The *nodD* genes of rhizobia which nodulate the non-legume *Parasponia*

Kathryn K. Le Strange

A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

July 1996
Declaration

The research described in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree.

K. K. Le Strange
18 July 1996
Acknowledgements

Thanks must go to:

😊 Murray Badger, and my supervisors who read the revised thesis;

😊 my supervisors Barry Rolfe, Michael Djordjevic and John Redmond whose efforts ensured this thesis was initially submitted on time;

😊 Greg Bender, who provided supervision and support;

😊 Marie Oakes and Lynette Preston, who provided much practical molecular biology advice;

😊 Jan McIver, Elena Gartner, Brigid Roberts and Anne Moten for technical assistance;

😊 all the students of PMI over the years for friendship and mutual support;

❤️ my family for their help and support over the years;

❤️ my husband Paul, who suffered through my protracted illness with encouragement and support, and who worked late many nights by my side to make sure this thesis was finally completed; and

❤️ our young son Nathaniel, who provided the final impetus for completion.
Abstract

The central aim of this study was to define the characteristics of the \textit{nodD} genes in strains of rhizobia nodulating the non-legume \textit{Parasponia}. \textit{NodD1} of \textit{Rhizobium} strain NGR234, a fast-growing strain which nodulates \textit{Parasponia}, was found to be activated by a wide range of monyclic and polycyclic phenolic compounds to induce the transcription of a \textit{nodA::lacZ} fusion. These \textit{NodD}-dependent \textit{nod} gene induction assays revealed that a hydroxyl group \textit{para} to an electron-withdrawing group, or the presence of a cluster of oxygen functions are the prime structural requisites of phenolic compounds for the transcriptional activation of \textit{NodD}-dependent \textit{nod} genes. Furthermore, hydroxylation of the B ring of flavonoids increases their potency as \textit{NodD} activators.

\textit{NodD1} of strain NGR234 was shown also to be activated by extracts of various non-legumes, including \textit{Parasponia}, wheat and rice. The monyclic phenolic compounds vanillin and isovanillin which activate \textit{NodD1} of NGR234 were isolated from wheat seedling extracts and were identified in 5 of the 7 Australian wheat varieties examined. \textit{Bradyrhizobium} strain CP279, a slow-growing strain which is virtually \textit{Parasponia} specific, was found to have three putative \textit{nodD} genes. Complementation assays revealed that the putative CP279 \textit{nodD1} is responsible for \textit{Parasponia}-specificity, whereas the putative \textit{nodD2} gene enables nodulation of a broad range of host plants, and the putative \textit{nodD3} gene was not involved in the nodulation of the plant species tested.

Each of the CP279 putative \textit{nodD} genes was activated by a variety of phenolic compounds, including flavonoids and monyclic phenolics. Assays of \textit{NodD}-dependent \textit{nod} gene expression induced by the putative CP279 \textit{NodDs} showed that 7,4'-dihydroxyflavone and vanillin activated \textit{NodD1} and \textit{NodD3}, but not \textit{NodD2}, isovanillin activated \textit{NodD1} and \textit{NodD2}, hesperetin and apigenin activated \textit{NodD2} and \textit{NodD3}, and coumarin activated \textit{NodD1} and \textit{NodD3}. Specific flavonoid compounds were also found to enhance the growth of the \textit{Bradyrhizobium} strains examined, but do not necessarily activate \textit{NodD} to induce \textit{nod} gene transcription.

The \textit{nodD} genes of both \textit{Parasponia} nodulating strains examined were activated by phenolic compounds with a variety of structural features, supporting the hypothesis that the induction of \textit{nod}-gene transcription by a large suite of plant-derived phenolic compounds represents an important component of the capacity to nodulate \textit{Parasponia}. However this trait does not correlate with the narrow host range of the strain CP279, possibly due to regulation of the expression of \textit{nodD} genes within the wild-type strain.

The results presented in this thesis show that the interaction of \textit{NodD} with simple phenolic compounds is a common trait of strains NGR234 and CP279, both of which nodulate \textit{Parasponia}, and may contribute to the ability to nodulate a non-legume.
Table of Contents

Declaration iii
Acknowledgements v
Abstract vii
Table of Contents ix
List of Tables xiv
List of Figures xv
Abbreviations xvi

Chapter One Introduction
1.1 The importance of combined nitrogen to plants............................................. 1
1.2 Establishment of the Rhizobium/legume symbiosis ........................................ 2
1.3 Rhizobia also form nodules on the non-legume tree Parasponia ......................... 3
1.4 Plant defence against pathogenic micro-organisms ........................................... 4
1.5 The role of flavonoids in plants ...................................................................... 5
1.6 Rhizobia in the rhizosphere ........................................................................... 6
1.7 Flavonoids activate NodD ............................................................................. 7
1.8 Signal molecules in Agrobacterium infection ................................................... 8
1.9 Nod factors - nod genes and the initiation of infection .................................. 8
1.10 The roles of nod genes in Nod factor production ......................................... 9
1.11 NodD and the induction of nod gene transcription ....................................... 10
1.12 NodD and host range .................................................................................... 11
1.13 NodD Regulation ......................................................................................... 12
1.14 NodD and the nodulation of the non-legume Parasponia ............................... 13
1.15 Project aims .................................................................................................. 15
Table 1.1 The roles of Nod proteins in Nod factor production ............................... 17
Figure 1.1 Signal exchange between rhizobia and legumes during nodulation .......... 18
Figure 1.2 Model of the infection of Parasponia roots, and some of the Rhizobium genes known to be involved................................................................. 20
Figure 1.3 The phenyl-propanoid pathway, focusing on the simplified chalcone synthase branch. .................. 22
Figure 1.4 Structures of Nod factors produced by strains NGR234 and Bradyrhizobium japonicum .......... 24
Figure 1.5 Model for the mechanism of activation of NodD by flavonoid compounds and the initiation of transcription of nod genes ...... 26

Chapter Two Materials and Methods
2.1 Bacterial culture ............................................................................................... 29
2.1.1 Strains and plasmids used ......................................................................... 29
2.1.2 Media ......................................................................................................... 29
2.1.3 Media supplements .................................................................................... 30
2.1.4 Preparation of cultures ............................................................................. 30
2.1.5 Storage of bacterial strains ....................................................................... 30
2.1.6 β-Galactosidase assays ............................................................................. 31
2.1.6.1 β-galactosidase assays in Rhizobium strains ....................................... 31
2.1.6.2 β-galactosidase assays modified for Bradyrhizobium strains ............. 32
2.1.6.3 X-gal agar plates................................................................. 32
2.2 Chemistry.................................................................................. 32
  2.2.1 Chemical materials.................................................................. 32
  2.2.2 High performance liquid chromatography............................... 33
2.3 Plant Culture.............................................................................. 33
  2.3.1 Plant species used................................................................. 33
  2.3.2 Parasponia seed germination.................................................. 33
  2.3.3 Germination of other non-legumes......................................... 34
  2.3.4 Siratro seed germination........................................................ 34
  2.3.5 White clover seed germination............................................... 34
  2.3.6 Subterranean clover seed germination................................. 35
  2.3.7 The agar plate assay.............................................................. 35
  2.3.8 The Magenta jar pot assay.................................................... 35
  2.3.9 Plant culture media............................................................... 36
  2.3.10 Isolation of bacteria from root nodules............................... 36
2.4 Molecular biology....................................................................... 37
  2.4.1 General................................................................................. 37
    2.4.1.1 Preparation of materials.................................................... 37
    2.4.1.2 Storage of materials......................................................... 37
    2.4.1.3 General buffer solutions.................................................. 37
  2.4.2 Isolation of DNA................................................................... 38
    2.4.2.1 DNA isolation solutions................................................... 38
    2.4.2.2 Small scale isolation of E. coli plasmid DNA...................... 38
    2.4.2.3 Large scale isolation of E. coli plasmid DNA...................... 38
    2.4.2.4 Large scale isolation of total DNA from rhizobia................. 39
    2.4.2.5 Isolation of DNA from agarose gels.................................. 40
    2.4.2.6 Purification of DNA from agarose gels using GENECLEAN DNA Purification Kit......................................................... 40
  2.4.3 General DNA manipulations.................................................... 41
    2.4.3.1 DNA manipulation solutions............................................. 41
    2.4.3.2 Ethanol precipitation of DNA............................................. 41
    2.4.3.3 DNA digests with restriction endonucleases........................ 41
    2.4.3.4 Fractionation of DNA by sucrose gradient centrifugation .... 42
    2.4.3.5 Alkaline phosphatase treatment......................................... 42
    2.4.3.6 DNA ligations................................................................. 43
    2.4.3.7 Amplification of DNA fragments by polymerase chain reaction 43
  2.4.4 Agarose gel electrophoresis of DNA fragments........................ 44
  2.4.5 DNA transfer and hybridisation techniques............................ 44
    2.4.5.1 DNA transfer and hybridisation solutions.......................... 44
    2.4.5.2 Colony blot................................................................. 45
    2.4.5.3 Transfer of electrophoresed DNA onto membranes for hybridisation................................................................. 45
    2.4.5.4 Radiolabelling DNA fragments......................................... 46
    2.4.5.5 DNA hybridisation - rotating bottle method...................... 46
    2.4.5.6 DNA hybridisation - plastic bag method............................ 47
    2.4.5.7 Deprobing Hybond-N membranes..................................... 47
  2.4.6 Electroporation of E. coli with DNA....................................... 47
    2.4.6.1 Preparation of E. coli cells for electroporation.................. 47
    2.4.6.2 Electroporation of E. coli cells........................................ 47
2.4.7 Conjugation of bacteria by tri-parental patch mating 48
2.5 Chemicals and reagents 49
Table 2.1 Bacterial Strains Used In This Study 51
Table 2.2 Plasmids Used In This Study 52
Table 2.3 Antibiotic Concentrations Used in Bacterial Culture 53
Figure 2.1 The Magenta jar assay 54
Figure 2.2 Tri-parental patch mate 56

Chapter Three - Compounds inducing nod gene transcription in Rhizobium strain NGR234.
3.1 Introduction 59
3.2 Experimental procedure 60
3.2.1 Construction of plasmid pMD1 61

Results
3.3 Phenolic compounds inducing nodD-dependent transcription 62
3.3.1 Negative controls for β-galactosidase assays 62
3.4 Key features of inducing compounds 63
3.5 Discussion 63
Table 3.1 Phenolic compounds assessed for biological activity with the nodD1 gene of strain NGR234 68
Table 3.2 Relative induction levels of compounds inducing Rhizobium strain NGR234 NodD1-dependent nod-gene expression 69
Figure 3.1 Map of plasmid pMD1 70
Figure 3.2 The phenyl-propanoid pathway, highlighting compound types found to activate NodD1 of strain NGR234 72
Figure 3.3 Representative substances that induce Rhizobium strain NGR234 NodD1-dependent nod gene transcription in strain ANU265(pMD1) 74
Figure 3.4 The occurrence of lignin on root hairs 76

Chapter Four Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.
4.1 Introduction 79
4.2 Experimental procedure 80
4.2.1 Collection of plant extracts (general method) 81
4.2.1.1 Acid hydrolysis of glycosides (in the H2O phase) 81
4.2.2 Imbibed wheat extracts 81
4.2.3 Siratro seed extract 81
4.2.4 Preparation of plant extracts for chromatography 82
4.2.5 Thin layer chromatography (TLC) 82
4.2.6 Ascending paper chromatography 83
4.2.7 Diazotized sulphanilic acid spray 83
4.2.8 Development of methods for large scale extracts of wheat seedlings 83
4.2.9 Preparative chromatography of wheat seedling extract 85

Results
4.3 Extracts of non-legumes activate NodD1-dependent nod genes 85
4.4 Glycosides of activating flavonoids are not present in extracts of wheat, siratro and Parasponia 86
4.5 Chromatographic analysis of plant extracts 86
4.5.1 Thin layer and paper chromatography ....................................................... 86
4.5.2 Column chromatography ........................................................................... 87
4.6 Development of preparative scale extracts from wheat seedlings ................. 87
4.7 Chromatographic analysis of wheat seedling extracts-characterisation of simple phenolic compounds ............................................. 88
4.8 Vanillin, a compound activating NGR234 NodD1, is found in Australian wheat varieties 89
4.9 Wheat exudate co-activates nod gene transcription ........................................ 89
4.10 Discussion ......................................................................................................... 90

Table 4.1 Response by R. I. bv trifolii strain ANU894, carrying the nodD1 gene from strain NGR234 to seedling extracts 94
Table 4.2 Characteristics Of Australian Wheat Varieties Examined ........................ 95
Figure 4.1 Map of plasmid pMN40 ................................................................. 96
Figure 4.2 Summary of the fractionation procedure for Australian Standard White (ASW) wheat seedling extracts 98
Figure 4.3 HPLC elution profile of fraction C2 of the of ASW wheat seedling extract .............................................. 100
Figure 4.4 UV spectrum analysis of peak A of fraction C2 of ASW wheat seedling extract .............................................. 102

Chapter Five The role of Bradyrhizobium strain CP279 putative NodDs in the nodulation of plant hosts and their activation by phenolic compounds.

5.1 Introduction ........................................................................................................ 105
5.2 Experimental Procedure .................................................................................. 108
5.2.1 Identification of nodD homologues in strain CP279 ..................................... 109
5.2.2 Cloning of the putative nodD genes from strain CP279 ............................ 110
5.2.3 Subcloning putative CP279 nodD clones for nodD complementation assays .... 110
5.2.4 Subcloning the 10kb BamHI fragment (containing a nodY::lacZ fusion, inducible by NodD activation) from pZB32 to pKT240 ... 110
5.2.5 β-galactosidase assays ................................................................................ 111
5.2.6 The effect of flavonoid compounds on the growth of Bradyrhizobia ......... 111

Results
5.3 CP279 has at least three regions that show homology to NGR234 nodD1 ...... 112
5.4 Plant nodulation assays to assess complementation by the putative nodD genes of strain CP279 ........ 113
5.4.1 CP279 clone D17 ..................................................................................... 114
5.4.2 CP279 clone D20 ..................................................................................... 115
5.4.3 CP279 clone D40 ..................................................................................... 115
5.5 NonD-dependent nod gene assays in bradyrhizobia using reporter plasmids pRT311 and pZB32 ................................................................. 115
5.6 Subcloning the 10kb BamHI fragment (containing a NodD-dependent nodY::lacZ fusion,) from pZB32 to pKT240 ..................... 116
5.7 Analysis of phenolics activating native NodDs in bradyrhizobia ....................... 116
5.8 Analysis of phenolics activating the cloned putative NodDs from CP279 in nodD deficient backgrounds ................. 118
5.8.1 The activation of CP279 putative NodDs, measured by reporter plasmid pLSC17 ................................. 119
5.8.2 The activation of CP279 putative NodDs, measured by reporter plasmid pRT311................. 119
5.9 Bacterial growth enhancement by phenolic compounds....................................................... 120
5.10 Discussion....................................................................................................................... 121
Table 5.1 Approximate sizes of the hybridizing bands in each lane, in kb............................... 113
Table 5.2 The endogenous antibiotic resistance of *Bradyrhizobium* strains ......................... 116
Table 5.3 A Summary of NodD activation .............................................................................. 125
Table 5.3 B Phenolic compounds used in *nod*-gene induction assays .................................. 125
Table 5.4 The nodulation of *Parasponia* by CP279 *nodD* clones......................................... 130
Table 5.5 The nodulation of siratro by CP279 *nodD* clones.................................................. 131
Table 5.6 The nodulation of white clover by CP279 *nodD* clones ......................................... 132
Table 5.7 The nodulation of subterranean clover by CP279 *nodD* clones ......................... 133
Table 5.8 The growth effects of phenolic compounds on bradyrhizobia .............................. 134
Figure 5.1 Hybridisation of NGR234 *nodD1* probes to digests of strain CP279 total DNA .... 136
Figure 5.2 The generation of probe fragments from NGR234 *nodD1* by polymerase chain
reaction (PCR).................................................................................................................. 138
Figure 5.3 The fractionation of strain CP279 DNA................................................................ 140
Figure 5.4 Cloning of the CP279 DNA BgII fragments into plasmid pJJ358 ..................... 142
Figure 5.5 The subcloning of CP279 *nodD* genes into plasmid pLAFR3 .......................... 144
Figure 5.6 Construction of plasmid pLSC17, showing the cloning of the
*nodY*:lacZ fusion from plasmid pZB32 to plasmid pKT240 .......................................... 146
Figure 5.7 Restriction maps of the three cloned CP279 *nodD* genes.................................. 148
Figure 5.8 Physical map of plasmid pRT311 ....................................................................... 150
Figure 5.9 The structures of phenolic compounds examined for activation of NodDs of bradyrhizobia................................................................. 152
Figure 10 β-galactosidase assays showing activation of bradyrhizobia NodDs .... 154

Chapter Six Discussion
6.1 Introduction .................................................................................................................. 159
6.2 Compounds activating NGR234 NodD1 ....................................................................... 160
6.3 The putative *nodD* genes of CP279 and the nodulation of *Parasponia* ...................... 162
6.4 The putative *nodD* genes of CP279 and the activation of NodD................................. 164
6.5 Regulation of *nodD* genes ......................................................................................... 165
6.6 Conclusions.................................................................................................................. 168
6.7 Future directions .......................................................................................................... 169
6.8 Publications arising from the research reported in this thesis..................................... 171
Table 6.1 Summary of the major findings reported in this thesis ........................................ 158
Figure 6.1 Model of the infection of *Parasponia* roots, and some of the
*Rhizobium* genes known to be involved......................................................................... 172

References ......................................................................................................................... 175
Published Papers .............................................................................................................. 195
List of Tables

Chapter One
Table 1.1 The roles of Nod proteins in Nod factor production ............................... 17

Chapter Two
Table 2.1 Bacterial Strains Used In This Study ................................................................ 51
Table 2.2 Plasmids Used In This Study ................................................................... 52
Table 2.3 Antibiotic Concentrations Used in Bacterial Culture........................................ 53

Chapter Three
Table 3.1 Phenolic compounds assessed for biological activity with the nodD1 gene of strain NGR234 .......................... 68
Table 3.2 Relative induction levels of compounds inducing Rhizobium strain NGR234 nodD1-dependent nod-gene expression .... 69

Chapter Four
Table 4.1 Response by R. l. bv trifolii strain ANU894, carrying the nodD1 gene from strain NGR234 to seedling extracts........ 94
Table 4.2 Characteristics Of Australian Wheat Varieties Examined .............................. 95

Chapter Five
Table 5.1 Approximate sizes of the hybridizing bands in each lane, in kb .................. 113
Table 5.2 The endogenous antibiotic resistance of Bradyrhizobium strains ................. 116
Table 5.3 A Summary of NodD activation ............................................................. 125
Table 5.3 B Phenolic compounds used in nod-gene induction assays .......... 125
Table 5.4 The nodulation of Parasponia by CP279 nodD clones ....................... 130
Table 5.5 The nodulation of siratro by CP279 nodD clones ............................. 131
Table 5.6 The nodulation of white clover by CP279 nodD clones ..................... 132
Table 5.7 The nodulation of subterranean clover by CP279 nodD clones ........ 133
Table 5.8 The growth effects of phenolic compounds on bradyrhizobia ............. 134

Chapter Six
Table 6.1 Summary of the major findings reported in this thesis ......................... 158
## List of Figures

### Chapter One

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Signal exchange between rhizobia and legumes during nodulation</td>
</tr>
<tr>
<td>1.2</td>
<td>Model of the infection of <em>Parasponia</em> roots, and some of the <em>Rhizobium</em> genes known to be involved</td>
</tr>
<tr>
<td>1.3</td>
<td>The phenyl-propanoid pathway, focusing on the simplified chalcone synthase branch</td>
</tr>
<tr>
<td>1.4</td>
<td>Structures of Nod factors produced by strains NGR234 and <em>Bradyrhizobium japonicum</em></td>
</tr>
<tr>
<td>1.5</td>
<td>Model for the mechanism of activation of NodD by flavonoid compounds and the initiation of transcription of <em>nod</em> genes</td>
</tr>
</tbody>
</table>

### Chapter Two

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The Magenta jar assay</td>
</tr>
<tr>
<td>2.2</td>
<td>Tri-parental patch mate</td>
</tr>
</tbody>
</table>

### Chapter Three

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Map of plasmid pMD1</td>
</tr>
<tr>
<td>3.2</td>
<td>The phenyl-propanoid pathway, highlighting compound types found to activate NodD1 of strain NGR234</td>
</tr>
<tr>
<td>3.3</td>
<td>Representative substances that induce <em>Rhizobium</em> strain NGR234 NodD1-dependent <em>nod</em> gene transcription in strain ANU265(pMD1)</td>
</tr>
<tr>
<td>3.4</td>
<td>The occurrence of lignin on root hairs</td>
</tr>
</tbody>
</table>

### Chapter Four

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Map of plasmid pMN40</td>
</tr>
<tr>
<td>4.2</td>
<td>Summary of the fractionation procedure for Australian Standard White (ASW) wheat seedling extracts</td>
</tr>
<tr>
<td>4.3</td>
<td>HPLC elution profile of fraction C2 of the of ASW wheat seedling extract</td>
</tr>
<tr>
<td>4.4</td>
<td>UV spectrum analysis of peak A of fraction C2 of ASW wheat seedling extract</td>
</tr>
</tbody>
</table>

### Chapter Five

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Hybridisation of NGR234 <em>nodD1</em> probes to digests of strain CP279 total DNA</td>
</tr>
<tr>
<td>5.2</td>
<td>The generation of probe fragments from NGR234 <em>nodD1</em> by polymerase chain reaction (PCR)</td>
</tr>
<tr>
<td>5.3</td>
<td>The fractionation of strain CP279 DNA</td>
</tr>
<tr>
<td>5.4</td>
<td>Cloning of the CP279 DNA BglII fragments into plasmid pJJ358</td>
</tr>
<tr>
<td>5.5</td>
<td>The subcloning of CP279 <em>nodD</em> genes into plasmid pLAFR3</td>
</tr>
<tr>
<td>5.6</td>
<td>Construction of plasmid pLSC17, showing the cloning of the <em>nodY::lacZ</em> fusion from plasmid pZB32 to plasmid pKT240</td>
</tr>
<tr>
<td>5.7</td>
<td>Restriction maps of the three cloned CP279 <em>nodD</em> genes</td>
</tr>
<tr>
<td>5.8</td>
<td>Physical map of plasmid pRT311</td>
</tr>
<tr>
<td>5.9</td>
<td>The structures of phenolic compounds examined for activation of NodDs of bradyrhizobia</td>
</tr>
</tbody>
</table>

### Chapter Six

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Model of the infection of <em>Parasponia</em> roots, and some of the <em>Rhizobium</em> genes known to be involved</td>
</tr>
</tbody>
</table>
Abbreviations

bp base pair
CLOS chito-lipo-oligosaccharide
DHF 7,4'-dihydroxyflavone
dH₂O distilled water
DNA deoxyribonucleic acid
DTT dithiothreitol
EDTA Na₂-ethylenediaminetetra-acetic acid
HPLC high pressure liquid chromatography
kb kilobase pairs
ONPG O-nitrophenyl β-D-galactopyranoside
PCR polymerase chain reaction
SDS sodium dodecyl sulphate
SSC salt, sodium citrate solution
SSPE salt, sodium phosphate and EDTA solution
TE Tris-EDTA buffer
Tris Tris[hydroxymethyl]aminomethane
UV ultra violet light
x-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
The advantage of the nitrogen fixing symbiosis with rhizobia to the non-legume tree *Parasponia andersonii*.

