i1_2	0.0
Bng202_2	6.0
B101_1	0.0
Bng179_1	3.8
Satt405(34)	0.7
Satt249(66)	2.6
Satt287(42)	6.4
Sct_046(46)	0.0
Satt285(58)	5.9
A060_1	2.8
B046_2	8.1
A204_1	1.6
B074_1	5.5
E107_1	0.0
mO109_1	1.7
pcr2_190	3.6
B166_1	1.8
K384_1	0.7
A450_1	5.6
p40_2_2	0.4
Bng044_1	0.0
BLT049_4	4.1
Sct_065(54)	2.3
Satt596(74)	0.0
Satt414(72)	0.0
Scaa003(18)	3.6
Satt280(42)	0.0
Satt456(54)	0.8
Satt406(72)	2.8
Satt380(58)	0.0
Sat_093(80)	1.8
Satt183(48)	1.8
Satt549(54)	1.4
Sct_001(54)	15.4
B122_1	0.0
Bng063_1	7.5
pcr2_135	5.7
K375_1	1.1
Satt244(74)	4.3
B032_1	0.8
G815_1	3.7
Sle_4m	3.7
Satt431(70)	1.9
A724_1	0.0
A233_1	4.9
A199_2	0.0
pcr2_176	----
	124.8

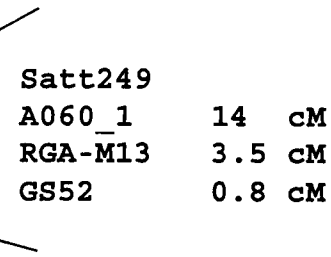


Figure 39. Genetic map of Major Linkage Group (MLG) J based upon alignments of the USDA/Iowa State Univ. *G. max* × *G. soja*, the Univ. of Utah Minsoy × Noir 1, and the Univ. of Nebraska Clark × Harosay molecular genetic maps. The number in parentheses following each SSR locus is the gene diversity × 100. SSR loci are in **bold** font, RAPD loci are in *italic* font. Linkage data modified from Cregan *et al.* (1999).

CHAPTER 5

DISCUSSION

The perception of chitin oligosaccharides has been shown to evoke a wide range of responses in a number of plant species (Cosio *et al.*, 1988; De Jong *et al.*, 1993; Minami *et al.*, 1996a; Minami *et al.*, 1996b; Minami *et al.*, 1996c). In soybean, Minami *et al.* (1996b) showed that chitin oligomers could induce the transient expression of the early nodulin ENOD40. This suggested that a chitin binding protein could be involved in the nodulation response in soybean. We sought to identify such a binding site using a ^{125}I-labeled chitin ligand. Two ligands were used, GN_5-APEA and GN_8-APEA. The first of these ligands matches in size the most active chitin oligomer involved in ENOD40 induction in soybean (Minami *et al.*, 1996b). The second ligand is identical to that shown to be the most active in the binding to the rice plasma membrane (Ito *et al.*, 1997). We analyzed the response of suspension-cultured cells to chitin since such cells are easily manipulated and have been widely used for studies of microbial elicitors. In addition, we analyzed tissue from 5-day-old roots since it is root tissue that responds to the Nod signals. In both cases, the binding of the ^{125}I-chitin ligands demonstrated high affinities (K_d) of 35 nM and 47 nM in suspension cells and root cells, respectively, using ^{125}I-$(GlcNAc)_8$-APEA as a ligand. Binding of ^{125}I-$(GlcNAc)_5$-APEA to plasma membrane-enriched fractions from both root tissue and suspension-cultured soybean cells also

demonstrated a high affinity (e.g., 75 nM, data not shown). The K_d determined compared favorably to those reported for chitin binding to rice (i.e., 29 nM) (Shibuya *et al.*, 1996) and tomato cells (i.e., 28 nM) (Baureithel *et al.*, 1994). These values also compared favorably to the lipo-chitin binding sites reported from the membrane preparations from *Medicago* (i.e., NFBS1, 86 nM and NFBS2, 2 nM, respectively) (Bono *et al.*, 1995; Gressent *et al.*, 1999).

To our knowledge, there are no published reports showing that chitin is an elicitor of a defense response in soybean cells. Clearly, soybean can be subjected to fungal pathogen attack and responds with a defense response (Okinaka *et al.*, 1995). Therefore, one might expect the presence of a chitin perception system that would help mediate such a response. As shown in Figure 15, chitin did elicit a potent oxidative burst response in soybean cells. Moreover, the specificity of this response matched nicely with the specificity of chitin binding shown by the plasma membrane chitin-binding protein. Similarly, perception of chitin oligosaccharides by suspension-cultured soybean cells induced a rapid alkalinization of the culture medium in a size-dependent fashion (Figure 16). The correlation between the structural specificity of chitin binding to the plasma membrane-localized 85 kDa protein, and the biological responses evoked upon perception of these elicitors is striking, and suggests a role for chitin oligosaccharides in an elicitor signaling pathway (He *et al.*, 1998).

There have been reports suggesting that an incompatible *Rhizobium* (i.e., inoculated onto a heterologous host) might induce a defense response that could

inhibit nodulation (Bhagwat *et al.*, 1999). Such a mechanism could play an important role in determining the host specificity of rhizobia. Although preferring the larger oligomers, the plasma membrane chitin-binding protein did recognize smaller chitin oligomers. Therefore, it is theoretically possible that incompatible Nod signals could induce a defense response.

Our data indicate that chitin molecules of a lesser degree of polymerization (i.e., d.p. =5) are relatively poor competitors for ligand binding. Therefore, it seems unlikely that the chitin binding protein found in the soybean plasma membrane mediates a nodulation-related response to Nod signals. However, the ability of the major lipo-chitin Nod signal from *B. japonicum* to inhibit chitin binding to the plasma membrane of soybean (Figure 12), albeit at a relatively low affinity, suggests that there might be some structural elements which are common to both Nod signal and chitin perception.

A 85 kDa chitin binding protein was identified in plasma membrane preparations from both suspension-cultured cells and root tissue. Competition experiments suggest that the chitin binding sites in both membranes preferred the larger chitin oligomers (i.e., d.p. =8). The K_d values obtained for both the GN_5-APEA and GN_8-APEA ligands were roughly comparable for both membrane preparations from roots and suspension-cultured cells. Moreover, Scatchard and Hill plot analysis supported the presence of a single binding site. However, other data argue for the possibility of two, distinct binding sites in the plasma membrane preparations from roots. For example, although giving qualitatively similar patterns

(e.g., d.p. = 8 better that d.p. =5), the quantitative ability of different chitin oligomers to inhibit GN_5 or GN_8-APEA binding did differ between the membrane preparations from suspension-cells and root tissue. As shown in Figure 10B, unlike the situation in suspension-culture cells (Figure 10A), the chitin octamer and heptamer were not significantly different in their ability to inhibit GN_8-APEA binding to root plasma membrane. These experiments were repeated many times with the same result. Therefore, there appears to be some subtle differences in the relative affinity of binding to plasma membrane from suspension-cultured cells and root tissue. This difference may simply reflect the expected heterogeneity of the membrane preparations from roots. Data supporting the presence of multiple binding sites stem from inhibition analysis using both the $(GN)_5$-APEA ligand and the $(GN)_8$-APEA ligand. When the lipo-chitin Nod signal produced by *B. japonicum* was used as a competitive inhibitor of APEA-ligand binding to the plasma membrane of soybean, an IC_{50} of approximately 10μM was observed. However, when $(GN)_8$-APEA was used as a ligand, the Nod signal showed no inhibitory effect at concentrations as high as 250μM. These results argue for the presence of two distinct chitin binding sites in the soybean plasma membrane. Additional evidence for the possibility of two binding sites in root membrane preparations stems from the observation that cross-linking of root plasma membrane with $(GN)_5$-APEA and $(GN)_8$-APEA resulted in complexes of different molecular weights (data not shown). We are currently repeating these experiments to determine if these results are reproducible, or if they are simply the

result of standard variation during SDS-PAGE analysis. These ligands differ only slightly in molecular weight and, therefore, the data are suggestive that two different binding proteins were labeled. However, we cannot rule out the possibility that structural changes, resulting from ligand binding, altered the migration of the binding protein so that it appeared to differ in molecular weight. Collectively, these data suggest that there may be two binding sites present on the root plasma membrane, only one of which recognizes the Nod signal.