All plants are 10 weeks old and were inoculated with the strains indicated approximately 5 days after germination.
The advantage of the nitrogen fixing symbiosis with rhizobia to the non-legume tree *Parasponia andersonii*.

All plants are 10 weeks old and were inoculated with the strains indicated approximately 5 days after germination.
Chapter One
Introduction

1.1 The importance of combined nitrogen to plants

In many soils nitrogen represents the limiting factor in crop production. Modern agriculture, particularly in developed economies, requires enormous expenditure on nitrogenous fertilisers and other plant nutrients to maintain farming efficiency. The production of nitrogen-based fertiliser requires large inputs of energy and is hence expensive. In less developed economies it is generally too costly to import and apply such plant nutrients on a large scale or to establish a local fertiliser industry (Gallon and Chaplin 1987). Some of the arguments in relation to the use of applied nitrogen fertilisers on the basis of a cost benefit analysis include: the variety of costs associated with the use of nitrogen fertilisers such as - the use of oil in production, the purchase price for farmers, the waste of the applied fertilisers via volatilisation and run-off, and the environmental costs of the ensuing pollution of waterways; versus the benefit of increased yield of crop plants.

A well known alternative to exogenously applied nitrogen fertiliser is the fixation of atmospheric nitrogen by prokaryotes in association with a plant partner, described by the following equation

\[ N_2 + 6 e^- + 12 \text{ATP} + 12 \text{H}_2\text{O} \rightarrow 2 \text{NH}_4^+ + 12 \text{ADP} + 12 \text{P}_i + 4 \text{H}^+. \]

Nitrogen fixing interactions between plants and prokaryotes are well documented in achieving great improvements in crop and pasture production and soil fertility. Unfortunately these interactions often fail to achieve their full potential. A wide variety of factors contribute to such failure, including: incompatible soil pH, limited soil nutrient availability, inappropriate soil temperature, under-inoculation with the prokaryotic partner, the failure of the inoculated species to establish in the soil, or to compete effectively with the native population.

Symbiotic nitrogen fixation occurs between the actinomycete Frankia and a diverse array of plant families, including woody trees and shrubs, such as Casuarina and Alnus. However, the Leguminoseae are the major family of plants which participate in nitrogen-fixing symbioses. Their symbiotic partners are bacteria of the family Rhizobiaceae, which include the genera Rhizobium, Bradyrhizobium and Azorhizobium. Rhizobium and Bradyrhizobium will be referred to as rhizobia throughout this thesis.

Research into the extension of the host range of rhizobia beyond the Leguminoseae has long been heralded as an opportunity to significantly reduce the reliance of agricultural production on nitrogen fertilisers. However, it should also be noted that the symbiotic
associations between rhizobia and legumes which occur when both pasture and grain legumes are cultivated contribute to increases in soil acidity, most particularly in poorly buffered soils (Mengel and Schubert 1987). Acid soils have a poor nutrient status and are unfavourable for the growth of the great majority of plant species (Ray, 1972), including legumes (Graham and Chattel 1983). Thus the extension of the host range of rhizobia to major crops such as wheat and corn may not be a wholly positive step.

1.2 Establishment of the *Rhizobium*/*legume* symbiosis

Strains of rhizobia generally do not fix atmospheric nitrogen in the free living state. Nitrogen fixation by rhizobia can usually only occur when the bacterial dinitrogenase/dinitrogenase reductase complex is protected from attack by oxygen. In symbioses with plant partners, such protection is provided by the nodule structure, and by the active removal of oxygen by rapid bacterial respiration (Werner 1992).

Nodule formation on the roots of leguminous plants occurs under the influence of their co-symbionts, rhizobia. The process of nodule formation involves an ongoing series of signals and responses between the plant and the bacterium, and this signal exchange is summarised in Figure 1.1. The signalling mechanisms between potential co-symbionts are initiated prior to seed germination, as flavonoid compounds present in the seed coat of legumes leach into the surrounding soil. As the seed germinates, a different array of flavonoid compounds are released. The initial response by rhizobia is chemotaxis towards flavonoid compounds, in a concentration-dependent manner (Caetano-Anolles et al. 1988a). Plant-produced flavonoid compounds also serve to induce the nodulation genes (*nod*) of *Rhizobium* species. Most of the *nod* genes are generally thought to be expressed at a very low level in the soil, and *nod* gene activation is a requisite for nodule initiation and formation (Kapulnik et al. 1987). Signal molecules to the plant, produced by the bacterium under the control of the *nod* genes (Nod factors), elicit root hair curling and stimulate cortical cell division (Lerouge et al. 1990; Truchet et al. 1991). The expression and regulation of *nod* genes and the production of Nod factors will be discussed in detail below in Section 1.9.

The details of nodulation described here are those that occur in the nodulation of soybean and clover species by rhizobia. A limited number of alternate routes of infection occur in other legume species, such as the utilisation of an emerging lateral root in *Sesbania* and peanut (reviewed by Rolfe and Gresshoff 1988).

Following the attachment by rhizobia to a root hair, marked cytoplasmic streaming occurs and the plant cell nucleus migrates to the site of bacterial attachment. The tip of the root hair cell begins to curl, apparently under the influence of Nod factors, which have been shown to induce depolarisation of root hairs (Ehrhardt et al. 1992), changes in the concentration of $H^+$ and $Ca^{2+}$ ions, and re-organisation of the cell's actin cytoskeleton (Allen et al. 1994).
The bacterium becomes trapped as the tip of the root hair cell curls. The trapped bacterium dissolves the plant cell wall, and as the cell membrane invaginates an infection thread of plant cell wall material is deposited around and in front of the invading bacteria. The infection thread is preceded by the host cell nucleus, attached by microtubules to the infection thread tip (Lloyd et al. 1987; Bakhuizen 1988). The infection thread grows to penetrate the plant cell layers, and on reaching the root cortex ramifies (Robertson and Lyttleton 1982). When the infection thread reaches the area of induced cortical cell division, the rhizobia contained in the infection thread are packaged into a plant membrane (the peribacteroid membrane), and the encapsulated bacteria released into the plant cells. The bacteria continue to grow inside the peribacteroid membrane (some divide, others expand), and eventually differentiate into bacteroids, in which form they fix atmospheric nitrogen.

There are two major forms of legume root nodule. The first type is referred to as indeterminate and these nodules form on temperate legumes, such as clovers, and contain a persistent nodule meristem. Initial cell divisions occur in the inner cortex, and the nodule grows by cell division, leading to an elongated shape. In contrast, the second nodule type forms on tropical and subtropical legumes, for example soybean, and is referred to as determinate. The determinate nodule grows by cell expansion following initial cell division in the outer cortex, resulting in a spherical shape (reviewed by Hirsch 1992).

1.3 Rhizobia also form nodules on the non-legume tree *Parasponia*

A number of rhizobia are also able to form nodules in association with non-legume trees of the genus *Parasponia* (Ulmaceae) (Trinick 1979), tropical species which grow as pioneer plants on volcanic soils in New Guinea, Indonesia and Malaysia. The natural nodulation of the non-legume *Parasponia* by rhizobia is an important area of research if the nodulation of cereal crops and other non-legumes of agronomic importance is to be achieved. This thesis focuses on rhizobia which nodulate *Parasponia* and the mechanism of recognition of *Parasponia* as a potential host.

The establishment of *Parasponia* nodules differs in a number of important ways from the establishment of nodules in legumes, described above in Section 1.2. The infection of *Parasponia* roots is described schematically in Figure 1.2. In the first stage of infection, rhizobia actively erode the mucilaginous layer of the root surface at the point of contact and induce the division of subepidermal cells (Bender et al. 1987a). This erosion of the root surface apparently does not involve Nod factors since strain ANU265, a non-nodulating strain devoid of the Sym plasmid that encodes the nod genes, is able to erode successfully (Bender et al. 1987b). However, *nodD* and *hsn* genes are required for the induction of cell division (Morrison et al. 1984; Bender et al. 1987b). Continued cell division results in localised swelling, which breaks through the epidermis thereby providing access for the bacterial invasion of the intercellular spaces
Chapter One

(Bender et al. 1987a). This second, prenodule, stage of infection requires the expression of the nodD and nodABC genes (Bender et al. 1987b). The third stage of infection requires the expression of nodD, nodABC, and hsn genes (Bender et al. 1987b). These latter stages therefore involve Nod factors which are presumably not involved in the first stage of infection.

During the third stage of infection, infection threads are initiated from intercellular colonies within the prenodule, (Lancelle and Torrey 1984), and a second cycle of cell division is induced, in the pericycle (Bender et al. 1987b). Bacteroids are retained in the infection threads, where some bacteria continue to divide. Nitrogen fixation occurs in these threads, referred to as fixation threads at this later stage of development. The Parasponia nodule structures are larger than legume nodules and are clearly modified lateral roots with a central vascular bundle (Trinick 1979), but bear some similarity to indeterminate legume nodules. These nodule structures bear some similarity to the indeterminate nodule described above in Section 1.2, in that they possess a nodule meristem (Bender 1987).

The rhizobia able to form nitrogen fixing nodules on Parasponia are mostly slow-growing bradyrhizobia including strains CP279 and CP283, however, the fast-growing Rhizobium strain NGR234 is also able to nodulate Parasponia.

1.4 Plant defence against pathogenic micro-organisms

Flavonoid compounds, which act as signal molecules inducing the expression of Rhizobium nod genes in the Rhizobium-legume symbiosis, are produced via a biosynthetic pathway which also produces a plethora of compounds involved in plant responses to pathogenic attack (reviewed by Koes et al. 1994).

In successfully establishing nodules on the roots of a host plant, rhizobia must avoid or prevent the elicitation of most of the defences a plant has in place to limit pathogens. One such strategy is the formation of infection threads which prevent rhizobia from entering the plant cell cytoplasm and perhaps eliciting a full scale plant defence response.

Plants adopt a wide variety of strategies to prevent the entry of pathogens into plant tissue. Structural defence mechanisms include lignification, suberization and the deposition of phenolic acids in cell walls (Ride 1986). These mechanisms are induced via transcriptional activation of defence genes (Dixon and Lamb 1990). However, the primary defence of plants against invasion by pathogens is the hypersensitive response, where rapid, localised defence responses result in the death of plant cells surrounding the infection site (reviewed by Koes et al. 1994). Other defence responses adopted include the reinforcement of cell walls by callose and lignin deposition, the induction of lytic enzymes such as chitinases and glucanases, and the synthesis of phytoalexins.
Phytoalexins are anti-microbial compounds of low molecular weight synthesised by plants after exposure to microorganisms (Dixon 1986; Ebel 1986). Phytoalexins encompass a diverse group of compounds, including flavonoids, terpenoids, stilbenes, pterocarpins and polyacetylenes. However, each plant family will produce only one or two types of phytoalexin. For example, the phytoalexins of the Solanaceae are sesquiterpene compounds, whereas in the Leguminosae the phytoalexins are predominantly derived from the isoflavonoid branch of the phenyl-propanoid pathway (VanEtten and Pueppke 1976).

1.5 The role of flavonoids in plants

The products of the phenyl-propanoid pathway play a wide variety of roles in plants (reviewed in Koes et al. 1994). Genes coding for the enzymes of this pathway are amongst those activated as part of a plant’s induced defence response to invading pathogens (Lawton and Lamb 1987; Hahlbrock and Scheel 1989). Flavonoid is a general term, and in this thesis is used to refer to phenyl-propanoid pathway compounds produced through the chalcone synthase branch of the pathway, illustrated in Figure 1.3. Flavonoids are poly-cyclic phenolic compounds based on two aromatic rings (A- and B-rings), joined by a 3 carbon unit (C-ring). The general structure and carbon numbering system for flavonoids is illustrated in the legend to Figure 1.3. Flavonoids are further divided into subclasses based on the oxidation of the C-ring. The flavonoid subclasses include flavanones, flavonols, isoflavonoids, chalcones, anthocyanins and tannins. Over 3500 different flavonoids have been characterised. The wide variety of structures amongst flavonoid compounds is due to the diversity of possible substitutions to the basic structures - for example, hydroxylation, methylation, acylation, and substitution/addition of sugars via glucosylation and glycosylation (Harborne 1988; Koes et al. 1994).

Flavonoids play a variety of roles in plants in addition to serving as phytoalexins in legumes (reviewed by Koes et al. 1994). Anthocyanin compounds function as visual signals for various animal species, in particular insects and birds, influencing reproduction through pollination. Flavonoids are also strongly UV absorbing, and serve as a protective shield to prevent damage to a number of physiological processes and to plant cell DNA. Flavonoid gene expression is induced by exposure to UV light, and the flavonoids accumulate mainly in epidermal cells (Schmelzer et al. 1988). Flavonoids also play an essential role in pollen tube development, although the mechanism involved is currently unknown (Coe et al. 1981; Mo et al. 1992).

In the context of *Rhizobium*/*legume* symbioses, flavonoids are initially involved as plant signals to rhizobia in relation to chemotaxis and *nod* gene induction. Both of these processes involve the transcriptional regulatory gene *nodD* (Spaink et al. 1987a; Caetano-Anolles et al. 1988a). Flavonoid biosynthetic genes are active in legumes in young root cap cells and in zones of emerging root hairs (Rommeswinkle et al. 1992;
Yang et al. (1992). Following release into the rhizosphere these compounds interact in an as yet unidentified manner with NodD, inducing nod gene transcription.

Flavonoids are also thought to play a role within the root during nodulation (Jacobs and Rubery 1988; Hirsch 1992; Schmidt et al. 1992; Yang et al. 1992). Flavonoids can function as modulators of polar auxin transport (Jacobs and Rubery 1988) and may therefore play a role as internal signal molecules in the alteration of plant development towards nodule meristem formation (Schmidt et al. 1994).

1.6 Rhizobia in the rhizosphere

Legumes exude a variety of compounds into the rhizosphere at a relatively high rate, and exudates may include amino acids, sugars and phenolic compounds (Werner 1992). These compounds are able to enhance the growth of rhizobia to the extent that rhizobia and other Gram-negative bacteria will predominate in the legume rhizosphere, despite Gram-positive bacteria dominating in the surrounding soil (Werner 1992). Catabolism of rhizosphere substituents extends beyond the amino acids and sugars to include a wide array of simple phenolic compounds which can be used as sole sources of carbon and energy (Chen et al. 1984; Parké and Ornston 1986). Flavonoids may also enhance the growth of rhizobia, although catabolism is not necessarily involved (Hartwig et al. 1991).

Flavonoids exuded into the plant’s rhizosphere are potentially absorbed by rhizobia. Hubac et al. (1993) studied the absorption by bacterial cells of flavonoid compounds by chromatographic analysis of cell extracts, and by measurement of the disappearance of the flavonoid compounds from the growth medium. Flavonoid compounds which induce the transcription of R. meliloti nod genes were absorbed at a higher rate than were non-inducers (luteolin > naringenin = quercetin). Luteolin accumulated preferentially in the outer membrane, but a small amount was also found in the inner membrane. There is some implication that the site of luteolin absorption (in the outer membrane) allows protection of the respiratory chain from an excess of flavonoids (Hubac et al. 1993). In contrast to these findings, naringenin was found to accumulate in Rhizobium leguminosarum bv. viciae at the inner membrane (Recourt et al. 1989).

The B. japonicum strain USDA110 has recently been shown to degrade isoflavone inducers of nod gene transcription (daidzein and genistein) via C-ring fission. The conserved single phenolic ring (A- and B-ring) products serve as weak nod gene inducers, but some also serve to inhibit induction by daidzein (Rao and Cooper 1994). The degradation of flavonoid inducers by rhizobia has strong implications for the regulation of nod gene induction in that the processing of flavonoids by rhizobia may affect their potential for activation of NodD.
1.7 Flavonoids activate NodD

Chemotaxis is the first response of rhizobia to the presence of flavonoids in the soil. Caetano-Anolles and co-workers (1988a) reported that rhizobia are able to detect flavonoids in the soil at concentrations of $10^{-7}$ M down to $10^{-10}$ M. The nodD gene product is essential for flavonoid induced chemotaxis, although a second chemotactic pathway for ordinary nutrients is also postulated to exist (Bergman et al. 1988; Caetano-Anolles et al. 1988a). In R. meliloti luteolin induces maximal chemotaxis at $10^{-8}$ M, but maximal nod gene induction occurs at $10^{-7}$ M (Caetano-Anolles et al. 1988a; Peters et al. 1986). In B. japonicum, however, chemotaxis to simple phenolics was shown but not to nodD-activating flavonoids (Kape et al. 1991). Chemotactic ability is not essential for successful nodulation, although mutants defective in chemotaxis or motility have a reduced efficiency of nodulation, reduced adsorption to root surfaces, and are less effective competitors for nodule occupancy. This reduced effectiveness has been demonstrated by competition assays between mutant and non-mutant strains (Caetano-Anolles et al. 1988b; Soby and Bergman 1983).

A major role of flavonoids in the Rhizobium/legume symbiosis is the induction of transcription of the nod genes, in conjunction with the nodD gene product. The flavonoids activating NodD have been identified largely by plasmid reporter gene constructs indicating the induction of transcription of the NodD-dependent nod genes. The compounds identified include the activating compounds luteolin, isolated from alfalfa (Peters et al. 1986) and 7,4’-dihydroxyflavone (DHF) from clover (Redmond et al. 1986), and anti-inducers such as coumarin, flavonol, and some isoflavones (Redmond et al. 1986; Firmin et al. 1986; Djordjevic et al. 1987). Anti-inducers appear to competitively inhibit NodD-dependent nod gene induction by the activating flavonoid compounds (Djordjevic et al. 1987). However, anti-inducer compounds in one strain may function as inducers of NodD-dependent nod genes in another strain. For example, the isoflavone daidzein has been isolated from soybean and shown to activate NodD of B. japonicum (Kossak et al. 1987), whereas in R. l. bv. trifolii daidzein behaves as an anti-inducer (Djordjevic et al. 1987).

Flavonoids may also play other roles in the establishment of symbioses. Recently a nod gene inducing flavonoid (apigenin) was shown to modulate the production of some cell-associated R. fredii polysaccharides (Reuhs et al. 1994). The mechanism and biological significance of this modulation is unknown. Furthermore, nod-gene inducing flavonoids have been shown to stimulate R. fredii to excrete proteins. This response is Sym plasmid dependent, but is not necessarily directly related to nodulation (Krishnan and Pueppke 1993).
1.8 Signal molecules in Agrobacterium infection

In the infection of plants by another member of the Rhizobiaceae, Agrobacterium tumefaciens, plant compounds also play a role in activating the key infection genes, the virulence genes (vir genes) (reviewed in Winans 1992). Signal molecules able to activate vir genes include substituted phenolics and various sugars, and a low pH environment is also necessary. In addition, sugar molecules are sensed by the chvE gene product. ChvE has been shown to interact with VirA, altering the ability of VirA to respond to phenolics. There is no physical evidence that VirA interacts directly with signal molecules although neither can such direct interaction be ruled out (Machida et al. 1993; Binns et al. 1993). The signal detected by VirA is transduced via VirA autophosphorylation, followed by the transfer of the phosphate to VirG, activating VirG which then functions as a transcriptional regulator of the other vir genes (Reviewed in Stock et al. 1990; Winans 1992).

1.9 Nod factors - nod genes and the initiation of infection

Specific groups of nod genes are essential for the successful nodulation by a Rhizobium strain of its plant host. The nod genes are located on the Sym(biosis) plasmid of the fast-growing Rhizobium species, and are chromosomally located in the slow-growing Bradyrhizobium species. The nod genes are generally under the transcriptional control of the nodD gene product, and most contribute to the production of the signal molecules (Nod factors) responsible for root hair curling and induction of cortical cell division. The inducible nod genes are not expressed in the rhizosphere (or are expressed at a very low level), and their expression again ceases once bacteria are released from the infection thread (Sharma and Singer 1990; Schlaman et al. 1991).

The first Nod factor identified was that of R. meliloti, designated Nod Rm 1 (Lerouge et al. 1990). This phytohormone-like substance is a chito-lipo-oligosaccharide (CLOS). Since the identification of this substance, related molecules produced by other rhizobia have also been identified. These molecules contain an oligosaccharide backbone of β-1,4-linked N-acteyl-D-glucosamine, varying in length from three to five sugar units (chitin oligomers). A lipid moiety, an N-acyl group, is located on the non-reducing terminal glucosamine. Fatty acid groups are of C16, C18 or C20. Variations to the basic structure include:

i) a sulphate group on the C-6 of the reducing terminal sugar in R. meliloti (Schultze et al. 1992; and Lerouge et al. 1990);
ii) an O-acetyl group on the C-6 of the non-reducing sugar in R. leguminosarum bv viciae (Spaink et al. 1991);
iii) a 2-O-methylfucose substituent on the reducing sugar end, and a C16 or C18 fatty acid on the non-reducing end in B. japonicum (Spaink et al. 1991; Stacey et al. 1993); and
iv) an N-methyl group in addition to an acyl group and carbamoyl groups on the non-reducing sugar moiety in strain *Rhizobium* sp. NGR234, and a fucose group, either O-methyl substituted or not, on the reducing sugar end (Price *et al.* 1992).

Figure 1.4 shows the structure of Nod factors produced by strain NGR234 (Price *et al.* 1992) and by *B. japonicum* (Sanjuan *et al.* 1992).

More recently identified Nod factors have shown yet other substitutions to the CLOS molecules. *Bradyrhizobium* strains isolated from *Acacia* species produce Nod factors which are sulphated, bear a methyl fucose group, and have one or two O-carbamoyl groups and one N-methyl group at the non-reducing end (Boivin *et al.* 1994). These structures are similar to some Nod factors produced by NGR234. A 4-O-acetyl fucose has also been identified as a reducing terminal end substituent in *R. loti* (Lopez Lara *et al.* 1994). In *R. leguminosarum* host specificity apparently lies in the nature of the highly unsaturated fatty acyl moiety which appears to play a role in the delivery of the CLOS molecule into plant tissue (Spaink *et al.* 1994).

Nod factors have been found to induce the differentiation of epidermal cells into root hair cells, the formation of pre-infection threads and the formation of nodule primordia (Lerouge *et al.* 1990; Spaink *et al.* 1991; reviewed in Dénarié and Cullimore 1993). Nod factors of *R. meliloti* have also been shown to rapidly depolarise alfalfa root hairs (Ehrhardt *et al.* 1992) and to induce re-organisation of the actin cytoskeleton (Allen *et al.* 1994).

Current research is focusing on the exact effects of the Nod factors on host plants, including the genes and/or pathways activated. In particular, chalcone synthase (Lawson *et al.* 1994), early nodulin (Kondorosi *et al.* 1994) and *cdc2* (Verma 1994) genes are receiving attention. Although CLOS are clearly excreted into the rhizosphere (at low concentrations since activity is seen at $10^{-9}$ M), it has recently been demonstrated that CLOS of *R. l. bv trifolii* strain ANU843 accumulate in bacterial cell membranes (McKay and Djordjevic 1993; Orgambide *et al.* 1994). As yet no role within rhizobia for CLOS molecules (membrane bound or otherwise) has been demonstrated.

### 1.10 The roles of *nod* genes in Nod factor production

Research into the capacity of particular *Rhizobium* species to nodulate specific plant hosts has shown that host specificity genes (*hsn*) are a key factor (reviewed in Djordjevic and Weinman 1991). These genes are *nod* genes whose functions have more recently been identified in producing modifications to the chito-lipo-oligosaccharide, Nod factor molecules. Such modifications result in the variety of substitution patterns identified for Nod factors and detailed above. The roles (or proposed roles) of *nod* genes in the production of Nod factors are detailed in Table 1.1.
1.11 NodD and the induction of nod gene transcription

The nod genes are regulated by the positive transcriptional activator NodD which has been classified as a member of the LysR family of transcriptional regulators on the basis of DNA and protein homology (Henikoff et al. 1988). Most members of this family are transcriptional activators, although some are repressors. The key properties of these proteins are listed below (Reviewed in Schlamann et al. 1992a and Schell 1993).

(i) The proteins require an inducing compound for activation.

(ii) They have a helix-turn-helix DNA-binding motif in the amino terminus region, the region of highest sequence conservation.

(iii) They lack sequence homology in the C-terminal region.

(iv) They are often subject to negative autoregulation.

(v) In vitro, their binding characteristics to their target DNA are usually not altered by the presence or absence of inducers.

(vi) Mutants which activate transcription in the absence of inducing compounds have been described for several members.

Consistent with the function of NodD as a positive gene regulator, this protein has been shown to bind to the nod-box promoter regions identified adjacent to most nod gene operons (Kondorosi et al. 1989; Fisher and Long 1989; Long et al. 1991; Fisher and Long 1992). Not surprisingly, nod-box sequences are highly conserved in rhizobia (Rostas et al. 1986; Schofield and Watson 1986). The nod-box may contribute to nod gene regulation and also to nodD autoregulation (Burn et al. 1988; Kondorosi et al. 1989).

The specific mechanism of action of NodD has not yet been fully elucidated although a detailed model was proposed in 1989 (Spaink et al. 1989). This model proposed that NodD, being an amphitropic protein (Burn et al. 1988), spans the cytoplasmic membrane, and a domain sited in the periplasmic space interacts with incoming (activator) molecules. This domain was postulated to correspond largely to the carboxy-terminal region of the nodD product (Horvath et al. 1987), although certain areas of the amino-terminal region were also postulated to be involved in flavonoid recognition (Spaink et al. 1989). The model further proposed that a cytoplasmic domain, corresponding largely to the amino-terminal region of NodD, is bound constitutively to the nod box, although not in a conformation to enable transcription to proceed. When the membrane sited domain interacts with an activating compound, a conformational change occurs in the cytoplasmic domain and transcription of the nod genes can occur.

This model was based on previous data which had shown the amino-terminal region of the nodD product to be highly conserved between different rhizobia (Horvath et al. 1987). The amino-terminal region was also shown to be interacting with DNA at the
nod-box (Fisher and Long 1989). The carboxy-terminal region shows greater divergence between nodD products from different rhizobia (Horvath et al. 1987), and was postulated to be the membrane sited region, interacting with phenolic signal molecules, thus providing ample latitude for variation between species. A particular difficulty not addressed by this model was the implications for DNA replication by the nodD product remaining continually bound to nod-box regions.

A more refined model of NodD action has resulted from recently published research. The nodD product has been demonstrated unequivocally to bind to the nod-box, which precedes most nod gene operons (Fisher and Long 1993). In addition, work on the LysR family of transcriptional regulators has more clearly identified NodD functional domains (Reviewed in Schell 1993). A DNA binding domain has been identified at the amino terminus of the protein from residues 1-65, two domains involved in co-inducer recognition have been identified, situated at residues 100-173 and 196-206, and a domain required for both DNA binding and co-inducer response has been identified towards the C-terminus of the protein, between residues 227-253 (Schell 1993).

NodD both responds to inducing compounds and binds to DNA at the nod-box. Flavonoids are thought to interact directly with NodD although there has been no evidence of direct interaction to date (see reviews Fisher and Long 1992; Schlaman et al. 1992a). NodD has also been shown to move between the cytoplasmic membrane and a soluble, cytoplasmic form, and is thought to be bound to nod-boxes only briefly, and in a dynamic fashion (Schlaman 1992), which addresses the problem of DNA replication in the earlier model, referred to above.

Figure 1.5 presents a model incorporating recent research in relation to the proposed mechanism of action of NodD.

1.12 NodD and host range

A number of rhizobia carry more than one copy of the nodD gene, whereas R. leguminosarum bv. viciae and R. tropici carry only one nodD gene each. In R. meliloti, the three nodD genes identified have been shown to be activated by different suites of flavonoid compounds (Honma and Ausubel 1987; Fisher and Long 1989; Hartwig et al. 1990). Further, different combinations of these genes are necessary for optimal nodulation of different host plants (Györgypal et al. 1988; Honma et al. 1990). In some cases, an identified nodD gene has no recognisable role in nodulation (Appelbaum et al. 1988; Bassam et al. 1988; Göttfert et al. 1992). It has been suggested that multiple nodDs may have evolved to optimise the specific interaction with the flavonoid inducer mix exuded by the root system of different hosts plants.

Recently, a flavonoid-dependent nod gene, nolI, has been identified in R. fredii. No nod-box sequence has been found, yet a functional nodD1 gene is required for
flavonoid-mediated transcriptional induction of this gene. This data implies that NodD is able to effect the transcriptional activation of this gene by a mechanism different to that involved for most of the nod genes, probably via a different promoter (Boundy-Mills et al. 1994). The function of noU has not yet been determined.

1.13 NodD Regulation

The regulation of nodD genes differs between species. In many rhizobia nodD is constitutively expressed, or autogenously regulates its own expression (Rossen et al. 1985; Spaink et al. 1987b; Bassam et al. 1988). However, in B. japonicum, nodD transcription can be enhanced by the same inducer compounds co-activating the nodYABC operon in conjunction with NodD (Banfalvi et al. 1988). In R. meliloti the regulation pattern is more complex, involving each of the three nodD genes and the syrM gene (Mulligan and Long 1989; Long et al. 1991).

A model for NodD/nod-box binding has recently been proposed by Fisher and Long (1993) where NodD binds as two monomers to the two NodD binding sites on the nod-box, and a bend induced in the DNA by the complex is thought to play a role in the interaction with RNA polymerase. Activation of the NodD protein could therefore be related to the degree of bend mediated upon the DNA by interaction with the flavonoid co-inducer (Fisher and Long 1993). This proposed mechanism of action is incorporated into the model illustrated in Figure 1.5. Such binding has also been postulated for other members of the LysR family and their target DNA sequences (Schell 1993).

In addition to autoregulating in some strains, NodD is subject to regulation by various other factors. A repressor of nodD, NolR, has been identified in 80% of R. meliloti strains. NolR has been shown to be involved in the regulation of nodD transcription, and binds to the promoters of nodD1 and nodD2 (Kondorosi et al. 1989, Kondorosi 1991). At least one activator has been identified amongst other proteins shown to bind to some nod-box sequences (Schlaman et al. 1992b).

Ammonia has been demonstrated to regulate some nod genes, including nodD in some species. Ammonia regulation of the common nod genes (nodABC) of R. meliloti has been shown to be mediated by nitrogen regulatory genes ntrC and ntrA (Dusha et al. 1989), and then transmitted to the syrM-nodD3 genes. At low ammonia concentration the activator protein NtrC exerts its effect via nodD3. Where nitrogen excess is present, ntrR, involved in the repression of nod genes, may function in co-ordination with the syrM gene. It is also thought that the NodD3 protein may relay the nitrogen status signal to the transcriptional control of the nodABC genes (Dusha et al. 1989). In B. japonicum the nodD1 gene mediates ammonia repression of both the nodD1 and nodYABC operons, while the nitrogen regulatory protein ntrC is apparently not involved (Wang and Stacey 1990). In contrast, the nodD and nodABC operons of R. l. bv. viciae are not regulated by combined nitrogen (Baev et al. 1992).
NodD is also involved in the regulation of transcription of other genes. Rhi is one of the most abundant proteins in *R. l. bv. viciae*, and is produced in free-living bacteria in the rhizosphere, but scarcely at all in the bacteroid. NodD is involved in the repression of transcription of *rhiA*, either directly by interaction with the *rhiA* promoter or indirectly via the *rhiR* promoter (Economou *et al.* 1989). A nodulation gene whose function has not yet been determined, *nolR*, is apparently both regulated by *nodD* and involved in the regulation of Rhi production (Economou *et al.* 1989).

Other *nod* genes also appear to contribute to the regulation of nodulation of specific hosts. Göttfert *et al.* (1990) identified the *nodV* and *nodW* genes of *B. japonicum*, proposed members of a two component regulatory system (like that of *A. tumefaciens virA* and *virG*). NodV is the predicted sensor, and NodW the predicted regulator. The *nodV* and *nodW* gene products influence host specific nodulation since they are necessary for the nodulation of hosts other than soybean. NodW has recently been shown to be involved in the expression of common *nod* genes in response to flavonoid inducers in *B. japonicum* in combination with NodD (Sanjuan *et al.* 1994).

### 1.14 NodD and the nodulation of the non-legume *Parasponia*

One of the most interesting and possibly important observations of *Rhizobium* research is that certain strains of *Rhizobium* and *Bradyrhizobium* are able to nodulate the non-legume *Parasponia* (Trinick 1979). All of the research described above regarding NodD was carried out in relation to rhizobia and legume hosts. Far less research has been completed on the role of NodD in the nodulation of the non-legume *Parasponia*.

Strain NGR234 (a fast growing *Rhizobium* strain) has the widest host range of any *Rhizobium*, nodulating over 75 genera of legumes (Relic *et al.* 1994), but does not fix nitrogen in *Parasponia* nodules. Two *nodD* genes have been identified in strain NGR234 (Rodriguez-Quinones *et al.* 1987), but only one (*nodD1*) is directly involved in nodulation (Bassam *et al.* 1988). Bassam *et al.* (1988) also found the NGR234 *nodD1* gene to be constitutively expressed at a high level. A mutation of the *nodD1* gene eliminates nodulation of all hosts, and the *nodD2* gene is unable to complement for the loss of *nodD1* on any host examined (Bassam *et al.* 1986; 1988). The function of the second *nodD* gene remains unknown.

The *nodD1* gene of strain NGR234, when examined for flavonoid activation leading to the induction of *nod* gene transcription, was activated by a wider array of flavonoid compounds than the narrow host range rhizobia, including some flavonoids which have been identified as anti-inducers in other rhizobia (Bassam *et al.* 1988). Bender *et al.* (1988) demonstrated that the introduction of the NGR234 *nodD1* gene allows induction of transcription of ANU843 *nod* genes by a wider range of flavonoids than does the native *nodD* gene of that species. Compounds activating *nod* gene induction included
coumestans and isoflavones, and at least one compound identified as an anti-inducer in strain ANU843, umbelliferone (Redmond et al. 1986).

The nodD1 gene of strain NGR234 shows a high level of molecular conservation with the nodD genes of other rhizobia, but is apparently less specific in host recognition than the nodDs from more widely characterised, narrow host range rhizobia (Horvath et al. 1987; Bender et al. 1988; Bassam et al. 1988). By transferring the nodD1 gene of strain NGR234 to R. meliloti and R. l. bv trifolii the host range of both of these species could be extended to legumes not nodulated by the recipient strains (Horvath et al. 1987; Bassam et al. 1988). The transfer of the same gene to R. l. bv trifolii strain ANU843 extended the host range to include the non-legume Parasponia (Bender et al. 1988). This extension of host range required the introduction of only the nodD1 gene from NGR234 into strain ANU843. The nodD1 gene is therefore an absolute requirement for Parasponia nodulation by strain NGR234 (Bender et al. 1988).

The common nod genes nodABC (and by extension Nod factors) are essential for Parasponia nodulation (Scott et al. 1987; Marvel et al. 1987). However, Nod factors are apparently not involved in the induction of cortical cell division in Parasponia infection, since nodA mutants of strain CP283 are still able to induce cortical cell division (Bender et al. 1987b). In contrast, the nodABC genes are required for pre-nodule development (Bender et al. 1987b). This situation implies a second signal molecule is involved in the induction of cortical cell division in Parasponia, and is illustrated in the model in Figure 1.2.

A mutant of strain ANU843, with two point mutations to the nodD gene resulting in an altered amino acid residue in the central and C-terminal regions of the nodD protein (mutant C58), was able to extend the host range of strain ANU843 to Parasponia (McIver et al. 1989). Both of these mutated regions of the nodD gene are implicated in flavonoid recognition (Schell 1993), and this nodD mutant strain also showed an altered flavonoid response (McIver et al. 1989). Other single site mutants of ANU843 nodD were also found to initiate small nodules on Parasponia. When these other ANU843 nodD mutants were introduced into strain NGR234 (nodD::Tn5) they were able to complement the NGR234 nodD mutation on Parasponia efficiently, where mutant C58 could not (McIver et al. 1989). Based on subsequent research it is likely that mutant C58, in an ANU843 background, induces the production of Nod factors with appropriate decorations to initiate nodulation in Parasponia. However, this mutant is unable to induce the necessary operons to produce the appropriate signals required for Parasponia nodulation in the strain NGR234 background (B. Rolfe, pers. comm.).

Bradyrhizobium strain CP279 (a slow growing rhizobia) nodulates Parasponia to form nitrogen fixing nodules with high efficiency, (Trinick 1980a) and nodulates only a few other tropical legumes with varying efficiency. Strain CP279 is therefore considered a Parasponia-specific strain. Hybridisation analysis using the NGR234 nodD1 gene as a
probe identified two putative $nodD$ genes in strain CP279 (Bender 1987). Thus, whilst both strains NGR234 and CP279 nodulate Parasponia, strain NGR234 has a single functional $nodD$ gene and a broad host range, whilst strain CP279 has a narrow host range and two putative $nodD$ genes which have not been further studied.

1.15 Project aims

The increase in our general understanding of the basis for the nodulation of the non-legume Parasponia may be critical in any future success in reliably extending the host range of rhizobia to non-legumes of agricultural importance. The $nodD$ gene is an essential determinant of nodulation success on a particular host plant in both legumes (Spaink et al. 1987b) and the non-legume Parasponia (Bender et al. 1988). The central aim of this study was to further delineate the role of $nodD$ genes in the nodulation of Parasponia.

Since Rhizobium sp. NGR234 has the widest host range known, yet only one functional $nodD$ gene (Bassam et al. 1988), I sought to determine if the broad host range of this strain was reflected in the suite of phenolic compounds (both flavonoid and non-flavonoid) able to activate NGR234 NodD1 to induce $nod$ gene transcription. The results of this research are presented in Chapter 3.

Based on the hypothesis that the interaction of NGR234 NodD1 with activator compounds was less specific than for narrow host range rhizobia (Horvath et al. 1987), I further sought to determine if extracts of non-host plants would co-induce $nod$ gene transcription in conjunction with NGR234 $nodD1$, and to isolate activating compounds from non-host plants. This work is presented in Chapter 4.

To correlate the nodulation of Parasponia with the activation of NodD, I further sought to examine the putative $nodD$ genes of the Parasponia-nodulating, narrow host range Bradyrhizobium strain CP279, to establish the functionality of these genes, their role in Parasponia nodulation and the range of compounds able to induce $nod$ gene transcription. The results of this research are presented in Chapter 5.

Selected results of the research presented in this thesis have been published in Bender et al. 1988 (part of Chapter 4), and Le Strange et al. 1990 (Chapter 3 and much of the remainder of Chapter 4).
This page is intended to be left blank.
Table 1.1 The roles of Nod proteins in Nod factor production.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Functions of gene products</th>
</tr>
</thead>
<tbody>
<tr>
<td>NodABC</td>
<td>Synthesises the backbone of a phytohormone-like substance, the Nod factor. N-acetylglucosamine residues linked β-1-4 and a fatty acid group (C16, C18 or C20) with varying degrees of unsaturation N-linked at the non-reducing end of the chitin oligomer.</td>
</tr>
<tr>
<td>NodC</td>
<td>Chitin synthase, which links N-acetyl glucosamine in β-1-4 linkages into chitin.</td>
</tr>
<tr>
<td>NodB</td>
<td>Chitin deacetylase, removing the acetyl group from the non-reducing end of the chitin oligomer.</td>
</tr>
<tr>
<td>NodA</td>
<td>Catalyses the addition of the acyl (fatty acid) group to the deacylated chitin oligomer.</td>
</tr>
<tr>
<td>NodD1, D2, D3</td>
<td>Transcriptional regulator required for the expression of the other nod genes.</td>
</tr>
<tr>
<td>NodE</td>
<td>A fab condensing enzyme, is thought to affect the degree of fatty acid saturation by interfering with normal fatty acid biosynthesis.</td>
</tr>
<tr>
<td>NodF</td>
<td>Acyl carrier protein. Binds an acyl group and delivers it to the extending chitin oligomer chain.</td>
</tr>
<tr>
<td>NodG</td>
<td>Host range, reported sequence similar to dehydrogenases.</td>
</tr>
<tr>
<td>NodH</td>
<td>Sulphotransferase. Adds a sulphate group to the R. meliloti CLOS in conjunction with Nod P and Q to the C6 of the reducing end N-acetylglucosamine.</td>
</tr>
<tr>
<td>NodI, J, T</td>
<td>Apparently form a pore, thought to export CLOS.</td>
</tr>
<tr>
<td>NodK</td>
<td>Reported in B. sp (Parasponia). Function unknown.</td>
</tr>
<tr>
<td>NodL</td>
<td>Acetyltransferase- adds acetyl group to C6 at non reducing end of the CLOS.</td>
</tr>
<tr>
<td>NodM</td>
<td>Glucosamine synthase, providing substrates for NodC.</td>
</tr>
<tr>
<td>NodN</td>
<td>Function unknown.</td>
</tr>
<tr>
<td>NodO</td>
<td>Involved in the formation of transmembrane ion channels in plant plasma membrane.</td>
</tr>
<tr>
<td>NodP</td>
<td>ATP sulphurylase. See NodH.</td>
</tr>
<tr>
<td>NodQ</td>
<td>APS kinase. See NodH.</td>
</tr>
<tr>
<td>NodR</td>
<td>Reported only in R. leguminosarum bv. trifolii.</td>
</tr>
<tr>
<td>NodS</td>
<td>Function unknown. MW 23 kDa.</td>
</tr>
<tr>
<td>NodT</td>
<td>Functions with Nod IJ.</td>
</tr>
<tr>
<td>NodU</td>
<td>Function unknown. MW 62 kDa.</td>
</tr>
<tr>
<td>NodX</td>
<td>Acetyl transferase, adds an acetyl group to the C6 of the reducing end sugar.</td>
</tr>
<tr>
<td>NodY</td>
<td>Reported only in B. japonicum, but found in all rhizobia by hybridisation. Function unknown.</td>
</tr>
<tr>
<td>NodZ</td>
<td>Adds 2-O-methyl fucose to CLOS of B. japonicum. Not regulated by NodD.</td>
</tr>
<tr>
<td>Nod1A</td>
<td>Host range. Essential for nodulation of selected soybean cultivars. Reported only in B. japonicum. Shows sequence similarity to MerR, a transcriptional regulatory protein.</td>
</tr>
</tbody>
</table>

(noID, E, F, G, Host range in R. meliloti.)