At present, the data do not allow a firm conclusion as to whether one or two binding sites are being recognized by chitin oligomers in root membrane preparations. It may well be that there are two sites localized in different cellular fractions. Further work will be required to resolve this issue. However, if two binding sites are present, then one site could be similar to the EBP identified by Shibuya *et al.* (1996) in rice plasma membrane. In rice, chitin recognition by this site paralleled the ability of chitin oligomers to induce a variety of defense responses. Likewise, our data indicate that soybean responds to larger chitin oligomers by inducing ROX and medium alkalinization. Thus, this type of binding site may be widely conserved in plants as a mechanism to respond to fungal pathogen attack.

As shown in Figures 17 and 18, ENOD40 induction responded optimally to the addition of smaller chitin oligomers (d.p.=4-5). The levels of chitin used in these experiments (1μM) were relatively high in relation to the measured K_d (70 nM) for the GN_5-APEA, or GN_8-APEA (40 nM) ligand. Therefore, it may be that this

concentration was far beyond saturation for the larger chitin oligomers. If this were the case, then reducing the concentration of chitin used in this experiment may give a different sensitivity profile. However, in the absence of additional data, these results support the existence of a chitin binding activity in soybean that prefers the smaller chitin oligomers.

The next obvious step in the characterization of the chitin binding site in soybean is to purify the binding protein from plasma membrane-enriched fractions. Once purified, the protein could be N-terminal sequenced, and a cDNA could be obtained. The Shibuya laboratory is currently pursuing an affinity-chromatography-based approach to isolate the EBP from rice membrane preparations (Ito *et al.*, 1999; Kaku *et al.*, 1999). Given the relative abundance of the EBP from rice (e.g., B_{max}=33 pmol/μg protein), as compared to that of the chitin binding protein identified in soybean (e.g., B_{max}=7.5 pmol/μg protein, Figure 8), isolation of the soybean chitin binding protein will require significantly more tissue that that used to isolate the rice EBP (Ito *et al.*, 1999; Kaku *et al.*, 1999).

Recently, Kim *et al.* (1998) reported the identification of a cDNA from tobacco predicted to encode a membrane-associated protein of approximately 75 kDa. The interesting feature of this protein is that its N-terminal half showed significant similarity to plant chitinases. However, specific amino acids known to be required for the catalytic activity were absent. Conversely, the C-terminus showed significant sequence similarity to a family of receptor-like protein kinases. This protein

represents a promising candidate for a chitin binding, protein kinase receptor. Its predicted molecular weight is very close to the EBP from rice and to the chitin binding protein identified in this study. A homologue of this protein does exist in soybean, and efforts are underway to isolate the cDNA for this protein.

Minami *et al.* (1996b) presented evidence suggesting that signal perception in the soybean-*Bradyrhizobium* symbiosis involves two discrete signaling events. The first, as described above, involves a less-stringent, non-specific recognition event, possibly mediated by the recognition of the chitin backbone of the rhizobial Nod signal. The second, a more specific event, would be mediated by a host-specific Nod signal binding protein capable of distinguishing among the multitude of modifications to the lipo-chitin backbone (Figure 2). The identification of a Nod signal binding protein which exhibits the same specificity as the nodulation response has been of interest for a number of years. Historically, legume lectins have been of interest for their proposed role in nodulation; yet to date, a precise role for these proteins has not been defined. Therefore, there was a great interest in the report of the ability of the DB46 lectin to specifically bind the lipo-chitin Nod signals produced by a variety of rhizobia (Etzler *et al.*, 1999). Interestingly, DB46 was found to bind Nod signals from a variety of rhizobia, and showed the highest affinity for the Nod signal produced by *B. japonicum* and *Rhizobium* sp. NGR234, both of which can nodulate *D. biflorus.* In an effort to define the possible role of apyrases in soybean nodulation, we sought to isolate a putative ortholog to LNP from soybean (*G. soja*) roots. PCR amplification using primers to conserved motifs found in all apyrase proteins (Figure 21) identified

two apyrases (GS50 and GS52, Figure 19 and 20), both of which share a high degree of sequence similarity to LNP. The identification of two orthologs in soybean prompted us to explore the possibility that one, or both, of these apyrases might be involved in nodulation.