Figure 1.1

Signal exchange between rhizobia and legumes during nodulation.

Note the flavonoid inducer released by the plant root, and the Nod factor generated by the bacterium.
RHIZOSPHERE

Cortical Cell Division

SOIL

developing root hairs

Phenolic plant signals

Root cap

LEGUME ROOT

Cortical Cell Division

soil rhizobia

nodulation genes are activated by plant signals and Nod factors are generated

pSym

Model *Rhizobium* Cell

Flavone Inducer

Model *Rhizobium* Cell

Flavone Inducer
Figure 1.2

Model of the infection of *Parasponia andersonii* roots, and some of the *Rhizobium* genes known to be involved.

IT - infection thread; En - endoderm; P - pericycle
Known *Rhizobium* genes required

nod D hsn

Erosion of epidermal cells

First cell division signal

Subepidermal cells induced to divide

nod D nod ABC

Prenodule

structure formed of dividing cells

Second cell division signal required

It
Figure 1.3

The phenyl-propanoid pathway, focusing on the simplified chalcone synthase branch.

PAL - phenylalanine ammonia-lyase; C4H - cinnamate 4-hydroxylase; 4CL - 4-coumaroyl co-enzyme; CHS - chalcone synthase; CHI - chalcone flavone isomerase; F3H - flavanone 3β-hydroxylase; DFR - dihydroflavonol 4-reductase; IFS - isoflavonoid synthase; FLS - flavonol synthase.


Below: the ring structure and numbering convention for flavonoids.
Figure 1.4

Structures of Nod factors produced by strains NGR234 and *Bradyrhizobium japonicum*.

**Rhizobium sp. NGR234**

- $R_1 = \text{C18:1(\Delta)}$ or C16:0
- $R_2 = \text{CH}_3$
- $R_3 = \text{carbamoyl or H}$
- $R_4 = \text{carbamoyl or H}$
- $R_5 = \text{acetate or H}$
- $R_6 = \text{sulphate or H}$

**Bradyrhizobium japonicum**

- $R_1 = \text{C18:1 (\Delta9)}$
- $R_2 = \text{H}$
- $R_3 = \text{H}$
- $R_4 = \text{H}$
- $R_5 = \text{H}$
- $R_6 = \text{H}$
Figure 1.5
Model for the mechanism of activation of NodD by flavonoid compounds and the initiation of transcription of nod genes.

a) NodD in soluble form in the cytosol, bound to nod-box DNA.

b) NodD integrated into the cytoplasmic membrane, bound to nod-box DNA. NodD is in the inactive form, preventing transcription of nod genes.

c) NodD, activated by flavonoid compounds, induces a bend in the nod-box DNA, allowing transcription of nod genes.

Note: there is no direct evidence that flavonoid molecules interact directly with NodD, however, no intermediate signal been identified.

Chapter Two
Materials And Methods

2.1 Bacterial culture

2.1.1 Strains and plasmids used

All bacterial strains, their characteristics and the sources from which they were obtained are listed in Table 2.1. Plasmids are similarly listed in Table 2.2. Plasmids were stored both in recombination deficient Escherichia coli strains and as purified DNA suspended in TE buffer at 4°C.

2.1.2 Media

All media were prepared in distilled water and autoclaved at 138 kPa pressure (121°C) for 20 min. Solid media were dispensed into sterile Petri dishes. Liquid media were dispensed in 5 ml or 10 ml quantities into capped test tubes or McCartney bottles, as appropriate, before autoclaving. The pH of all media was 6.8-7.0 and solid media contained 2% agar.

GMM minimal medium (Bender and Rolfe 1985) was routinely used for culture of Rhizobium strains and contained: K$_2$HPO$_4$, 348 mg/L; KH$_2$PO$_4$, 272 mg/L; MgSO$_4$.7H$_2$O, 40 mg/L; NaCl, 20 mg/L; CaCl$_2$.2H$_2$O, FeCl$_3$.6H$_2$O, 5 mg/L; Gamborgs trace elements, 1 ml/L; D-mannitol, 5 g/L; monosodium glutamate, 0.5 g/L. Gamborgs trace elements (Gamborg and Eveleigh 1968) contained: MnSO$_4$.4H$_2$O, 10 g/L; H$_3$BO$_3$, 3 g/L; ZnSO$_4$.7H$_2$O, 3 g/L; Na$_2$MoO$_4$.H$_2$O, 250 mg/L; CuSO$_4$.5H$_2$O, 250 mg/L; CoCl$_2$.6H$_2$O, 250 mg/L.

GMY complete medium was identical to GMM with the addition of 0.5 g/L Bacto-yeast extract.

Slow G, an adaptation of GMM, was used for the culture of Bradyrhizobium strains. D-mannitol and monosodium glutamate were replaced with sodium gluconate, 5 g/L and (NH$_4$)$_2$SO$_4$, 5 g/L.

BMM - Bergersen's modified medium (Bergersen 1961): was used as a general purpose Rhizobium growth medium and for storage and contained: Na$_2$HPO$_4$.12 H$_2$O, 0.36 g/L; MgSO$_4$.7H$_2$O, 0.08 g/L; FeCl$_3$, 0.003 g/L; CaCl$_2$.2H$_2$O, 0.04 g/L; Gamborgs trace elements 1.0 ml/L (see GMM for composition); Thiamine-HCl, 2 mg/L; Biotin 0.2 mg/L; Monosodium glutamate, 0.5 g/L; Mannitol, 3.0 g/L; Yeast extract 0.5 g/L.
L - Luria medium (Miller 1972) was used for the routine culture of E. coli strains and contained Bacto-tryptone, 10 g/L; Yeast extract, 5 g/L; NaCl, 5 g/L.

Tryptone medium -TY (Beringer 1974) is a salt-free medium and was used for culture and patch mating of E. coli and rhizobia or bradyrhizobia, and contained: Bacto-tryptone, 5 g/L; Yeast extract, 3 g/L; CaCl₂·2H₂O, 1 g/L.

E. coli minimal medium - M9 (Miller 1972) was used for culture of E. coli strain NM522 to maintain F plasmid, and contained: M9 salts, 10 g/L; MgSO₄, 0.24 g/L; CaCl₂, 0.011 g/L; Thiamine, 1 mg/L.

Nutrient agar - NA was used for the long term storage of E. coli strains and contained: 4.25% Oxoid Blood Agar Base; 0.5% Oxoid Yeast Extract.

2.1.3 Media supplements

The concentrations of antibiotics used for different bacterial species in solid media are listed in Table 2.3. All antibiotics were freshly prepared as concentrated solutions, filter sterilised, then added to autoclaved media which had been pre-cooled to 65°C. Sm, Sp, Km, Tc and Cb are water soluble. All antibiotics were obtained from Sigma Chemical Co. and stored at 4°C in solid form.

2.1.4 Preparation of cultures

Rhizobium and Bradyrhizobium strains were streaked for single colonies on GMM, slow GMM or TY agar plates (as appropriate), with or without antibiotics, and incubated at 28°C for 3 and 8 days, respectively. E. coli strains were streaked for single colonies on LB, TY or M9 agar plates (as appropriate), with or without antibiotics, and incubated overnight at 37°C. For liquid culture a loopful of cells was taken from an agar plate and suspended in 50 ml of medium in a sterile 250 ml conical flask. Cultures were shaken at 200 rpm at either 28°C or 37°C. Smaller volumes of exponential phase cultures of E. coli were obtained by diluting 1 ml of a fresh overnight L broth culture into 10 ml of the same medium which was then incubated with shaking for 2-3 h; under these conditions exponential phase cultures had cell densities of approximately $5 \times 10^8$ bacterial cells per ml. A Haeruaus Christ centrifuge, which developed a force of 1000 g, was used to sediment bacterial cells. Bacterial cells were resuspended in solution, after sedimentation, by vigorous shaking on a bench vortex.

2.1.5 Storage of bacterial strains

E. coli strains were maintained on L-agar, with antibiotics where necessary (see Table 2.3). These bacteria were grown overnight at 37°C. E. coli strains were stored on NA medium, or kept for several years at -20°C as a suspension in 33% glycerol and 67% L medium.
Materials and Methods

*Rhizobium* strains were maintained on BMM or GMM medium containing the appropriate level of antibiotics where necessary (see Table 2.3). These bacteria were grown at 28°C on solid medium for 3 days or in liquid medium for 16 h. *Rhizobium* strains were stored for periods of 12 to 24 months at room temperature, in stab cultures in vials containing BMM medium, and for longer periods as a thick suspension of stationary phase cells in a solution containing 12% glycerol and 10% sucrose. The suspension was incubated at 28°C overnight before being stored at -20°C.

*Bradyrhizobium* strains were maintained on Slow GMM. *Bradyrhizobium* strains were stored for periods of 12 to 24 months at room temperature, in stab cultures in vials containing BMM medium, and for longer periods as a thick suspension of stationary phase cells in a solution containing 12% glycerol and 10% sucrose. The suspension was incubated at 28°C overnight before being stored at -20°C.

2.1.6 **β-Galactosidase assays**


Induction of β-galactosidase activity:
Aliquots of test compound (compounds as 10 mM or 0.1 mM stock in 100% methanol), or plant preparations were dried under vacuum at room temperature. Sterile distilled water (1.2 ml) was added and the solution vortexed vigorously. To this solution was added 0.4 ml of bacterial cells from an early log phase liquid culture (OD 600 of 0.20 to 0.25). Following light mixing, cultures were incubated without shaking at 28°C in darkness for a standard induction period of 2 h.

Processing of the induced cell culture:
To 0.4 ml of induced culture was added 0.4 ml of Z buffer, 25 µl of 1% SDS and 10 µl of chloroform (the absorbance of the remaining 1.2 ml of induced culture was read at 600 nm (OD 600) using a Pye Unicam spectrophotometer, then discarded). After being thoroughly mixed, 150 µl of o-nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml in 0.1M phosphate buffer, obtained from Sigma Chemical company, St. Louis, MI) was added. The solution was mixed then placed at 28°C in darkness for colour development. Depending on the assay strain colour development generally took from 5 min to 2 h. The reaction was stopped by the addition of 0.4 ml of 1M sodium carbonate after an appropriate period of colour development (t). The solutions were spun in an Eppendorf centrifuge for 5 min then the absorbance read at 420 nm (OD 420).

Calculation of β-galactosidase activity:
β-galactosidase activity was calculated as in Miller (1972), using the formula:

\[
\text{units } \beta\text{-galactosidase} = \frac{1000 \times \text{OD}_{420}}{t \times v \times \text{OD}_{600}}
\]

where t is the time in minutes for development of colour; and v is the volume of cell culture used (for all assays reported in this thesis v = 0.4 ml).
Z buffer contained (per litre): Na$_2$HPO$_4$.7H$_2$O, 16.1 g; NaH$_2$PO$_4$.H$_2$O, 5.5 g; KCl, 0.75 g; MgSO$_4$.7H$_2$O, 0.246 g; 2.7 ml of a 50 mM solution of β-mercaptoethanol; adjusted to pH 7.0.

0.1 M Phosphate buffer, pH 7.0, contained per 100 ml: 39 ml 0.1 M KH$_2$PO$_4$, and 61 ml 0.1 M K$_2$HPO$_4$.

2.1.6.2 β-Galactosidase assay modified for *Bradyrhizobium* strains

Induction of β-galactosidase activity:
Cultures were grown in BMM to an OD$_{600}$ of 0.1 to 0.2. The inducing compound was added to an empty tube (compounds as 10 mM or 0.1 mM stock in 100% methanol), and dried under vacuum. 2 ml of culture was added, and the tubes incubated for induction periods of 8-16 h with shaking at 28°C.

Processing of the induced cell culture:
An 0.4 ml aliquot of induced culture was removed for processing as described in Section 2.1.6.1.

Calculation of β-galactosidase activity:
As in Section 2.1.6.1.

2.1.6.3 X-gal agar plates (Miller 1972)

Agar plates containing X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) and IPTG (isopropyl β-D-thiogalactopyranoside) were poured after the addition of filter sterilised stock solutions to sterilized L agar (Section 2.1.2). X-gal was added to a final concentration of 40 µg/ml and IPTG to 0.2 mM.

X-gal stock solution: 100 mg/ml in dimethyl formamide.

IPTG stock solution (0.1M): 23.8 mg/ml in sterile distilled water.

2.2 Chemistry

2.2.1 Chemical materials

Phenolic compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co (St. Louis, MI). Methanolic solutions were stored at -18°C. Matrex Silica C18 (90-130 µm) was from Amicon Corporation (Danvers, MA) and silicic acid (Keiselgel 60, 70-230 mesh) from E. Merck (Darmstadt, Federal Republic of Germany). Thin layer chromatography was performed using DC Alufolien Keiselgel 60 F254, obtained from E. Merck (Darmstadt, Federal Republic of Germany). Chromatography paper (Whatman No.1 Chromatography paper) and filter paper
(Whatman No. 4 filter paper) were obtained from Whatman International Ltd. (Maidstone, UK).

2.2.2 High performance liquid chromatography

Reverse phase high performance liquid chromatography (HPLC) was conducted using an RP-8 column (MPL analytical cartridge, 100 x 4.6 mm, 5 µm) obtained from Brownlee Labs (Santa Clara, CA) eluted with a gradient generated from solvents A (0.2% trifluoroacetic acid in MilliQ water) and B (methanol), and monitored by a Waters 490 Multiwavelength detector; data were acquired with a Shimadzu Chromatopak CR-4. Peaks were characterised by retention time and ultra-violet (UV) profiles in the stop-scan mode.

2.3 Plant culture

2.3.1 Plant species used

*Parasponia andersonii* was the *Parasponia* species used for this work and is referred to throughout the text as *Parasponia*. The tropical legume *Macroptilium atropurpureum* (siratro) was used as a representative legume host. Clover species used were *Trifolium repens* (white clover) and *Trifolium subterraneum* (subterranean clover) and were obtained from Cleanseeds, Bungendore, Australia.

*Trema aspera* seed was collected from trees growing on the central coast of eastern Australia in the Mullumbimby district. *Casuarina cunninghamiana* seed was obtained from the Forestry division of the CSIRO in Canberra. Seed for sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) were obtained from Dr Peter Dart.

Australian Standard White (ASW) was used as the principal test wheat for identification of inducing compounds. All Australian wheat varieties were obtained as a gift from the CSIRO Bread Research Institute, North Ryde, Australia.

2.3.2 *Parasponia seed germination* (Bender and Rolfe 1985)

Seeds were removed from fruits by gentle rubbing between fine wire gauze and a rubber stopper. Seeds were then shaken vigorously for 10 min in 96% ethanol to remove traces of the oily fruit. The ethanol was decanted off and replaced with fresh 12.5% sodium hypochlorite (full commercial strength). Seeds were shaken vigorously for a further 30 min then washed with sterile distilled water before being arranged on H agar plates (Section 2.3.9), approximately 40 seeds per plate. Each seed was individually secured using a drop of 2% distilled water agar. The plate was sealed with Nescofilm into which was punched a small hole for gas exchange. Plates were placed vertically and
spatially separated in a growth cabinet with a 12 h diurnal temperature variation of from 35°C (day) to 25°C (night). Using this procedure germination began after 6 days and continued for a further 10 days.

*Parasponia* seedlings showed persistent fungal contamination during germination prior to plant assays. The contaminants were thought to be underneath the seed coat and were found to affect all seed stocks available, grown in the previous 2 seasons. In an attempt to reduce this contamination an overnight wash of Cefotaxime was included after the normal germination procedure and prior to placing of seeds on plates. Cefotaxime (obtained from Roussel Uclaf, Australia) was used at a concentration of 33 mg per 100 ml sterile distilled H₂O. This treatment reduced germination rate slightly, but was effective at significantly reducing fungal contamination.

### 2.3.3 Germination of other non-legumes

Seeds of *Trema aspera* were treated as for *Parasponia*. Seeds of *Casuarina cunninghamiana*, sunflower, wheat, rice and maize were gently shaken in a 4% solution of sodium hypochlorite, with a few drops of 96% ethanol, for 5 min. Seeds were thoroughly washed with sterile distilled water (6-10 washes) then arranged on the surface of H agar plates (Section 2.3.9). A layer of 2% distilled water agar was used to secure seeds after which plates were incubated vertically in darkness at 28°C for 2 to 5 days.

### 2.3.4 Siratro seed germination (Cen et al. 1982)

Seeds were shaken in concentrated sulphuric acid for 5 min then rinsed in tap water. Fresh 12.5% sodium hypochlorite (full commercial strength) was then added and the seeds shaken for a further 5 min or until a few seeds had lost their dark coats. Seeds were then rinsed 5 times in sterile distilled water. Seeds were arranged on F agar plates (Section 2.3.9) and individually secured with a drop of 2% distilled water agar. Plates were then sealed with Nescofilm and placed vertically in the dark in an incubator at 28°C. Seedlings were ready for use after 40 h, when the radicles had reached about 2 cm in length.

### 2.3.5 White clover seed germination

Seeds were washed in distilled water, then soaked in 75% ethanol for 5 min. After rinsing three times with sterile distilled water, the seeds were soaked for 15 min in 5% sodium hypochlorite (40% of commercial concentration of 12.5%) with occasional shaking. The seeds were rinsed three times in sterile distilled water and placed onto a BMM plate (Section 2.1.2) with approximately 100 seeds per plate so that each seed had no contact with any other. The plate was wrapped in foil and refrigerated at least overnight. To induce germination, the plate was transferred to a 28°C incubator for 1-2 days.
2.3.6 **Subterranean clover seed germination**

Seeds were washed in 96% ethanol then rinsed 3 times with sterile distilled water. The seeds were soaked for 15 min in 6.5% sodium hypochlorite (50% of commercial concentration of 12.5%) with occasional shaking. The seeds were rinsed 3 times with sterile distilled water and soaked in sterile distilled water for 15-30 min with gentle shaking. The seeds were then transferred to a BMM plate (Section 2.1.2), with approximately 50 seeds per plate so that each seed had no contact with any other, and secured with a drop of 2% water agar. The plate was wrapped in foil and refrigerated at least overnight. To induce germination the plate was transferred to a 28°C incubator for 1-2 days.

2.3.7 **The agar plate assay** (Bender and Rolfe 1985)

The lower half of a 93 mm diameter agar plate (H medium for non-legumes, F medium for legumes, Section 2.3.9) was streaked with a loopful of fresh cells (3 days old for rhizobia, 8 days old for bradyrhizobia). 4 or 5 pre-germinated sterile seedlings were placed on the plate such that the roots of each seedling were in contact with the inoculum. The plate was then sealed with Nescofilm into which were punched 2 holes for gas exchange. For siratro and *Parasponia*, the plates were placed vertically in a growth cabinet with fluorescent tubes as a light source. Light intensity at the centre of the cabinet at a height of 10 cm was 300 µE/m²/s. The cabinet was adjusted to provide a 12 h day with a constant temperature of 28°C. Internal plate temperature rose to maximum of 31°C during the day photoperiod. For clovers the bottom half of each plate was covered with a brown paper "skirt" to minimise exposure of the roots to light. Plates were placed vertically in the controlled environment of a growth cabinet for 3 weeks. Fluorescent tubes and incandescent bulbs with a light intensity of 400 µE/m²/s at a distance of 20 cm were used as a light source. The cabinet had a 17 h day with day/night root temperatures of 22°C/19°C and humidity of 60%.

2.3.8 **The Magenta jar pot assay**

This method was originally developed by E. R. Appelbaum as a variation of the Leonard Jar Assay (Vincent 1970). It was used primarily for nodulation assays and is illustrated in Figure 2.1. Magenta jars were purchased from Magenta Corp., USA. These jars are made of clear autoclavable plastic, 76 x 76 x 103 mm (with a volume of approximately 400 ml) and are slightly tapered at the base such that one jar can be placed partially inside the other. A hole of about 7 mm diameter was drilled in one pot, and a cotton wick about 15 cm long was inserted. This pot was then placed inside another, so that half the wick was contained in each. Vermiculite, soaked in plant nutrient solution and autoclaved at 138 kPa pressure (121°C) for 20 min, was used to fill the upper pot while nutrient solution alone was added to the lower pot. A plastic cap was placed on the upper pot and the entire apparatus sterilised by autoclaving at 138 kPa pressure (121°C)
for 20 min. 5 pre-germinated sterile seedlings were planted in the vermiculite, inoculated with the appropriate bacterial strain (prepared from a mid-log phase liquid culture, centrifuged and resuspended in 1/2 volume of plant culture medium) and the cap replaced. Plants were incubated under the conditions described for the plate assay (Section 2.3.7). When seedlings had grown to reach the cap of the apparatus the cap was removed and sterile aquarium gravel was added around the base of the seedlings to cover the entire vermiculite surface. Incubation was continued without the cap and sterile nutrient solution was added to the lower pot as needed. For all parts of this procedure H (Parasponia and siratro) or one half strength F (clovers) nutrient solutions were used (Section 2.3.9).

Jars were placed in the controlled environment of a growth cabinet: clover plants were incubated typically for 4 weeks, siratro for 6 weeks, and Parasponia for 8 - 12 weeks. The cabinet conditions for clovers were: a 17 h day with day/night root temperatures of 22°C/19°C and humidity of 60%, with a light intensity of 400 µE/m²/s at a distance of 20 cm produced by both fluorescent tubes and incandescent bulbs. The cabinet conditions for Parasponia and siratro were: 12 h day with a 12 h diurnal temperature variation of from 35°C (day) to 25°C (night), with a light intensity of 300 µE/m²/s at a distance of 10 cm, produced by fluorescent tubes.