As shown in Figure 25, GS50 and GS52 mRNA is differentially expressed in soybean tissues. Due to the high degree of sequence similarity between *gs50* and *gs52*, we opted to use RT-PCR, in lieu of northern blot analysis, to differentiate the expression pattern of the two mRNAs. RT-PCR results indicated that GS50 mRNA is optimally expressed in the stem of *G. soja*, with minimal expression in the root. Conversely, GS52 mRNA is optimally expressed in the roots of soybean, with a reduced expression in the hypocotyl. Neither mRNA was detected in the leaf. Interestingly, both mRNAs were expressed at relatively high levels in the flowers of *G. soja*, suggesting that GS50 and GS52 are not exclusively localized to a single tissue. Likewise, LNP was also detected in the flowers of *D. biflorus* (Roberts *et al.*, 1999).

The differential tissue localization of GS50 and GS52 mRNA that these proteins likely play different roles in the plant. As shown in Table 3, a number of legume genes thought to be involved nodulation are up-regulated upon perception of the invading rhizobia. To determine if *gs50* and/or *gs52* mRNA expression was enhanced upon inoculation of soybean roots with *B. japonicum*, RT-PCR was utilized to monitor the time course of expression of both *gs50* and *gs52* upon inoculation of soybean roots with *B. japonicum*. As shown in Figure 26 (*gs50*) and Figure 28 (*gs52*)

maximal expression was observed at 3 days. Interestingly, this expression pattern coincided with the development of the zone of the first-emergent root hairs, the region of the root considered most susceptible to rhizobial infection (Bhuvaneswari *et al.*, 1981). By nine days post-imbibition, expression of both *gs50* and *gs52* mRNA was reduced significantly. Previous reports support the hypothesis that the differentiating epidermal cells that lie just behind the rapidly growing root tip is the region of the root which is most susceptible to rhizobial infection (reviewed in Caetano-Anollés and Gresshoff, 1991). Thus, the expression of both apyrases appears to parallel the susceptibility of the developing root to rhizobial infection.

A number of host-responsive genes expressed in the roots of legumes have been extensively characterized for their temporal and spatial expression patterns upon rhizobial inoculation (Table 2). Among these, the early nodulins are the best characterized. For example, Minami *et al.* (1996b) demonstrated the rapid accumulation of ENOD40 mRNA in response to root inoculation with *B. japonicum* cells or Nod signal. RT-PCR analysis was used to determine if *gs50* and/or *gs52* mRNA levels increased upon inoculation of soybean roots with *B. japonicum*. After inoculation of soybean roots, expression of GS50 was unchanged (Figure 26). However, levels of GS52 mRNA rapidly increased within 3 hours post-inoculation, with sustained, enhanced expression detectable beyond 120 hours post-inoculation (Figure 28). These results coincide with reports suggesting agonist-induced expression of membrane-localized receptors in a number of biological systems (Ng *et*

al., 1997; Perera *et al.*, 1999; Potenza *et al.*, 1999). Moreover, these data justify classifying GS52 as an early nodulin, and therefore likely involved in the onset of nodulation. Although *gs50* mRNA levels were unresponsive to rhizobial inoculation, we cannot exclude the possibility that this protein may also be involved in the nodulation response.

Although the mRNA data indicated that GS50 and GS52 are differentially expressed in soybean tissues, these data do not necessarily reflect the protein expression pattern. Therefore, using purified antibodies to both GS50 and GS52, we sought to define the tissue localization of each protein in the various tissues of *G. soja*. In general, the protein expression patterns of both GS50 and GS52 were similar to the mRNA expression data (i.e., compare Fig. 34 to Fig. 26 and 28). However, there were several slight differences between the protein localization and the RT-PCR results. First, we were unable to detect GS52 mRNA in the cotyledon using RT-PCR (Figure 25), yet western blot analysis clearly showed expression of GS52 in 5-day-old cotyledons from *G. soja* (Figure 34). One possible explanation for this is that GS52 mRNA undergoes a rapid turnover in these tissues, and RT-PCR failed to detect a signal. A second possibility is that anti-GS52 antibodies are cross-reacting with an additional LNP ortholog present in the cotyledon of soybean.