2.3.9 Plant culture media

H - Herridge's medium (Delves et al. 1986) was used for growth of siratro and Parasponia and contained: KH₂PO₄, 17.0 mg/L; K₂HPO₄, 21.8 mg/L; KCl, 18.7 mg/L; MgSO₄·7H₂O, 123.3 mg/L; CaCl₂, 27.7 mg/L; FeNaEDTA, 8.7 mg/L; H₃BO₃, 71.5 x 10⁻² mg/L; MnCl₂·4H₂O, 45.3 x 10⁻² mg/L; ZnCl₂, 2.8 x 10⁻² mg/L; CuCl₂·2H₂O, 1.3 x 10⁻² mg/L; NaMoO₄·2H₂O, 0.6 x 10⁻² mg/L. The pH was 7.0 and 2% agar was added for solid media.

F - Fähræus medium (Fähræus 1957, modified): F medium was used as a nitrogen free medium for growth of clover plants and contained: Na₂HPO₄·12H₂O, 150 mg/L; KH₂PO₄, 100 mg/L; MgSO₄·7H₂O, 120 mg/L; CaCl₂·2H₂O, 100 mg/L; Fe citrate 5 mg/L; Gibbon's trace elements, 1 ml/L. The pH was 7.0 and 2% agar was added for solid medium. Gibbon's trace elements contained H₃BO₃, 2.86 mg/L; MnSO₄·4H₂O, 2.03 mg/L; ZnSO₄·7H₂O, 220 mg/L; CuSO₄·5H₂O, 80 mg/L; H₂MoO₄·H₂O, 90 mg/L.

2.3.10 Isolation of bacteria from root nodules

Nodules were removed from the plant so as to include a short length of the attached root. This was to prevent entry of subsequent treatment solutions into the interior of the nodule which would kill the resident bacteroids. Individual nodules were placed in a drop of 96% ethanol for 5 min to remove surface tension and act as a preliminary sterilant. Each nodule was then transferred to a drop of 1% sodium hypochlorite for 15 min, then to 3 consecutive drops of sterile distilled water in order to remove the
sterilant. The intact nodule was rolled on the surface of a GMM agar plate (Section 2.1.2) which was then incubated to check for surface contaminants. The nodule was placed in a drop of protoplast dilution buffer then crushed using the blunt end of a flamed glass rod. The rod was used to streak the suspension onto a GMM agar plate. After growth was obtained bacteria were then tested for relevant antibiotic markers. Protoplast dilution buffer contained: sorbitol 45.5 g/L; mannitol, 45.5 g/L; CaCl₂.2H₂O, 294 mg/L; NaH₂PO₄.2H₂O, 312 mg/L.

2.4 Molecular biology

2.4.1 General

2.4.1.1 Preparation of materials

All tubes used, such as Eppendorf and Nunc tubes, were boiled in TE buffer for a minimum of 2 h, rinsed thoroughly in glass distilled water and sterilised by autoclaving at a pressure of 138 kPa (121°C) for 20 min. They were then dried at 37°C prior to use. Plastic tips used for micropipettes were sterilised by autoclaving then dried at 37°C prior to use. Polyallomer tubes used for ultracentrifugation were rinsed with AR grade ethanol and then dried prior to use. Dialysis tubing (Selbys Scientific Co.) was boiled in TE buffer for approximately 1 h, transferred into fresh TE buffer then stored at 4°C. Phenol was melted at 68°C, and 8-hydroxyquinolone added to a final concentration of 0.1%. Phenol was then extracted twice with an equal volume of 1.0 M Tris (pH 8), and twice with 0.1 M Tris (pH 8) containing 0.2% β-mercaptoethanol. Aliquots of prepared phenol were stored under 0.1 M Tris (pH 8) containing 0.2% β-mercaptoethanol at -20°C.

2.4.1.2 Storage of materials

DNA was stored in 2 ml screw top Nunc tubes (previously treated as described in Section 2.4.1.1) at a constant temperature of 4°C. Restriction endonucleases, T4 DNA ligase and DNA polymerase I were stored at a constant temperature of -20°C. All solutions were prepared with distilled water, and stored in plastic or glass containers. Solutions were stored at room temperature unless otherwise specified.

2.4.1.3 General buffer solutions

**TE x 10 Buffer**: Tris, 12.1 g/L; Na₂EDTA, 3.7 g/L; pH 8.0.

**TES x 10 Buffer**: Tris, 12.1 g/L; Na₂EDTA, 3.7 g/L; NaCl, 99.4 g/L; pH 8.0.

**TBE x 4 Buffer**: Tris, 43.1 g/L; Na₂EDTA, 3.7 g/L; Boric acid, 22.0 g/L.
2.4.2 Isolation of DNA

2.4.2.1 DNA isolation solutions

Alkaline lysis buffer: Tris, 3.0 g/L; Glucose, 9.0 g/L; Na₂EDTA, 3.7 g/L. The solution was stored at 4°C.

40% PEG-8000 solution: Tris, 6.06 g/L; Na₂EDTA, 7.45 g/L; Polyethylene glycol-8000, 400 g/L; pH 8.0.

2% Triton X 100 solution: Tris, 6.0 g/L; Na₂EDTA, 22.3 g/L; Triton X 100, 20 ml/L.

2.4.2.2 Small scale isolation of *E. coli* plasmid DNA

Small scale preparations (modified from Birnboim and Doly 1979) were used for rapid screening of plasmids, for transformations and for some cloning experiments. A single colony was inoculated into 5 ml of L liquid medium (Section 2.1.2) and grown for 16 h at 37°C with shaking (only 3 h incubation was necessary for Bluescript-derived clones). 1.5 ml of dense *E. coli* culture was centrifuged in an Eppendorf tube for 30 sec. The cell pellet was then thoroughly resuspended in 80 µl Alkaline lysis buffer (Section 2.4.2.1), with lysozyme added at 4 mg/ml and left at room temp for 5 min. 200 µl of freshly made 1% SDS in 0.2 M NaOH was added, the tube inverted several times to initiate lysis and the tube placed on ice for 10 min. 150 µl of potassium acetate solution (made up as 3 M CH₃COOK in 5 M CH₃COOH) was then added. Following gentle vortexing to precipitate cell debris, the tube was centrifuged for 5 min and the supernatant transferred to a clean tube. The supernatant was purified by extraction twice with phenol/chloroform/isoamyl alcohol (25:24:1, phenol prepared as in Section 2.4.1.1, then equilibrated against TE, Section 2.4.1.3), and once with chloroform. The DNA was then ethanol precipitated and finally resuspended in 30 µl TE buffer.

2.4.2.3 Large scale isolation of *E. coli* plasmid DNA

This method (modified from Clewell and Helinski, 1969) was used for obtaining several milligram quantities of ultra-pure supercoiled plasmid DNA for cloning. 2 ml of an overnight culture was spread onto 2 large (30 ml) L medium plates (Section 2.1.2). The plates were allowed to dry at room temperature and incubated at 37°C for 16 h. Cells on each plate were washed off the agar surface with 4 ml 25% sucrose in 0.05 M Tris (pH 8.0) using a glass rod that had been bent into a loop, and placed into an SS34 Sorvall tube. 4.5 ml of 0.25 M Na₂EDTA (pH 8.0) was then added, followed by 0.5 ml of freshly made 40 mg/ml lysozyme in water. The cells were swirled on ice for 5 min, and lysis was completed by addition of 4.5 ml 2% Triton X 100 solution (Section 2.4.2.1) and chilling on ice for 30 min. Lysed cells were then spun at 18000 rpm in an SS34 rotor (Sorvall RC5 centrifuge) for 60 min at 4°C. To the supernatant was added 6 ml of 5 M NaCl and 7.5 ml of 40% PEG-8000 solution (Section 2.4.2.1) and the
mixture left at 4°C overnight. The tubes were balanced by the addition of 25% sucrose in 0.05 M Tris (pH 8.0) and centrifuged for 10 min at 5000 rpm. Following centrifugation the precipitate was resuspended in 3.1 ml TES buffer (Section 2.4.1.3). The contents of 1 or 2 Sorvall tubes were combined into 1 Corex tube, with 7.00 g caesium chloride and 1.0 ml of a 10 mg/ml ethidium bromide solution. The total volume was adjusted to 7.5 ml with TES buffer (Section 2.4.1.3), and the tube gently rocked to dissolve the CsCl. After chilling the tube in the dark on ice for 30 min, it was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was transferred to a Beckman polyallomer ultracentrifuge tube and the remaining volume of the polyallomer tube was filled with liquid paraffin to a point just below the neck of the tube. The sample was balanced with another polyallomer tube (containing CsCl solution and paraffin) to within 5 mg using liquid paraffin, or when necessary 1.6 g/ml CsCl in TES, and the tubes sealed using a Beckman "Quickseal" apparatus. Each gradient was spun at 45,000 rpm for 40 to 72 h, in a Ti60 or Ti70 rotor, at 18 - 25°C before the plasmid band was extracted with a large bore needle (19G) and syringe under 302 nm UV light. 2 bands of DNA are visible; the lower band is supercoiled plasmid DNA and the upper band is largely chromosomal DNA. Ethidium bromide was extracted 3 times against an equal volume of n-butanol. The DNA solution was placed into dialysis tubing (equilibrated against TE buffer, Section 2.4.1.1) and dialysed at 4°C against a 4 litre volume of TE buffer (Section 2.4.1.1) with constant stirring. The buffer was replaced after 1 h and 3 h with fresh TE buffer. After a further 16 h dialysis the DNA solution was transferred to 1.8 ml screw-capped plastic tubes (Nunc) and stored at 4°C. A 20 µl sample was electrophoresed on an agarose gel to estimate the concentration of DNA.

2.4.2.4 Large scale isolation of total DNA from rhizobia

This method (modified from Chesney et al. 1979) produced large quantities of DNA (several mg) from both _Rhizobium_ and _Bradyrhizobium_ strains. 100 ml of freshly grown cells (in TY broth, Section 2.1.2) were centrifuged in Sorvall tubes at 8000 rpm (SS34 rotor) for 10 min, 20°C. The pelleted cells were washed in 5 ml 0.1 M Tris.HCl (pH 8) and recentrifuged. The cells were then resuspended in 4 ml 25% sucrose in 0.05 M Tris.HCl (pH 8), to which was added 0.5 ml freshly prepared lysozyme solution (10 mg/ml in water), and 1.2 ml 0.1 M Na₂EDTA (pH 8). After gentle mixing the solution was placed on ice for 10 min. 0.6 ml freshly prepared SDS solution (10% in water) was added and the mixture vortexed vigorously for 10 seconds, followed by the addition of 0.25 ml RNAse [2 mg/ml ribonuclease A (Sigma)]. Following incubation at 37°C for 30 min, 0.25 ml Proteinase K (Sigma) (1 mg/ml in water) was added and the mixture incubated at 65°C for 2 h. The solution was transferred to a Corex tube, and 0.5 ml ethidium bromide (10 mg/ml in water) and 7.0 g CsCl added. CsCl was dissolved by gentle inversion of the tube. The Corex tubes were centrifuged at 7000 rpm for 15 min, and the clear solution transferred to a Beckmann polyallomer tube. The procedure was then identical to that described in 2.4.2.3, excepting that both DNA bands were removed following ultracentrifugation. A 5 µl sample was taken to check
the concentration and purity of DNA by digestion with a restriction endonuclease and electrophoresis on an agarose gel.

**2.4.2.5 Isolation of DNA from agarose gels (Dretzen et al. 1981)**

The DNA of interest was electrophoresed and ethidium bromide-stained as normal in a 0.8% agarose gel (Section 2.4.4). A piece of nitrocellulose membrane (Type S+S NA45, Schliecher and Schuell, Germany) was cut to a size of 2.0 x 0.5 cm, washed in 10 mM Na$_2$EDTA (10 min), then in 0.5 M NaOH (5 min) and then rinsed well in water. A slit was cut in the gel just down from the position of the wanted band, and the nitrocellulose inserted. The gel was then electrophoresed at 80 V for 10 min and viewed again under UV light to confirm DNA transfer to the membrane. The membrane was removed and rinsed in NET buffer to remove adhering agarose. The membrane was then transferred to an Eppendorf tube. 250 µl of high-salt NET buffer was added and the tube centrifuged for several seconds to force the membrane to the bottom of the tube. After then leaving the tube at 65°C for 45 min, the DNA in the solution was purified by ethanol precipitation, and the pellet resuspended in 20 µl of TE buffer (Section 2.4.1.3). A 2 µl sample was then run on a gel to estimate its concentration.

NET buffer: Na$_2$EDTA, 0.4 g/L; Tris, 2.4 g/L; NaCl, 8.76 g/L; pH 8.0.

High salt NET buffer: Na$_2$EDTA, 0.4 g/L; Tris, 2.4 g/L; NaCl, 58.4 g/L; pH 8.0.

**2.4.2.6 Purification of DNA from agarose gels using GENECLEAN DNA Purification Kit**

Following electrophoresis of restricted DNA, the agarose gel was stained with ethidium bromide and the DNA bands of interest identified (Section 2.4.4). The excised agarose slice containing the DNA band was place in a 1.5 ml microcentrifuge tube, 2.5 volumes of the supplied NaI stock solution added to the tube and the agarose melted at 55°C. 5 µl of the supplied GLASSMILK suspension was then added to the tube, mixed well and placed on ice for 5 min. Following centrifugation for 5 sec the supernatant was discarded and the pellet carefully washed in 10 to 50 vol cold NEW solution and recentrifuged. Following two repeat NEW washes, all the supernatant was removed, the silica particles resuspended in 10 µl TE buffer and incubated at 45 to 55°C for 2 min. The tube was centrifuged for 30 sec and the eluted DNA solution carefully removed to a clean tube. The elution procedure was repeated and this eluate added to the previously eluted DNA. DNA was then ready for restriction digestion or other manipulations.
2.4.3 General DNA manipulations

2.4.3.1 DNA manipulation solutions

10 x TA buffer: 1 ml = Solution A, 800 µl; Solution B, 100 µl; Solution C, 100 µl. All solutions were stored at -20°C.

Solution A = Tris, 5.0 g/100 ml; Potassium acetate, 8.1 g/100 ml; Magnesium acetate, 2.6 g/100 ml; pH 7.8.
Solution B = Dithiothreitol, 77 mg/10 ml; MgCl₂.6H₂O, 10 mg/10 ml.
Solution C = Bovine Serum Albumen, 10 mg/10 ml.

10 x SAP buffer: Tris.HCl, 31.52 g/L, pH 8.0; MgCl₂, 20.33 g/L. The solution was stored at -20°C.

Stop solution: Urea, 24.02 g/100 ml; Sucrose, 50.0 g/100 ml; Na₂EDTA, 1.86 g/100 ml; pH 7.0. Bromophenol blue (0.05% w/v) was then added.

Sucrose gradient buffer NaCl, 0.5 g/100 ml; Tris, 0.2 g/100 ml; Na₂EDTA, 0.4 g/100 ml.

10 x PCR buffer KCl, 3.7 g/100 ml; Tris, 1.57 g/100 ml, pH 8.3; MgCl₂, 14.25 mg/100 ml. The solution was stored at -20°C.

2.4.3.2 Ethanol precipitation of DNA

One half volume of 7.5 M ammonium acetate, and 2½ volumes of 96% ethanol were added to the solution containing DNA, and the tube was left at 20°C for 15 min, then centrifuged for 15 min at room temp. The DNA pellet was washed in 70% ethanol and subsequently in 96% ethanol before being dried under vacuum for 12 min. Finally the dried pellet was resuspended in 20 µl TE buffer, and a 2 µl aliquot electrophoresed on an agarose gel to determine the approximate concentration of DNA, relative to known amounts of DNA size markers.

2.4.3.3 DNA digests with restriction endonucleases

Restriction enzymes were used according to the supplier’s recommendations. Typically, 2 units of enzyme were mixed with 0.5 µg of DNA in a volume of 10-30 µl in a sterile Eppendorf tube. Most digestions were carried out in the manufacturer’s recommended buffer (Pharmacia - One-Phor-All buffer; Boehringer Mannheim GmbH - A, B, L, M, H buffers) at 37°C for a minimum of 1 h. If a multiple digest was to be performed, the restriction enzyme requiring the lowest NaCl concentration buffer was used first, then the reaction volume doubled and fresh suitable buffer added with the following enzyme.

If the sample was to be electrophoresed only, the digestion was terminated by adding one tenth volume of stop solution (Section 2.4.3.1). If digested DNA was to be ligated,
the restriction enzymes were inactivated by either heating for 20 min at 65°C or by phenol/chloroform/isoamyl alcohol extraction (according to the manufacturer's recommendations). In the latter case, 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1 and the phenol prepared as described in Section 2.4.1.1 and equilibrated against TE buffer, Section 2.4.1.3) was added, the tube vortexed briefly and then centrifuged for 3 min. The aqueous layer was extracted against an equal volume of chloroform/isoamyl alcohol and the DNA in the purified aqueous phase ethanol precipitated and resuspended in 20 µl TE or sterile water.

2.4.3.4 Fractionation of DNA by sucrose gradient centrifugation

Four sucrose solutions were prepared at 10%, 20%, 30% and 40% concentrations in Sucrose gradient buffer (Section 2.4.3.1) in a total volume of 2.7 ml each. The solutions were gently overlayed on each other in a polyallomer tube so that the 40% solution formed the bottom layer, with the 30% solution on top of it, then the 20% solution overlayed and finally the 10% solution as top layer. The gradient was left to settle at room temperature for 3 h before the fragmented DNA solution in a 200 µl volume of 10% sucrose was loaded onto the top layer. The tube was balanced with another sucrose gradient tube and centrifuged in a SW41 rotor at 34000 rpm (196,500 g) at 20°C for 16 h. The tube was then clamped in a retort stand and a small hole pierced in the bottom of the tube. Fractions of approximately 200 µl were continuously collected until the column had completely run through. 5-10 µl of each fraction was electrophoresed on an agarose gel to confirm fractionation had occurred. Fractions containing the desired 20-30 kb sized fragments for cloning were then purified by ethanol precipitation, after first diluting the sucrose by adding 400 µl TE. The final pellet was resuspended in 20 µl TE and 1 µl electrophoresed to estimate the concentration of DNA (Section 2.4.4).

2.4.3.5 Alkaline phosphatase treatment

The alkaline phosphatase treatment prevents DNA which has been digested with a single restriction enzyme only from religating to itself. Shrimp alkaline phosphatase (SAP, United States Biochemical, United States) was used due to its heat lability when compared to other alkaline phosphatase preparations. Alkaline phosphatase catalyses the removal of 5'-phosphate residues from DNA. The plasmid DNA to be phosphatased was first digested as normal. The sample was then heated at 65°C for 10 min to inactivate the restriction enzyme(s), and the volume of the reaction mixture was increased to 200 µl, including the addition of 20 µl of a 10 x SAP buffer (Section 2.4.3.1). Four units of SAP was added and the reactions left at 37°C for 1 h. The reaction mixture was then heated to 65°C for 15 min to inactivate the SAP.
2.4.3.6 DNA ligations

DNA Ligations were carried out as recommended by the manufacturer of the T4 DNA ligase used, Pharmacia LKB Biotechnology. Donor and recipient DNA were digested separately as normal, then the restriction enzymes inactivated by heating at 65°C for 20 min. Alkaline phosphatase treatment was carried out on the recipient vector if necessary (Section 2.4.3.5). A ratio of approximately 1:5 of vector:insert was then mixed in an Eppendorf tube. A one tenth volume of 10 x One-Phor-All PLUS buffer (Pharmacia - 100 mM Tris-acetate, 100 mM magnesium acetate, 500 mM potassium acetate) was added, and rATP added to give a final concentration of 1.0 mM. For blunt end ligations, the final concentration of rATP was reduced to 0.1 mM. T4 DNA ligase (5 units for cohesive ends, 15 units for blunt ends) was then added. T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3'-OH and 5'-phosphate termini in DNA. The typical reaction volume for the ligation of 2 fragments was 30 µl. The mixes were left at 14°C for 16 h. The reaction was stopped by heating to 65°C for 10 min. 4 µl of the sample was electrophoresed on an agarose gel (Section 2.4.4) prior to electroporation (Section 2.4.6.1) to check for the presence of large molecular weight ligated DNA.

2.4.3.7 Amplification of DNA fragments by polymerase chain reaction (based on Sambrook et al 1989, pp 14.15 - 14.19)

The polymerase chain reaction (PCR) method was used to amplify a small insert of DNA which was cloned in the vector Bluescript, for use as a hybridisation probe. This protocol exploits the stability of Taq polymerase enzyme at high temperatures by repeatedly performing DNA denaturing, annealing and extension cycles at different set temperatures. The reaction mixture containing 10 µl PCR buffer (Section 2.4.3.1), 1 µg of an appropriate primer(s), deoxynucleotides dATP, dCTP, dGTP and dTTP (each to a final concentration of 20 µM) in a final volume of 50 µl was prepared and added to 50 µl of water containing up to 2 µg of linearised plasmid DNA. Following heating to 94°C for 5 min (to denature DNA), 2.5 units of Taq polymerase enzyme ("Amplitaq", Perkin Elmer Cetus) was added to the mixture. The reaction mixture was then overlayed with 100 µl paraffin oil. The amplification reaction was conducted using a preprogrammed heating block (MJ Research Minicycler), with denaturation at 94°C for 30 sec, annealing at appropriate temperature (see below) for 30 sec and extension at 70°C for 2 min. This cycle was repeated 20 times. The DNA solution was separated from the paraffin and removed to a fresh 1.5 ml Eppendorf tube. To precipitate the DNA, 100 µl of 4 M ammonium acetate, then 400 µl 96% ethanol were added, and left for 16 h at 4°C. Following centrifugation for 20 min at 4°C the DNA pellet was allowed to dry and resuspended in 50 µl TE. A 5 µl sample was electrophoresed on an agarose gel to estimate the concentration of amplified insert (Section 2.4.4).
The appropriate temperature for the annealing reaction was calculated from the sequence of the primer used, as follows:

\[
\text{Annealing temperature (°C)} = 4 \times (G+C) + 2 \times (A+T) - 5.
\]

### 2.4.4 Agarose gel electrophoresis of DNA fragments

Horizontal agarose slab gel electrophoresis apparatus were used for all gel electrophoresis involving digested or ligated DNA or for estimations of DNA concentration. 0.8% w/v agarose gels were made by boiling agarose in 1 x TBE buffer, then pouring the molten agarose into a slab mould. Horizontal agarose gel electrophoresis was carried out essentially as described by Maniatis et al. (1982, p 150-162). 2 gel slab dimensions were used: maxi gels for genomic DNA; 190 x 130 x 7 mm, and the mini gel for plasmid DNA; 85 x 53 x 7 mm or Pharmacia mini apparatus. Typically, 1.5 mg of DNA in a volume of 30 µl was loaded into a maxi gel well, and 0.5 mg of DNA in a volume of 10 µl was loaded into a mini gel well. A one-tenth volume of stop solution was added to each sample before loading. Maxi gels were electrophoresed at 25 V, 16 mA (usually 16 h), and mini gels at 80 V, 30 mA (approximately 60 min). Following electrophoresis, gels were stained in a 3 µg/ml ethidium bromide solution for 3-5 min (minigels) or 15 min (maxigels) and destained in running water for 15-30 min. Gels were photographed on a 302 nm wave length UV transilluminator (Ultra Violet Products, Inc.) using a Polaroid MP4 Land camera with Polaroid type 667 film and a Wratten gelatin yellow filter (Kodak).