A second, more striking observation was the reactivity of anti-GS52 antibodies with a protein of smaller molecular weight (e.g., 50 kDa) in nodule extracts. Whether GS50 and GS52 exist *in vivo* as an integral membrane protein or as a peripheral protein remains to be determined. The sequence of this protein, and the

absence of any predicted transmembrane domain other than its signal sequence suggests that if GS50 or GS52 is bound to the membrane via its signal sequence, it would have little, if any, cytoplasmic tail. Thus, if GS50 and/or GS52 do serve as specific receptors for rhizobial Nod signals, they likely function in association with other membrane-localized proteins. The detection of a smaller molecular weight protein (e.g., 50 kDa) present in nodules (Figure 34) suggests several interesting hypotheses. We previously determined that anti-GS50 antibody does not cross-react with GS52 and, likewise, anti-GS52 antibody does not recognize GS50. Therefore, we exclude the possibility that anti-GS52 antibodies are detecting GS50 in nodule extracts. One possibility is that the signal we are detecting is in fact GS52, and the protein is simply post-transitionally modified (e.g., cleaved signal sequence) in nodule tissues. Potato apyrase exists in both soluble and insoluble states that differ in their catalytic properties, isoelectric points, and molecular weights (Handa and Guidotti, 1996). Similarly, reports suggest that both the pea apyrase and LNP exist in both an insoluble and soluble state (Chen and Roux, 1986; Etzler *et al.*, 1999). However, we only detected this smaller protein in nodule extracts. Therefore, if cleavage of the GS52 signal sequence is producing this smaller, cross-reacting band, it appears specific to nodule development. The physiological consequence of such an event is still unknown. An alternative hypothesis for the appearance of this smaller protein in nodule extracts is that the anti-GS52 antibody is cross-reacting with an unidentified ortholog to GS50 and GS52. However, we do not favor this hypothesis since the antibody to GS52 is highly specific and does not recognize GS50.

However, we cannot rule out this possibility since evidence suggests that multiple (e.g., >4) apyrase proteins are present *Medicago truncatula* (Cohn and Stacey, unpubl.), and likely soybean.

The ability of antiserum to LNP to block nodulation of *D. biflorus* by *Bradyrhizobium* (Etzler *et al.*, 1999) prompted us to test the ability of both anti-GS50 and anti-GS52 to block nodulation of soybean. Interestingly, antiserum to GS50 had no effect on the nodulation of soybean by *B. japonicum*. However, pre-treatment of 2-day-old soybean roots with anti-GS52 serum showed a marked decrease in the formation of nodules. Control plants (e.g., no treatment or pre-immune sera) showed normal nodulation phenotypes. The inhibition in nodulation by pre-treatment of the roots with anti-GS52 serum suggests that GS52 plays an important role in soybean nodulation.

The subcellular localization of apyrases is largely undefined. Two yeast orthologs, GDA1 and YND1, have been identified as Golgi-localized apyrases (Abeijon *et al.*, 1993; Gao *et al.*, 1999), while the localization of the pea apyrase is still unclear. The Nod signal binding protein, LNP, has been localized to the surface of root hairs in *D. biflorus* by immunofluorescence. However it remains to be determined if LNP is membrane associated (Etzler *et al.*, 1999). Our data clearly localized GS52 to sucrose gradient fractions enriched in plasma membrane. This localization would be consistent with a role as a membrane-associated receptor for the Nod signal. Based on cellular localization of GS50, we hypothesize that it is likely an

endo-apyrase that functions in a capacity similar to that of GDA1 or YND1 from *S. cerevisiae* (Abeijon *et al.*, 1993; Gao *et al.*, 1999).

GS52 appears the most likely candidate of the two soybean apyrases described in the present study to function in nodulation. The tissue localization data suggest that GS52 is localized in the root, and more specifically, within the region of the root known to be susceptible to rhizobial infection (e.g., root hairs). Localization of GS52 to the plasma membrane suggests that it may function as an ecto-apyrase, with its catalytic domain positioned in the extracellular matrix. A similar arrangement has been suggested for the pea apyrase (Thomas *et al.*, 1999). Induction of GS52 mRNA in response to inoculation with *Bradyrhizobium* provides perhaps the most compelling evidence that GS52 plays some role in nodulation. The ability of anti-GS52 antibodies to block nodulation also argues for the involvement of GS52 as an essential component of the soybean nodulation response.

A weakness in the present study is the failure to demonstrate apyrase enzymatic activity by GS50 or GS52. After repeated efforts, we were unable to isolate active proteins after expression of GS50 or GS52 in *E. coli*. In contrast, Etzler *et al.* (1999) were able to recover active LNP after expression in *E. coli*. Therefore, further efforts with GS50 and GS52 are likely warranted. Based on the homology of GS50 and GS52 to active apyrases from a variety of species, as well as the low enzymatic activity detected using the recombinant proteins (data not shown), we are confident that *gs50* and *gs52* do in fact encode active apyrases. It will be critical in

the future to conduct a thorough analysis of GS50 and GS52 enzymatic activity, and to determine whether or not their activity is modulated by addition of Nod signals.