DNA size standards typically used were *HindIII* and *HindIII* + *EcoRI* restricted bacteriophage lambda DNA fragments, which were run in parallel to the experimental samples. The rate of migration of a DNA fragment during gel electrophoresis is inversely proportional to the logarithm of its length.

### 2.4.5 DNA transfer and hybridisation techniques

#### 2.4.5.1 DNA transfer and hybridisation solutions

**Denaturing solution**: NaCl, 87.7 g/L; NaOH, 20.0 g/L.

**Neutralising solution**: NaCl, 87.7 g/L; Tris, 60.5 g/L; Na₂EDTA, 0.37 g/L; pH 7.2.

**Alkali transfer solution**: NaCl, 87.7 g/L; NaOH, 10.0 g/L.

**Deprobe solution**: SSC (20 x stock), 50 ml/L; Sodium dodecyl sulphate, 1 g/L; Tris, 24.2 g/L; pH 7.5.

**SSC x 20 buffer**: NaCl, 175 g/L; Sodium citrate, 87.5 g/L.
Hybridisation solution I: SSC (20 x stock), 334 ml/L; Sodium dodecyl sulphate, 10 g/L; Polyvinyl-pyrrolidone, 0.2 g/L; Ficoll Type 400, 0.2 g/L; Gelatine, 0.2 g/L.

Hybridisation solution II: SSC (20 x stock), 150 ml/L; Sodium dodecyl sulphate, 70 g/L; Skim milk powder, 0.05 g/L; PEG-8000, 10 g/L.

2.4.5.2 Colony blot

The method used was that recommended by the manufacturer of Hybond-N membranes, Amersham, UK, and was used to verify *E. coli* transformants or when screening a gene bank for a particular clone. Colonies were transferred to a Hybond-N membrane by placing the membrane directly onto a solid medium plate which contained 50 to 150 freshly grown colonies. Where plates were too crowded, single colonies were first spotted onto fresh L medium plates (Section 2.1.2) and regrown. The membrane was then placed (colony side up) on a piece of 3MM Whatman blotting paper which had been saturated with the denaturing solution (Section 2.4.5.1) for 7 min. The membrane was treated similarly with neutralising solution (Section 2.4.5.1), twice for 3 min, and then washed twice in 2 x SSC (Section 2.4.5.1). The membrane was air dried, wrapped in plastic film and exposed to 302 nm UV light for 4 min with colonies facing the UV source. This procedure cross-linked the DNA to the membrane.

2.4.5.3 Transfer of electrophoresed DNA onto membranes for hybridisation

A quick and efficient procedure for transferring electrophoresed DNA directly onto a Hybond-N membrane for hybridisation was adapted from the method of Southern (1975), as recommended by Amersham. The gel containing electrophoresed DNA was gently washed in 0.25 M HCl for 15 min, then rinsed twice with distilled water. The gel was then washed with denaturing solution (Section 2.4.5.1) for 30 min, followed by alkali transfer solution (Section 2.4.5.1) for 15 min. The treated gel was transferred upside-down to a glass plate. A piece of Hybond-N membrane paper was cut to the exact size of the gel, was pre-wet in alkali transfer solution, and placed onto the gel in a single motion. Whatman 3MM blotting paper (2 pieces) were cut to the same size as the membrane and soaked in alkali transfer solution. Each piece was placed over the membrane, ensuring no air bubbles were present between the layers. A stack of paper towels 3 cm high was placed over the blotting paper and weighted down lightly. The membrane was typically left to blot for 16 h before being removed and rinsed in 2 x SSC solution (Section 2.4.5.1). The agarose gel was stained with ethidium bromide solution (3 µg/ml) for 3 min, destained in running water for 10 min and inspected on a UV transilluminator to confirm the transfer of DNA. After drying for 15 min at room temperature the membrane was wrapped in plastic film and exposed to 302 nm UV light for 4 min. This procedure cross-linked the DNA to the membrane.
2.4.5.4 Radiolabelling DNA fragments

Radioactively-labelled hybridisation probes were prepared by randomly primed synthesis of DNA using *E. coli* DNA polymerase I (Klenow fragment) using the Multiprime Kit (Amersham, UK). 5 μl DNA to be labelled (in TE) was denatured by heating in a boiling water bath for 2 min, and then placed on ice. To the DNA was added 4 μl each of 20 mM dCTP, dGTP and dTTP, 5 μl buffer solution, 5 μl random hexamers, distilled H₂O to 43 μl, 5 μl [α³²P]-dATP (3000 Ci/mM), and finally 2 μl enzyme solution (DNA polymerase I). The reaction mixture was gently pipetted back and forth twice to thoroughly mix, and stray droplets centrifuged into the reaction mixture for 5 sec. Following incubation at 37°C for 30 - 60 min, 1.2 μl of 0.25 M spermine tetrachloride solution was added, mixed gently, and the tube placed on ice for 15 min. The mixture was then centrifuged for 10 min in a microcentrifuge and the supernatant carefully removed using a micropipette. To the pellet was added 100 μl resuspension buffer (0.5% SDS in 10 mM Na₂EDTA) and mixed by tapping the tube. Immediately prior to addition of the probe to hybridisation mixtures, the probe was denatured by placing in a boiling water bath for 4 min (after puncturing a hole in the microcentrifuge tube lid to relieve pressure build up). The DNA was then immediately placed on ice for 5 min, and centrifuged briefly to remove condensation from the lid of the tube.

2.4.5.5 DNA hybridisation - rotating bottle method

This method involved rotating a bottle containing the membrane to be hybridised in a rotisserie hybridisation oven (Hybaid Ltd., UK) as directed by the manufacturers. The membrane was placed between two mesh sheets, and inserted into a specially designed glass bottle with approximately 15 ml Hybridisation Solution I (Section 2.4.5.1). The bottle was transferred to the oven and left at 65°C for at least 1 h. Up to 5 membranes could be placed in the 1 bottle if required. Denatured, radioactively labelled probe, and denatured radioactively labelled λ DNA (Section 2.4.5.4), if required, was added directly to the bottle, which was allowed to rotate at 65°C (for homologous probes) or 50°C (for heterologous probes) for 16 h. The hybridisation solution was poured off, the bottle half filled with pre-warmed 2 x SSC (Section 2.4.5.1), and rotated in the oven for 15 min. The wash solution was poured off, and the wash repeated. A further wash in 1 x SSC followed. Where high stringency wash conditions were required to remove probes from non-homologous sequences, 1 wash in 0.1 x SSC, at 65°C for 10 min was included. After draining off excess moisture, the membrane was wrapped in plastic wrap, and exposed to XAR-5 film at -70°C overnight with 1 or 2 intensifying screens (Dupont). Longer exposures (up to 7 days) were needed in some cases with genomic blots, although typically 3 days was sufficient.
2.4.5.6 DNA hybridisation - plastic bag method

This method was routinely used before the acquisition of the hybridisation oven. Filters were placed in a heavy duty, heat-sealable plastic bag (usually 1 or 2 membranes per bag) to which approximately 5 ml of Hybridisation Solution II (Section 2.4.5.1) was added. The bag was sealed ensuring no air bubbles were present, and placed in a waterbath at 65°C for at least 1 h. Radioactively labelled probe was added to the contents of the bag after cutting off a corner, the bag resealed and left incubating in the waterbath for 16 h at an appropriate temperature (Section 2.4.5.5). Washing and autoradiography procedures were as in 2.4.5.5, except that membranes were washed in plastic boxes.

2.4.5.7 Deprobing Hybond-N membranes

Hybond-N membranes (Amersham, UK) could be deprobed and reused provided they remained moist. The membrane was placed in 100 ml of 0.4 M NaOH in a plastic box and incubated with gentle shaking at 65°C for 30 min. The membrane was transferred to 100 ml of deprobe solution and incubated again at 65°C for 30 min. After a final rinse in 2 x SSC (Section 2.4.5.1), excess moisture was blotted off the membrane, which was then ready for further hybridisation.

2.4.6 Electroporation of E. coli with DNA

2.4.6.1 Preparation of E. coli cells for electroporation

E. coli strain NM522 cells were grown overnight in L broth (Section 2.1.2). A 1.5 ml Eppendorf tube full of culture was centrifuged at 12,000 rpm for 1 min. The cells were then washed in 1.2 ml sterile MilliQ H$_2$O, and centrifuged again. The wash was repeated a further 3 times. The cells were finally resuspended in 100 µl sterile MilliQ H$_2$O.

2.4.6.2 Electroporation of E. coli cells

Following ligation (Section 2.4.3.6), DNA was precipitated from ligation mixture in a 1.5 ml Eppendorf tube by ethanol precipitation, and centrifuged at 12,000 rpm at 4°C for 10 min. The ethanol was decanted and the DNA pellet was washed in 70% ethanol (well mixed by vortexing). Following centrifugation at 12,000 rpm for 5 min, the DNA pellet was desiccated under vacuum. DNA was resuspended in 20 µl sterile MilliQ H$_2$O. An appropriate aliquot containing 0.1 to 0.5 µg of DNA was transferred to a sterile Eppendorf tube, 100 µl prepared E. coli cells (Section 2.4.6.1) added, and the mixture held on ice until required. Electroporation cuvettes (Biorad, 0.2 cm electrode separation) were prepared by rinsing in ethanol, rinsing in sterile MilliQ H$_2$O 5 times, and chilling on ice. The DNA/cell mixture was added to the cold cuvette so as to avoid air bubbles being trapped. The cuvette was placed in the chilled cuvette holder, and
voltage applied at settings of: voltage, 2.5 kV; capacitance, 25 µFD; in a Biorad Gene Pulser apparatus. 1 ml of L broth (Section 2.1.2) was added to the cuvette, and the broth/DNA/cell mix was transferred to a sterile 15 ml capped polycarbonate tube, incubated with rapid shaking at 37°C for at least 1 h. 150 µl aliquots of the transformed cells were plated onto selective media and grown overnight at 37°C.

When plasmid Bluescript (Stratagene, USA) derivative pJJ358 was used as the cloning vector, 5 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) and 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in dimethylformamide) were incorporated into the molten L medium (Section 2.1.2) before pouring. Colonies carrying recombinant plasmids are unable to cleave the galactosyl residue from the synthetic X-gal substrate and consequently form white colonies, while those transformants carrying vector (without insert) form blue colonies. Potential transformants were screened by colony blot on Hybond-N membrane (Section 2.4.5.2), and hybridisation to an \( \alpha^{32}P \) probe prepared from the appropriate DNA fragments (Sections 2.4.5.4 & 2.4.5.5), and confirmed by small scale DNA isolation and digestion with restriction endonucleases (sections 2.4.2.2 & 2.4.3.3).

### 2.4.7 Conjugation of bacteria by tri-parental patch mating

The patch mating procedure of Sinclair and Holloway (1982) was used to transfer the constructed plasmids into the recipient strain by tri-parental mating. The helper strain contained plasmid pRK2013 (Ditta et al. 1980) which can mobilise incP plasmids containing the appropriate mob region to other bacteria. Patch matings were performed by putting several loopfuls of freshly grown recipient *Rhizobium* cells on a TY plate (Section 2.1.2), and adding a small loopful of donor *E. coli* cells. Finally, a small loopful of cells of the helper strain was added and all three bacterial strains were mixed thoroughly with a sterile stick. Control patches of each strain spotted separately and in paired combinations were also set up on the same plate to detect any contamination between the parental strains, see Figure 2.2. After incubating the plates at 28°C overnight, the cells were replica-plated onto selective medium (GMM containing antibiotics (Sections 2.1.2, 2.1.3, Table 2.3)) and grown at 28°C for 3 days. Single colonies were purified by restreaking onto the selective medium. A check for any *E. coli* contamination was done by plating purified colonies onto L agar (Section 2.1.2) and incubating at 37°C overnight. *Rhizobium* transconjugants are unable to grow on L agar.
2.5 Chemicals and reagents

Special chemicals and reagents were obtained from the following sources:

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'(α^{32}P)-dATP</td>
<td>Amersham</td>
</tr>
<tr>
<td>Agar</td>
<td>Difco Laboratories</td>
</tr>
<tr>
<td>Agarose</td>
<td>SeaKem</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Sigma</td>
</tr>
<tr>
<td>Biotin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Black &amp; white film (type 667)</td>
<td>Polaroid</td>
</tr>
<tr>
<td>Caesium chloride</td>
<td>Metallgesellschaft</td>
</tr>
<tr>
<td>Deoxyribonucleotides</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ficoll Type 400</td>
<td>Sigma</td>
</tr>
<tr>
<td>GENECLEAN DNA Purification Kit</td>
<td>BRESA, Australia</td>
</tr>
<tr>
<td>Hybond-N membranes</td>
<td>Amersham</td>
</tr>
<tr>
<td>Klenow fragment</td>
<td>BRESA, Australia</td>
</tr>
<tr>
<td>M9 Minimal Salts</td>
<td>Gibco</td>
</tr>
<tr>
<td>Multiprime DNA labelling kit</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Nitrocellulose (0.45μm)</td>
<td>Millipore Inc., U.S.A.</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>Difco Laboratories</td>
</tr>
<tr>
<td>o-nitrophenyl-beta-D-galactoside (ONPG)</td>
<td>Sigma Chemical Company, St. Louis, MO</td>
</tr>
<tr>
<td>Polyethylene glycol-8000</td>
<td>Selby and Co. Ltd</td>
</tr>
<tr>
<td>Phenol</td>
<td>Wako</td>
</tr>
</tbody>
</table>
## 2.5 Chemicals and reagents - continued

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>rATP</td>
<td>Sigma</td>
</tr>
<tr>
<td>Restriction endonucleases</td>
<td>Boehringer Mannheim, Promega, Pharmacia, Biolabs</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Thiamine</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tris</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Difco Laboratories</td>
</tr>
<tr>
<td>X-ray film (type XAR-5)</td>
<td>Kodak</td>
</tr>
<tr>
<td>X-gal</td>
<td>Sigma</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Difco Laboratories</td>
</tr>
</tbody>
</table>
Table 2.1  Bacterial Strains Used In This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU1073</td>
<td>Strain HB101 containing the mobilisation helper plasmid pRK2013. Km', Sm'.</td>
<td>Ditta et al. 1980</td>
</tr>
<tr>
<td>NM522</td>
<td>(lac-pro) F' lacZM15 lacI hsd-5.</td>
<td>Gough and Murray 1983</td>
</tr>
<tr>
<td><em>Bradyrhizobium:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP279</td>
<td>Wild-type, narrow host range, <em>Parasponia</em>-specific strain. Nod⁺, Fix⁺ on <em>Parasponia</em>.</td>
<td>Trinick 1980a</td>
</tr>
<tr>
<td>CP283</td>
<td>Wild-type, broad host range cowpea strain. Nod⁺, Fix⁺ on <em>Parasponia</em>.</td>
<td>Trinick 1980a</td>
</tr>
<tr>
<td>ANU289</td>
<td>Sm' derivative of strain CP283</td>
<td>Trinick and Galbraith, 1980</td>
</tr>
<tr>
<td>USDA110</td>
<td>Wild-type, slow-growing <em>Bradyrhizobium japonicum</em>, soybean inoculant.</td>
<td>Sadowsky et al. 1991</td>
</tr>
<tr>
<td><em>Miscellaneous Rhizobium:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGR234</td>
<td>Wild-type, fast-growing, broad-host-range <em>Rhizobium</em> strain infecting tropical legumes.</td>
<td>Trinick 1980b</td>
</tr>
<tr>
<td>ANU265</td>
<td>Sym plasmid-cured derivative of strain NGR234. Sm', Sp', Nod⁻.</td>
<td>Morrison et al. 1983</td>
</tr>
<tr>
<td>ANU1255</td>
<td>NGR234, nodD::Tn5, Km'.</td>
<td>Morrison et al. 1984</td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum</em> biovar. trifolii:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>wild-type, Nod⁺ on all clovers.</td>
<td>Rolfe et al. 1980</td>
</tr>
<tr>
<td>ANU851</td>
<td>ANU843, nodD::Tn5, Km'.</td>
<td>Djordjevic et al. 1985</td>
</tr>
<tr>
<td>ANU894</td>
<td>ANU843 with a translational fusion of <em>nodA</em> (pSym) and the lac operon (MudII1734)</td>
<td>M. Djordjevic, this laboratory</td>
</tr>
<tr>
<td><em>Rhizobium fredii:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU1182</td>
<td>Sm' derivative of USDA 191, Nod⁺, Fix⁺ on soybean species.</td>
<td>Appelbaum et al 1985</td>
</tr>
</tbody>
</table>

Nod⁺ = ability to nodulate; Nod⁻ = inability to nodulate; Fix⁺ = Fixes nitrogen; Fix⁻ = fails to fix nitrogen; Km' = kanamycin resistant; Sm' = streptomycin resistant; Sp' = spectinomycin resistant.
Table 2.2 Plasmids Used In This Study

<table>
<thead>
<tr>
<th>Plasmids:</th>
<th>Relevant Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBS, SK&lt;sup&gt;−&lt;/sup&gt; Bluescript</td>
<td>Cloning and sequencing vector, Cb&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Stratagene, La Jolla, CA</td>
</tr>
<tr>
<td>pJJ252</td>
<td>Bluescript SK&lt;sup&gt;−&lt;/sup&gt; carrying a 1.9 kb PstI fragment containing NGR234 nodD1.</td>
<td>J. Weinman, this laboratory</td>
</tr>
<tr>
<td>pJJ358</td>
<td>Derived from Bluescript, multiple cloning site replaced, IncP1, Cb&lt;sup&gt;−&lt;/sup&gt;.</td>
<td>J. Weinman, this laboratory</td>
</tr>
<tr>
<td>pKS1701</td>
<td>nodD1 from CP279, in pLAFR3. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>pKS4001</td>
<td>nodD2 from CP279, in pLAFR3. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>pKS2001</td>
<td>nodD3 from CP279, in pLAFR3. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>pKT240</td>
<td>cloning vector, Cb&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;, promoterless Sm IncQ, mob&lt;sup&gt;+&lt;/sup&gt;, tra&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Bagdasarian et al. 1983</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>broad host range cloning vector, derived from pLAFR1, mob&lt;sup&gt;+&lt;/sup&gt;, tra&lt;sup&gt;+&lt;/sup&gt;, allows blue/white selection in appropriate E. coli strains. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Staskawicz et al. 1987</td>
</tr>
<tr>
<td>pLSC17</td>
<td>10 kb BamHI fragment from pZB32 inserted into pKT240</td>
<td>This study</td>
</tr>
<tr>
<td>pMD1</td>
<td>pMP220 carrying a 2.9 kb EcoRI fragment containing NGR234 nodD1 gene, and a 0.7 kb PstI fragment containing the nodA promoter of R. l. bv. trifolii strain ANU843.</td>
<td>This study, Le Strange et al. 1990</td>
</tr>
<tr>
<td>pMN40</td>
<td>pSUP106 Cm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; carrying 2.1 kb PstI fragment spanning the nodD1 gene from strain NGR234</td>
<td>Bender et al. 1988</td>
</tr>
<tr>
<td>pMP220</td>
<td>Broad-host-range IncP-1 promoter cloning vector, stably maintained in Rhizobium containing a promoterless E. coli lacZ gene. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Spaink et al. 1987a</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Narrow host range helper plasmid. Has Tra genes and ori-T of IncP1 plasmid RK2. Unable to be maintained in Rhizobium. Km&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Ditta et al. 1980</td>
</tr>
<tr>
<td>pRT311</td>
<td>pMP220 carrying an 0.7 kb BamHI fragment containing the nodA promoter of R. l. bv. trifolii strain ANU843.</td>
<td>McLiver et al. 1989</td>
</tr>
<tr>
<td>pZB32</td>
<td>Contains nodY::lacZ translational fusion from Bradyrhizobium japonicum. Tc&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>Banfalvi et al. 1988</td>
</tr>
</tbody>
</table>

Cb<sup>−</sup> = carbenicillin resistant; Km<sup>+</sup> = kanamycin resistant; Tc<sup>+</sup> = tetracycline resistant; Sm = streptomycin; mob<sup>+</sup> = mobilisable plasmid; tra<sup>+</sup> = plasmid unable to transfer.
### Table 2.3  Antibiotic Concentrations Used in Bacterial Culture

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentrations (µg/ml) for</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Rhizobium</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Carbenicillin (Cb)</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Kanamycin sulphate (Km)</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>Spectinomycin (Sp)</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>Streptomycin (Sm)</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>Tetracycline (Tc)</td>
<td>2-5</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 2.1

The Magenta jar assay, showing an eight week old *Parasponia andersonii* plant.

A - aquarium gravel; B - vermiculite;
C - plant nutrient solution; D - cotton wick.

The Magenta jar assay is describe in Section 2.3.8.
Figure 2.2

Tri-parental patch mate.

1 - patch mate mix of donor (D), recipient (R) and helper (H) strains
2 - 7 Controls: 2 - D; 3 - H; 4 - R; 5 - D + H; 6 - D + R; 7 - R + H

The tri-parental patch mate technique is described in Section 2.4.7.
Chapter Three

Compounds inducing nod gene transcription in *Rhizobium* strain NGR224.

3.1 Introduction

Extensive studies have shown that the *nodA* gene of *Rhizobium* is essential for the modulation of legume inoculation. It has been established that *nodA* is involved in the early stages of host recognize by the *Rhizobium* strain (Spezak et al. 1977). NodA is a transcriptional regulatory protein which binds to promoters of other nod genes. It has been shown that NodA binds to a specific region within the nod-box present in the *Rhizobium* strain (Barker and Long 1980). Plant synthesised flavonoids are known to induce nodulation in *Rhizobium* species (Kochhar et al. 1990; Lewinger et al. 1990; Amin et al. 1990).

Flavonoid compounds which interact with NodA have been identified in many studies. These include *Salvia* (Oras et al. 1980), 7,4'-Dihydroxyflavone (DFH) from *Vicia faba* (Redmond et al. 1982) and 3',4'-Dihydroxyflavone (DHF) from *Cicer arietinum* (Redmond et al. 1982). Anti-inhibitors such as orientalin, havenedol, and some isoflavones (Fitzke et al. 1986; Djemilevic et al. 1967), appear to competitively inhibit the induction of NodD-dependent nod genes by activating flavonoids (Ullman et al. 1987). In contrast, the isoflavone daidzein has been isolated from *Polaris* and shown to induce the NodD-dependent nod genes of *B. japonicum* (Kasahara et al. 1980).

*Rhizobium* strain NGR224 (Tani et al. 1980) is a fast-growing rhizobium strain and is one of the few strains capable of forming nodules with a non-legume host, the woody shrub *Paraseriocarpus* from the Ulmaceae family (Tani et al. 1980). Molecular characterization of the *nodC* gene of strain NGR224 shows it to be highly conserved at the DNA sequence level with the *nodC* genes of other rhizobia (J. J. Weisbein, personal communication).

Bussman et al. (1988) showed that a broad range of flavonoid compounds activated NodD1 of strain NGR224, including some which have been identified as anti-inducers in other studies. Bussman et al. (1988) further demonstrated that the introduction of the NGR224 nodD1 gene into *L. luteus* by Agrobacterium tumefaciens results in the induction of transcription of an *L. luteus nod* gene by a wider range of flavonoid compounds than
Figure 2.2
Tri-parental patch mate.

1. patch mate mix of donor (D), recipient (R) and helper (H) strains

The tri-parental patch mate technique is described in Section 2.4.7.

This page is intended to be left blank.
Chapter Three
Compounds inducing nod gene transcription in Rhizobium strain NGR234.

3.1 Introduction

Extensive studies have shown that the nodulation gene nodD is essential for the nodulation of legumes by rhizobia. The nodD product is involved in the earliest stages of host recognition between various rhizobia and legumes (Spaink et al. 1987b). NodD is a transcriptional regulating protein (Henikoff et al. 1988) which binds to promoters of other nod genes (Hong et al. 1987; Fisher et al. 1988) at a specific region within the nod-box promoter (Rostas et al. 1986; Long 1989; Fisher and Long 1993). Plant synthesised compounds, in concert with NodD, induce transcription of these nod genes. The products of the nod genes are involved in the initiation of plant nodulation (Mulligan and Long 1985; Innes et al. 1985; Rossen et al. 1985; Shearman et al. 1986; Spaink et al. 1987b; Bassam et al. 1988; Spaink et al. 1989), and they have been shown to synthesise species-specific Nod factors, the chito-lipo-oligosaccharides (CLOS) (Lerouge et al. 1990; Spaink et al. 1991; reviewed by Spaink 1992).

Flavonoid compounds which interact with NodD have been identified in many narrow host range Rhizobium-legume symbioses. Examples include luteolin, isolated from alfalfa (Peters et al. 1986) and 7,4'-dihydroxyflavone (DHF) from clover (Redmond et al. 1986). Anti-inducers such as coumarin, flavonol, and some isoflavones (Firmin et al. 1986; Djordjevic et al. 1987), appear to competitively inhibit the induction of NodD-dependent nod genes by activating flavonoids (Djordjevic et al. 1987). In contrast, the isoflavone daidzein has been isolated from soybeans and shown to induce the NodD-dependent nod genes of B. japonicum (Kossak et al. 1987).

Rhizobium strain NGR234 (Trinick 1980b) is a broad host range strain and is one of the few rhizobia capable of forming nodules with a non-legume host, the woody tree Parasponia from the Ulmaceae family (Trinick and Galbraith 1980). Molecular characterisation of the nodD1 gene of strain NGR234 shows it to be highly conserved at the DNA sequence level with the nodD genes of other rhizobia (J. J. Weinman, this laboratory, unpublished data).

Bassam et al. (1988) showed that a broad range of flavonoid compounds activated NodD1 of strain NGR234, including some which have been identified as anti-inducers in other rhizobia. Bender et al. (1988) further demonstrated that the introduction of the NGR234 nodD1 gene into R. l. bv trifolii strain ANU843 enables the induction of transcription of ANU843 nod genes by a wider range of flavonoid compounds than
occurs with the native ANU843 nodD gene. Compounds activating NGR234 NodD1-dependent nod gene induction include coumestans and isoflavones, and at least one compound identified as an anti-inducer in strain ANU843, umbelliferone (Djordjevic et al. 1987; Bender et al. 1988).

Despite the high level of molecular conservation with other nodD alleles, NGR234 NodD1 is apparently less specific in flavonoid recognition than the nodD genes from more widely characterised, narrow host range rhizobia (Bassam et al. 1988; Bender et al. 1988).

It was the aim of this research determine if the characteristics of the compounds capable of inducing nod gene transcription in conjunction with Rhizobium strain NGR234 NodD1 were indeed less specific than those of the narrow host range strains previously investigated, as proposed by Bender et al. (1988) and Bassam et al. (1988).

Furthermore, whereas flavonoids and isoflavonoids induce expression of Rhizobium nod genes (Djordjevic et al. 1987; Horvath et al. 1987), and simple phenolic compounds induce expression of A. tumefaciens vir genes (Stachel et al. 1985a; 1985b), simple phenolic compounds have not been shown to activate NodDs of the rhizobia investigated thus far. Parallel work on plant extracts, discussed in Chapter Four, indicated that simple phenolic compounds may also activate NodD dependent-nod gene transcription. Thus a further aim of this work was to investigate simple phenolic compounds as a group of potential NodD activators.

A large number of authentic, plant derived flavonoid and simple phenolic compounds were assayed for NodD-dependent nod gene induction using lacZ expression plasmids, and the common structural features of the NGR234 NodD1 activating compounds were identified. Unlike the activators of NodDs from other Rhizobium strains, simple phenolic compounds in addition to a very wide range of flavonoid compounds were found to activate NodD1 of strain NGR234. The implications of these findings are discussed.

The results of this research were published in Le Strange et al. 1990.

3.2 Experimental Procedure

The use of an E. coli lacZ expression vector driven by a nod gene promoter to measure the activation of NodD-dependent nod gene transcription was well established in the field (See Firmin et al. 1986; Peters et al. 1986; Redmond et al. 1986; Djordjevic et al. 1987; Horvath et al. 1987; Kossak et al. 1987; Spaink et al. 1987a). Thus similar plasmids for use in β-galactosidase assays were constructed.

In parallel with the work described in this chapter, Bender et al. (1988) studied the induction of transcription of strain ANU843 nod genes by NGR234 NodD1 (described
Compounds inducing *nod* gene transcription in *Rhizobium* strain NGR234.

in Chapter Four, Section 4.3) in a background which also contained the ANU843 *nodD* gene (strain ANU894(pMN40) - Table 2.1, & Figure 4.1). In contrast, strain ANU265, a pSym cured derivative of NGR234 (Table 2.1) was used as the host strain for the work described in this chapter in order to simulate more closely the wild-type NGR234 background. In addition a more refined expression vector became available to this laboratory and was used to construct plasmid pMD1 for use in the *nod* gene induction assays described in this chapter. The construction of pMD1 is described below in Section 3.2.1.

Commencing with commercially available compounds investigated in relation to NodD activation in other rhizobia (for example 7,4'-dihydroxyflavone (DHF) from clover, daidzein from soybean, and coumarin, flavonol, umbelliferone etc), β-galactosidase assays were used to assess NGR234 NodD1-dependent *nod* gene induction (protocol as per section 2.1.6.1).

Having confirmed that the same flavonoid structural features would induce activity as seen in *R. l. bv trifolii* and *B. japonicum*, but that particular anti-inducers of these strains would also activate NodD-dependent *nod*-gene transcription, I began to work systematically through commercially available flavonoid compounds of differing structural features to determine their capacity to activate NGR234 NodD1, and the influence of the differing structural characteristics on activation. I also investigated commercially available simple phenolic compounds, including some identified as inducers of *vir* genes, for example acetosyringone and syringealdehyde, for their activity in inducing NodD-dependent *nod* gene transcription in the test system. These compounds have strong structural similarity to the C-ring of flavonoid compounds. Refer to the legend to Figure 3.2 for the flavonoid numbering scheme.

β-galactosidase assays were performed in duplicate and repeated once at each concentration for assessment of activity in the test system (reported in Table 3.1), or repeated twice at each concentration for the calculation of log $A_{50}$ (reported in Table 3.2).

### 3.2.1 Construction of plasmid pMD1

A 0.7 kilobase (kb) BamHI DNA fragment containing the *nodD/nodA* intergenic region of *R. l. bv trifolii* ANU843 was cloned into the pBS+ vector (Table 2.2). This DNA fragment was derived from strain ANU843 containing an insertion of MudIII1734 in the 5' end of *nodA* and included 116 base pairs (bp) of the MudIII1734 DNA (McIver *et al.* 1989). Using a *PstI* site located 207 bp 3' to the initiation codon for *nodD*, and lying within the *nodD* coding region of this fragment (Schofield and Watson 1986), and the *PstI* site from the polylinker sequence of pBS+ vector, a 0.7 kb *PstI* fragment was excised and cloned (see Figure 3.1) into the *PstI* site of the plasmid pMP220 (Spaink *et al.* 1987a). This resulted in the fusion of the *nodA* promoter with the promoterless *lacZ*
gene of pMP220. This plasmid was isolated and linearised with EcoRI, and a 2.9 kb EcoRI DNA fragment containing the NGR234 nodD1 gene (Bassam et al. 1988) was cloned into the EcoRI site. The resulting plasmid pMD1 is shown in Figure 3.1.

Plasmid pMD1 was transferred to the pSym+ strain ANU265 by tri-parental patch mating using plasmid pRK2013 (Table 2.2) as the helper plasmid (by the method described in Section 2.4.7).

RESULTS

3.3 Phenolic compounds inducing nodD-dependent transcription

Some 70 phenolic compounds were examined for their capacity to induce the nodA::lacZ fusion of the NGR234 NodD1-dependent construct pMD1 in Rhizobium strain ANU265. Assays were conducted over a range of compound concentrations from $10^{-3}$ M to $10^{-9}$ M, as indicated in Table 3.1. Inhibition of bacterial cell growth was detected for a number of phenolic acids and is also indicated in Table 3.1. The log_{10} of the concentration at which nod gene induction is half-maximal (log A_{50}) was determined for 19 representative compounds, and the results are shown in Table 3.2. The comparison of log A_{50} gives a clearer picture of potent and weak inducers than does comparison of maximal induction (i_{max}).

The most potent inducers were daidzein, genistein, DHF, and apigenin, all of which have log A_{50} values of -8 or lower (Table 3.2). Other compounds able to induce activity at low concentrations (log A_{50} below -6) included: coumestrol, formononetin, biochanin A, quercetin, kaempferol, naringenin, hesperetin, and 7-hydroxy flavone. Those inducing at higher concentrations (log A_{50} above -6) included: vanillin, isovanillin, syringaldehyde, 7-hydroxychromone, and umbelliferone. Those compounds unable to induce detectable nodA expression included: 3,4-dimethoxybenzoic acid, 3,4-dimethoxybenzaldehyde, 4-methoxyacetophenone, o-hydroxyacetophenone, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and flavone (Table 3.1).

The plant pathways which synthesise the identified activating compounds are indicated in Figure 3.2.

3.3.1 Negative controls for these assays

The most appropriate negative control for the assay experiments presented in this chapter would be pMD1 with an inactivated nodD1 gene, in the ANU265 background. Unfortunately this construct was not available. However, this laboratory has previously reported on a construct almost identical to pMD1 derived from plasmid pMP220 (the same expression vector as pMD1); but lacking a nodD gene and containing a different nod-box promoter, from NGR234 rather than ANU843 (Bassam et al. 1988). This
Compounds inducing *nod* gene transcription in *Rhizobium* strain NGR234. 63

construct, pBB81, showed no significant induction of β-galactosidase activity with a wide range of flavonoid compounds and was shown to require a functional *nodD* gene to induce promoter activity (Bassam *et al.* 1988).

### 3.4 Key features of NGR234 NodD1 activating compounds

The key features of compounds able to induce expression of NGR234 NodD1-dependent *nod* genes in the test system used for these experiments were determined by comparison of the characteristics of potent inducers, weak inducers, and non-inducing compounds. These features are illustrated in Figure 3.3 and can be summarised as follows:

i) The presence of an hydroxyl (-OH) group *para* to an electron-withdrawing function increases biological activity, for example -OH *para* to a carbonyl function as in 4-hydroxybenzoic acid, or in 7-hydroxyflavone.

ii) The presence of a cluster of oxygen functions increases biological activity, for example pyrogallol.

iii) Hydroxylation of the B-ring increases potency of flavones (that is, decreases *A*₅₀).

### 3.5 Discussion

This chapter reports the first case of monocyclic, plant derived, phenolic compounds contributing to transcriptional activation of the NodD-dependent *nod* genes of *Rhizobium*. Furthermore, a wide variety of polycyclic, flavonoid compounds were found to induce transcription of the NodD-dependent *nod* genes.

Many of the flavonoid compounds examined here for induction of NGR234 NodD1-dependent *nod* gene transcription have been identified in legume sources: for example DHF, Geraldone, 4’-hydroxy-7-methoxy-flavone, formononetin and umbelliferone from white clover roots (Redmond *et al.* 1986); genistein, daidzein, coumestrol (from roots), kaempferol, naringenin, quercetin, genistein, and daidzein (from leaves) of soybean (Porter *et al.* 1986), are just a few. Of the monocyclic phenolic compounds examined in the experiments reported in this chapter, many are intermediates in the phenyl propanoid pathway, or breakdown products of flavonoid compounds.

Plant substances capable of inducing optimal expression of NodD-dependent *nod* genes identified for other rhizobia have specific structural characteristics, such as hydroxylation of the flavonoid skeleton on both the A- and B-rings, at the 7 and 4’ positions of both flavones and flavanones (refer to the legend to Figure 3.2 for the ring designations and numbering system for flavonoids) (Firmin *et al.* 1986; Redmond *et al.* 1986; Kapulnik *et al.* 1987; Györgypal *et al.* 1988). Induction of the *nod* genes of *R. leguminosarum* biovars also occurs with compounds hydroxylated at the 3’ position and
activity is further enhanced if a methoxy group is present at the 4' position. At least one hydroxyl group is required on the B-ring for activity (Firmin et al. 1986). Virtually any substitution at the 3 position results in inactive compounds (Firmin et al. 1986; Zaat et al. 1987).

Induction in *R. l. bv. trifolii* and *R. meliloti* Danegard is also observed with compounds hydroxylated at either or both the 5 and 3' positions (Peters et al. 1986). Antagonists of activation in these species are usually isoflavonoids (Djordjevic et al. 1987). In contrast, *B. japonicum*, a slow growing species, is activated by flavonoids similar to those described above and also by isoflavonoid compounds with the same general structures; however compounds hydroxylated at either or both the 3 and 5' positions are inactive (Kosslak et al. 1987; Göttfert et al. 1988).

In each class of naturally released bean flavonoids, increasing the number of free hydroxyl groups on the B ring decreased the $i_{\text{max}}$ value and increased the $I_{50}$ value, and the flavone molecule requires at least one hydroxyl group to activate *nodD* -dependent transcription in *R. l. bv. phaseoli* (Hungria et al. 1992).

The characteristics identified to govern activation of NodD in other rhizobia are in agreement with those identified in this work, however, they specify only a subset of the compounds capable of activating NodD1 of *Rhizobium* strain NGR234. Specifically, flavonoid compounds with C-ring substitutions at the 3 position for example hydroxyl (flavonols) or phenyl (isoflavonoids) groups also activate NGR234 NodD1. Further, a B ring hydroxyl is not absolutely essential for activity. The key features of compounds activating NGR234 NodD1 are detailed in Section 3.4. The identified features could be expected to have some value in predicting the structural features of compounds which will or will not be activators of NGR234 NodD1. For example, given its structure, shikimic acid (see Figure 3.2), despite a total of 3 hydroxyl groups would NOT be expected to be a NodD activator in this test system as it lacks the planar aromatic characteristics of the compounds with demonstrated activity.

Based on the results reported in this chapter, the range of compounds capable of activating NGR234 NodD1 extends to include a greater variety of complex, polycyclic phenolic compounds than identified for other rhizobia, in addition to the monocyclic phenolic compounds reported for the first time in this work. The product of the NGR234 *nodD1* gene may thus arise from a less specialised *nodD* allele, as postulated by Bender et al. (1988), perhaps retaining some aspects of an ancestral plant recognition gene. Alternatively, NodD1 from strain NGR234 may be a more versatile protein, incorporating the highly specialised interactive capacity of the narrow host range rhizobia along with the capacity to respond to simple phenolic compounds.

Chemically-induced single base substitution mutations to *R. l. bv. trifolii nodD* result in the extension of host range of the mutant strain to *Parasponia*, the non-legume host of strain NGR234 (McIver et al. 1989). Clearly *R. l. bv. trifolii* has most of the genes
Compounds inducing *nod* gene transcription in *Rhizobium* strain NGR234.

required to produce Nod metabolites which allow infection of this illegitimate host, since only point mutations of the *nodD* gene confer extended nodulation host range. The single base mutation presumably affects the final conformation of NodD, allowing activation of the protein and transcription of the *nod* genes (Fisher and Long, 1993).

Whilst a direct negative control of plasmid pMD1 (lacking the NGR234 *nodD1* gene) was not constructed, previous data from this laboratory in an almost identical construct indicated no significant induction of β-galactosidase activity in the absence of a functional *nodD* gene (Bassam *et al.* 1988). Although the two plasmids have different *nod*-box promoters, there have been no reports of substantial differences in promoter function between the highly conserved *nod*-boxes of different rhizobia, despite extensive investigation (Rostas *et al.* 1986; Schofield and Watson 1986; Scott 1986; Shearman *et al.* 1986; Fisher *et al.* 1987; Spank *et al.* 1987b; Fisher and Long 1989).

A significant DNA homology was noted between the *nodD* genes of several *Rhizobium* species and the *nahR* gene of *Pseudomonas putida* (Schell and Sukordhaman 1989). NahR is a transcriptional activator of the *nah* and *sal* operons, activating transcription of the *sal* promoter only in the presence of salicylate (a salt or ester of salicylic acid). It is interesting to note that NGR234 NodD1 is not activated by salicylic acid. Both *nahR* and *nodD* have been shown to belong to the LysR family of prokaryotic transcriptional regulators (Henikoff *et al.* 1988; Schell and Sukordhaman 1989). This family has over 50 members and the progenitor transcriptional regulator is postulated to have arisen early in prokaryotic evolution since there are large genetic distances between the members of the LysR family (Schell 1993).

The expression of *A. tumefaciens vir* genes (required for plant cell transformation and crown gall formation) is dependent upon a two component regulatory system. The *virA* gene product interacts with simple phenolic compounds and monosaccharides (via ChvE) to activate VirG, which induces expression of the remaining *vir* genes (Winans *et al.* 1986). High levels of induction do not occur at neutral pH, and induction is most efficient at acid pH (Stachel *et al.* 1986; Rogowsky *et al.* 1987). Phenolic inducers of the *vir* genes include acetosyringone, α-hydroxy acetosyringone, acetovanillone, syringaldehyde and sinapinic acid (Stachel *et al.* 1985a), all of which are monocyclic phenolic compounds. Acetosyringone production is significantly increased in wounded tissue (Stachel *et al.* 1985b) and other active compounds are found at sites of lignin production such as root elongation zones and tissue damage repair sites. Lignin has also been shown to be present on legume root hair surfaces in some circumstances (R. Ridge, personal communication) shown in Figure 3.4. *Rhizobium nod* genes are also induced by acetosyringone, acetovanillone, and syringaldehyde in the presence of the NGR234 *nodD1* gene, but not by sinapinic acid. Thus some wound-induced compounds, lignin production intermediates, and degradation products will induce NGR234 NodD1-dependent *nod* genes, and are likely to be found in the wound-like pre-nodule stage of *Parasponia* nodulation. Alfalfa tissues have been shown to produce the clover signal
compound 7,4'-dihydroxyflavone (DHF) in response to ozone wounding (Hurwitz et al. 1979) and thus, under certain circumstances, DHF can also be considered a wound product.