Through collaborative efforts with Dr. Nevin Young's laboratory (Univ. of Minnesota, St. Paul), we were able to map the location of *gs52*. Recent data have shown that the *M. truncatula* apyrase genes are clustered on the genetic map (Cohn and Stacey, unpubl.). Therefore, the mapping of *gs52* may allow us to identify other, closely linked, apyrase genes. Moreover, due to synteny between different legume species positioning of *gs52* on the soybean genome could allow for the identification of apyrase genes in a variety of legume species. An intriguing possibility would be to correlate the location of the apyrase gene with a mutation that results in a nodulation defect. Large scale mutagenesis efforts currently underway in *M. truncatula* and *Lotus japonicus* may ultimately make this possible.

In addition to their defined role in yeast, apyrases function in a broad range of cellular functions in both plants and animals. For example, apyrases have been implicated in neurotransmission, blood platelet aggregation and pressure regulation, protein glycosylation and sugar level control, and the regulation of membrane integrity (reviewed in Komoszynski and Wojtczak, 1996). A group of apyrases appear to have their catalytic activity exposed to the exterior of the cell and are termed ecto-apyrases (reviewed in Komoszynski and Wojtczak, 1996). The ability of the pea apyrase to scavenge extracellular phosphate from ATP suggests that this enzyme is an ecto-apyrase (Thomas *et al.*, 1999). The immunolocalization of DB46

on the surface of *D. biflorus* roots could also indicate that this enzyme is an ecto-apyrase (Etzler *et al.*, 1999).

Of all the proposed roles for apyrases in animals, the most intriguing with regard to nodulation is their function in neuro-synapses. (Sarkis and Salto, 1991). Apyrases are thought to act by rapidly degrading ATP to AMP. This releases the purinoreceptors due to their lower affinity for AMP. Moreover, by modulating the level of ATP + AMP/ ADP, apyrases also affect 5'-nucleotidase, an abundant enzyme in the synaptic space, which is inhibited by ADP and AMP, but activated by AMP. The final product of apyrase and 5'-nucleotidase action is adenosine that penetrates cell membranes and then can be phosphorylated, restoring the cellular ATP pool. Adenosine also has the effect of stimulating adenyl cyclase causing an increase in cAMP. Thus, apyrase activity can affect synaptic junction activity in many ways.

In terms of nodulation, the model of apyrase function in neural transmission is most interesting due to the demonstrated role of uridine in nodulation (Smit *et al.*, 1995; Schlaman *et al.*, 1997). In the presence of uridine, cell division in pea can be induced in every cortical cell upon addition of cytokinin (Libbenga and Harkes, 1973) and in inner cortical cells by chitin oligosaccharides following ballistic microtargeting in vetch (Schlaman *et al.*, 1997). Thus, we propose a model based on the function of apyrases to mediate the perception of Nod signals, and subsequent activation of nodulation via their ability to bind Nod signals. Binding of the Nod signal would stimulate the apyrase activity of the enzyme, resulting in the conversion of extracellular nucleotide tri- and di-phosphates to a pool of nucleosides, such as

Uridine. Such a model would be consistent with what is known about ecto-apyrase action during neurotransmission. However, at this point, such a model for legume nodulation is pure speculation. Further information on the importance of apyrases in nodulation is required before this or other models can be tested.

Based on our working hypothesis for the perception of lipo-chitin Nod signals in the *Bradyrhizobium*-symbiosis (Figure 4), the results described in this study have identified putative candidate proteins for each of the two proposed recognition events. The less stringent recognition event, is exemplified by the identification and characterization of a high affinity chitin binding site possessing the ability to recognize both chitin and lipo-chitin Nod signals. The identification of two soybean orthologs to a Nod signal binding protein from *D. biflorus*, represents candidate protein receptors for mediating the more stringent, Nod signal recognition event. In conclusion, this study serves as a basis for the further characterization of two independent recognition events involved in the establishment of the symbiotic association between *B. japonicum*, and its host legume, soybean.

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VITA

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