A number of phenolic acids were found to inhibit cell growth during β-galactosidase assay induction periods (of 2 h), despite some also being activating compounds (for example, vanillin and 2,4 dihydroxybenzoic acid). These and similar compounds are utilised for their antimicrobial, particularly phytotoxic, activity, and for their plant growth regulating properties in a variety of situations. For example, vanillin is used in sugar cane cultivation in Hawaii to reduce fungal contamination and as a sugar cane ripener (Nickell 1982); in food technology, vanillin can be used to prevent aflatoxin development in cereals (Bilgrami et al. 1982); and in silk cultivation, vanillin is used to prevent silkworm diseases caused by Lactobacillus and Streptococcus spp. (Ajinomoto 1983).

The activation of NodD1 from strain NGR234 by a wide variety of monocyclic and polycyclic phenolic compounds may reflect a requirement of NGR234 to induce NodD-dependent nod genes in several environmental regimes other than during symbioses. For example, nod genes are also involved in the chemotactic response of rhizobia to compounds such as cinnamic and coumaric acids (Kape et al. 1991). The ability to sense a broad range of monocyclic phenolics at higher concentrations than would normally be present during pre-infection indicates that NGR234 is probably a competent soil saprophyte.

Bender et al. (1988) hypothesised that NGR234 NodD1 was activated to induce nod gene transcription by flavonoids in a non-specific manner, but the results reported in this chapter show that the activation of this gene product by phenolic/flavonoid compounds is quite specific, but occurs in response to a wider variety of structural features than for most other rhizobia (Le Strange et al. 1990).

The identification of monocyclic phenolic compounds as inducers of NodD-dependent nod genes in Rhizobium strain NGR234 has contributed to a clearer definition of the chemical nature of the inducer:NodD interaction, which may contribute to the further development of a model for the mechanism of action of the nodD-encoded protein.
Compounds inducing \textit{nod} gene transcription in \textit{Rhizobium} strain NGR234.

This page is intended to be left blank.
Table 3.1: Phenolic compounds assessed for biological activity with the *nodD1* gene of strain NGR234

<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Active Compounds (range $10^{-3}$ - $10^{-6}$ M)</th>
<th>Inactive Compounds (range $10^{-3}$ - $10^{-6}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>4-OH benzoic acid (A), 2,3-diOH benzoic acid (B,I), 2,4-diOH benzoic acid (B,I), 2,6-diOH benzoic acid (B,I), 3,4-diOH benzoic acid (A,I), pyrogallic acid (B), acetyl salicylic acid (A)</td>
<td>Benzoic acid (I), salicylic acid (I), 3-OH benzoic acid, 3,5 diOH benzoic acid, 3,4 diOMe benzoic acid, cinnamic acid (I), 2-coumaric acid, 3-coumaric acid, 4-coumaric acid (I), sinapinic acid</td>
</tr>
<tr>
<td>Phenols</td>
<td>Catechol (C), resorcinol (A), vanillin (C,I), isovanillin (C), quinol (A)</td>
<td>Anisole</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Benzaldehyde (A), 3-OH benzaldehyde (B) 3,4-diOH benzaldehyde (C), 3,4-diOMe benzaldehyde (A), syringaldehyde (C)</td>
<td>Salicylaldehyde, 2,4-diOMe benzaldehyde, cinnamaldehyde (I), piperonal</td>
</tr>
<tr>
<td>Ketones</td>
<td>3-OH acetophenone (C), 4-OH acetophenone (C), 2',4'-diOH acetophenone (C), 2',6'-diOH acetophenone (B), 2,3,4-triOH acetophenone (C), 4-OH,3'-OMe acetophenone (B), 4-OH,3-Me acetophenone (C), acetovanillone (C), 2-OH,4-OMe acetophenone (A), acetylsyringone (B), 2,4,4,,-triOH chalcone (B)</td>
<td>Acetophenone, 2-OH acetophenone, 4-OMe acetophenone</td>
</tr>
<tr>
<td>Chromone</td>
<td>7-OH chromone (A)</td>
<td>Fraxetin</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Coumarin (A), umbelliferone (C)</td>
<td></td>
</tr>
<tr>
<td>Coumestan</td>
<td>Coumestrol (C)</td>
<td></td>
</tr>
<tr>
<td>Isoflavone</td>
<td>Daidzein (C), biochanin A (C), formononetin (C), genistein (C)</td>
<td>Bayin, taxifolin, naringin, 4'-OH flavanone</td>
</tr>
<tr>
<td>Flavonol</td>
<td>Quercetin (B), kaempferol (C)</td>
<td>Flavone, 3-OH flavone, 7,4'-diOMe flavone, 5-OH,7-OMe flavone, diosmin</td>
</tr>
<tr>
<td>Flavone</td>
<td>7,4'-diOH flavone (C), morin (C), 7-OH flavone (C), chrysin (C), apigenin (C)</td>
<td></td>
</tr>
</tbody>
</table>


Maximal levels of induction: A, low level of induction (approximately 400 - 700 units), B, intermediate level of induction (700 - 1500 units), AND C, high level of induction (1500 - 4500 units) (background, $[\text{H}_2\text{O}]$ 300 units). I = inhibition of bacterial cell growth noted at concentrations of $10^{-3}$ or $10^{-4}$ M

β-galactosidase assays were performed as described in Section 2.1.6.1, using strain ANU265(pMD1), described in Section 3.2.1. Assays were performed in duplicate and repeated once.
Table 3.2: Relative induction levels of compounds inducing *Rhizobium* strain NGR234 NodD1-dependent *nod*-gene expression. (Indicated by β-galactosidase activity).

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Compound</th>
<th>log A\textsubscript{50} \textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>4-hydroxybenzoic acid</td>
<td>-4.3</td>
</tr>
<tr>
<td>Phenols</td>
<td>Vanillin</td>
<td>-6.3</td>
</tr>
<tr>
<td></td>
<td>Isovanillin</td>
<td>-5.5</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>Syringaldehyde</td>
<td>-5.3</td>
</tr>
<tr>
<td>Ketone</td>
<td>Acetophenone</td>
<td>-4.3</td>
</tr>
<tr>
<td>Chromone</td>
<td>7-Hydroxyxochromone</td>
<td>-3.5</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Umbelliferone</td>
<td>-5.2</td>
</tr>
<tr>
<td>Coumestan</td>
<td>Coumestrol</td>
<td>-7.3</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>Daidzein</td>
<td>-8</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>-8</td>
</tr>
<tr>
<td></td>
<td>Formononetin</td>
<td>-6.3</td>
</tr>
<tr>
<td></td>
<td>Biochanin A</td>
<td>-6.3</td>
</tr>
<tr>
<td>Flavonol</td>
<td>Quercetin</td>
<td>-7</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>-7.5</td>
</tr>
<tr>
<td>Flavanone</td>
<td>Naringenin</td>
<td>-7.3</td>
</tr>
<tr>
<td></td>
<td>Hesperetin</td>
<td>-7</td>
</tr>
<tr>
<td>Flavone</td>
<td>7-Hydroxyflavone</td>
<td>-7.3</td>
</tr>
<tr>
<td></td>
<td>7,4'-Dihydroxyflavone</td>
<td>-8</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>-8.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} log A\textsubscript{50}, the log\textsubscript{10} of the concentration (M) at which induction is half-maximal for each compound. β-galactosidase assays were performed as described in Section 2.1.6.1, using strain ANU265(pMD1), described in Section 3.2.1. The assays from which this table is derived were performed in duplicate and repeated twice. Standard deviations were no greater than 25% for each assay.
Figure 3.1
Map of plasmid pMD1.

The locations of the 2.9 kb EcoRI fragment containing the nodD1 gene from 
*Rhizobium* strain NGR234 (Bassam *et al.* 1988) and the 0.7 kb PstI fragment 
containing the nodA promoter region from strain ANU843 (McIver *et al.* 1989) inserted into the multi-linker of plasmid pMP220 (Spaink *et al.* 1987a) are shown. The nodA promoter is positioned to drive transcription of 
the promoterless lacZ gene in the vector (arrow). The nodA promoter region 
contains 207 bp of the 5' region of the ANU843 nodD gene (stippled area); 
the nodD/nodA intergenic region (crosshatched area); the nod-box (black 
vertical bar); the nodA coding region (unshaded area); and 116 bp of 
MudIII1734 DNA (horizontal shaded area) inserted in the 5' end of the nodA 
coding region (McIver *et al.* 1989). Plasmid pMP220 is not drawn to the 
same scale as the inserted fragments. Other sites in the multi-linker are not 
shown. E = EcoRI; P = PstI; and H = HindIII.

Le Strange *et al.* 1990.
Figure 3.2
The phenyl-propanoid pathway, highlighting compound types found to activate NodD1 of strain NGR234.

* compound classes which activate NodD1 of strain NGR234, inducing the transcription of the nodA::lacZ fusion of plasmid pMD1.

PAL - phenylalanine ammonia-lyase; C4H - cinnamate 4-hydroxylase; 4CL - 4-coumaroyl co-enzyme; CHS - chalcone synthase; CHI - chalcone flavone isomerase; F3H - flavanone 3β-hydroxylase; DFR - dihydroflavonol 4-reductase; IFS - isoflavonoid synthase; FLS - flavonol synthase


Below: the ring structure and numbering convention for flavonoids.
Figure 3.3

Representative substances that induce *Rhizobium* strain NGR234 NodD1-dependent *nod* gene transcription in strain ANU265(pMD1).

Modifications to the basic structures are listed numerically as follows, with the effect on *nodA* induction also being indicated ($A_{50} = \text{concentration at which induction is half-maximal; increase in } A_{50} \text{ decreases potency as an inducer}$) - at circle position:

1. removal of the OH group increases $A_{50}$;
2. the OH group is essential for activity;
3. an OH group is necessary in at least one of the positions;
4. both functional groups are required for activity, but may be interchanged;
5. an OH group at this site decreases $A_{50}$;
6. reduction of the double bond does not significantly affect activity;
7. an OH group increases $A_{50}$;
8. an OH group at this site marginally lowers $A_{50}$; and
9. a methoxy (CH$_3$O) group at this site increases $A_{50}$. 
pyrogallol: $A_{50} = 1 \times 10^{-5}$ M

vanillin: $A_{50} = 5 \times 10^{-7}$ M

4-hydroxybenzoic acid: $A_{50} = 5 \times 10^{-5}$ M

umbelliferone: $A_{50} = 7 \times 10^{-6}$ M

acetophenone: $A_{50} = 5 \times 10^{-5}$ M

7-hydroxyflavone: $A_{50} = 5 \times 10^{-8}$ M

7-hydroxychrome: $A_{50} = 3 \times 10^{-4}$ M

daidzein: $A_{50} = 1 \times 10^{-8}$ M

coumestrol: $A_{50} = 5 \times 10^{-8}$ M
Figure 3.4

The occurrence of lignin on root hairs.

Section of a developing root nodule on subterranean clover. When nodules are formed as a result of infection by rhizobia, lignin is produced and is laid down in the cell wall of the developing nodule. Section stained with methylene blue, lignin shows as turquoise.

Photograph reprinted with the kind permission of Dr Robert Ridge.
Chapter Four

Compounds from non-host plants activate strain NGR24 KNO3DL, including simple phenolics isolated from wheat extract.
Chapter Four

Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

4.1 Introduction

Flavonoid compounds identified from extracts and exudates of leguminous plants have been shown to induce the transcription of NodD-dependent nod genes in rhizobia which normally nodulate these plants. The flavones 7,4'-dihydroxyflavone (DHF) and geraldone were isolated from white clover roots and activated NodD-dependent nod gene transcription in R. l. bv trifolii (Redmond et al. 1986); apigenin and eriodictyoil from pea extracts induced nod gene expression in R. leguminosarum (Firmin et al. 1986; Spaink et al. 1987b); luteolin from alfalfa induced nod gene expression in R. meliloti (Peters et al. 1986); and the isoflavone daidzein from soybean was shown to induce NodD-dependent nod gene transcription in B. japonicum (Kosselak et al. 1987).

The nodD1 gene from strain NGR234, when transferred to the narrow host range R l. bv trifolii strain ANU843, extended the host range of the mutant strain to include siratro, Vigna unguiculata, Glycine ussuriensis, Leucaena leucocephala (Bassam et al. 1988) and, when transferred to R. meliloti, extended host range to siratro (Horvath et al. 1987). Bender et al. (1988) demonstrated the extension of host range to the non-legume tree Parasponia by transferring the same nodD1 gene to a R. l. bv. trifolii nodD mutant.

Compounds capable of activating NGR234 NodD1-dependent nod gene transcription are clearly present in each of these plants, and the ability of NodD to recognise flavonoids is an integral part of host recognition and nodulation specificity.

The results reported in Chapter Three of this thesis indicated that a wide range of compounds, far wider than the range of NodD activating flavonoids reported for narrow host range rhizobia, activate NodD1 from strain NGR234. The aim of the research presented in this chapter was to establish whether extracts of non-host plants would also activate NodD1 from strain NGR234, particularly since monocyclic phenolic compounds are found in all plants.

A variety of plant extracts were assessed for activation of this transcriptional regulator protein. Extracts prepared from the roots of host and non-host plants were examined for nod gene inducing activity and the monocyclic phenolic compounds vanillin and isovanillin, which were shown to activate NGR234 NodD1-dependent nod gene transcription in Chapter Three, Table 3.1, were identified from fractionated wheat seedling extracts.
Selected results of the research presented in this chapter were published in Bender et al. 1988 and in Le Strange et al. 1990.

4.2 Experimental Procedure

The work described in this chapter was initiated in parallel with the work described in Chapter Three. A variety of plant species were selected for the preparation of plant extracts. The extracts were to be assessed for their capacity to activate NGR234 NodD1-dependent nod gene transcription in β-galactosidase assays. Plants were selected principally for the following reasons: siratro (Macroptilium atropurpureum) - a legume host of strain NGR234; Parasponia andersonii - a non-legume host of NGR234; Trema aspera - closely related to Parasponia, does not form nodules with any rhizobia but does contain plant haemoglobin genes; Casuarina cunninghamiana, a non-legume which forms nitrogen-fixing root nodule symbioses with Frankia; the crop plants sunflower, cotton, and the monocotyledonous crops wheat, rice, and maize for their agricultural importance. In addition, all plants were available at the Research School of Biological Sciences, Australian National University. Plant extracts were prepared as described in Section 4.2.1.

Plasmid pMN40 had been constructed in this laboratory for use in plant nodulation assays and was shown to have a functional copy of the NGR234 nodD1 gene (Bender et al. 1988). Plasmid pMN40 is shown in Figure 4.1. Introduction of plasmid pMN40 to strain ANU894, (containing an inducible, translational fusion of the E. coli lac operon with the nodA of the Sym plasmid of strain ANU843, by the method described in Section 2.4.7) enabled β-galactosidase assays to assess the induction of NodD-dependent nod genes in a R. l. bv. trifolii background, under the control of NGR234 NodD1. β-galactosidase assays were performed as described in Section 2.1.6.1.

Following the successful activation of NGR234 NodD1 by all of the prepared plant extracts, further extracts of Parasponia, siratro, and wheat were prepared (as described in Sections 4.2.1 to 4.2.4) and subjected to thin layer and paper chromatography (described in Sections 4.2.5 & 4.2.6) in an effort to make preliminary identification of the activating compounds. Eluted spots were assessed for NGR234 NodD1-dependent nod gene induction by β-galactosidase assay using strain ANU265(pMD1), described in Chapter Three, Section 3.2.1 and Figure 3.1 (Le Strange et al. 1990).

Various techniques were evaluated to develop a large scale method for extracting NGR234 NodD1 activating compounds from wheat seedlings (Section 4.2.8). Large scale extracts of wheat seedlings were prepared for analysis by HPLC (Section 4.2.9) to identify activating compounds, and then extracts of several varieties of wheat were prepared and examined by β-galactosidase assay and HPLC analysis (described in Section 2.2.2).

Finally, exudates (in contrast to extracts) from wheat were prepared and examined to
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

assess the likelihood of wheat exuding NGR234 NodD1 activating compounds into the rhizosphere.

4.2.1 Collection of plant extracts (general method)

Freshly germinated, undamaged, seedlings (wheat or siratro) or excised root tips (Parasponia) (approximately 5 g, prepared as described in Sections 2.3.2 to 2.3.4) were submerged in a minimum volume of 50% ethanol in a screw-topped vial (10 ml). Nitrogen was bubbled through the suspension (to eliminate oxygen and thus reduce the degradation of activating compounds) for 5 min, and the vial was sealed and placed in darkness for 2 h to facilitate extraction of activating compounds from the tissues. The suspension was then filtered through Whatman No. 4 filter paper to remove suspended matter. Nitrogen gas was again bubbled through the filtrate to eliminate oxygen. The filtrate was used immediately for β-galactosidase assays or stored at -20°C.

4.2.1.1 Acid hydrolysis of glycosides (in the H₂O phase).

The purpose of this technique was to achieve hydrolysis of any flavonoid glycosides present in the plant extract and thus release the flavonoid molecules. The extract sample was concentrated to half its original volume under reduced pressure and an equal volume of 4M trifluoroacetic acid (TFA) added in a glass test tube. The test tube was sealed under flame and heated in boiling water for 3 h. Following cooling, the tube was carefully broken open and the contents extracted twice with an equal volume of dichloromethane. The combined dichloromethane extracts were dried under N₂ and resuspended in methanol.

4.2.2 Imbibed wheat extracts

Wheat seeds (approximately 100 g) were washed in water and soaked in 96% ethanol for 5 min. After surface sterilisation, seeds were thoroughly rinsed in sterile distilled H₂O and left in sterile distilled H₂O (2 volumes) to imbibe overnight. Drained seeds were added to boiling ethanol and boiled for 10 min. When cool, the extract was filtered through Whatman No. 4 filter paper and the filtrate dried down completely under vacuum to eliminate remaining traces of water. The residue was redispersed in 100% ethanol, dried again, and redispersed in methanol. When fully dissolved, chloroform (3 volumes) was added and the mixture blended thoroughly, allowed to stand for 15 - 30 min and filtered to remove any remaining particulate matter.

4.2.3 Siratro seed extract

Siratro seeds (5,000) were prepared for germination (Section 2.3.4), and imbibed overnight in sterile distilled H₂O (3 volumes). The imbibed seeds were drained, boiled in ethanol in batches, and the mixtures pooled, cooled and filtered through Whatman
Chapter Four

No. 4 filter paper. The filtrate was concentrated under vacuum to half its original volume and 4 volumes of chloroform were added. The filtrate mixture was perfused with \( \text{N}_2 \) gas and left at room temperature for 16 h. The resulting mixture was adsorbed onto silicic acid (5 g) and applied to a column (100 x 20 mm) of silicic acid packed in 10% methanol/chloroform. Elution was performed with the same solvent (500 ml), 25% methanol/chloroform (500 ml), and 100% methanol (200 ml). Eleven fractions of 100 ml were collected. Each fraction was evaporated under vacuum to dryness and resuspended in HPLC grade methanol.

4.2.4 Preparation of plant extracts for chromatography

Extracts of plant materials were prepared from: Australian Standard White (ASW) wheat - imbibed seed and germinated seedlings (as described in Sections 4.2.1 & 4.2.2); Siratro - seedlings and roots (as described in Section 4.2.3); and Parasponia - roots and root tips (as described in Section 4.2.1). In addition, extracts were prepared from wheat seeds (imbibed only) and seedlings which had been bruised to induce wound response compounds by being encased in plastic and lightly pounded with a heavy, flat implement.

Each plant extract was evaporated under vacuum and resuspended methanol (3.5 ml). After adjusting pH to 2 with 4N HCl to prevent the ionization of the phenolic/flavonoid compounds, the mixture was re-evaporated to dryness under vacuum. The residue was resuspended thoroughly in methanol (300 µl), distilled \( \text{H}_2\text{O} \) (1.7 ml) added in aliquots (300 µl), with thorough mixing after the addition of each aliquot. Methylene chloride (2 ml) was added, the mixture thoroughly vortexed, and centrifuged for 10 min at 3000 rpm. The resulting residue was dried in a stream of \( \text{N}_2 \), and redispersed in methanol (400 µl).

4.2.5 Thin layer chromatography (TLC)

TLC plates (20 x 20 cm) (Section 2.2.1) were carefully cut to the required size (10 x 10 cm) with a guillotine. Any disturbed edges were smoothed with a sharp scalpel blade. A lead pencil was used to mark out the sample loading positions. A fine glass capillary tube was used to spot the prepared sample (up to 2 µl) by repeated applications of sample onto the plate, with drying between successive applications. When all the sample and authentic spots were dry the plate was carefully placed into a glass chromatography tank which had been pre-equilibrated with solvent vapours. Following development of the chromatogram, the plate was allowed to dry and examined under short and long wave ultra-violet light, then exposed to ammonia vapour and re-exposed to UV light. After the ammonia had completely evaporated from the plate, diazotized sulphanilic acid (Section 4.2.7) was sprayed onto the chromatogram and the spots again examined.

Solvent mix used for thin layer chromatography: 5%v/v Methanol in Chloroform.
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

4.2.6 Ascending paper chromatography

After preparation of the appropriate solvent, chromatography paper was cut to the appropriate size and washed by a descending solvent wash. Plant extract samples were spotted onto the dried paper from a 10 x concentrate in methanol, and applied from a 2 µl spot pipette. After the spots were dry, the paper was anchored within the chromatography tank. Following equilibration with solvent vapour in the sealed tank overnight, solvent was added to the lower tank reservoir and the chromatogram developed until the solvent front was near the top of the paper (1-2 days). After drying, the paper was examined under short and long wave ultra-violet light, then exposed to ammonia vapour and re-exposed to UV light. After the ammonia had completely evaporated from the paper, diazotized sulphanilic acid (described Section 4.2.7) was sprayed onto the chromatogram and the spots again examined.

Solvent mix used for paper chromatography: 1-butanol, glacial acetic acid and water were mixed in the ratio 4:1:5 in a separating funnel. Following separation the upper layer was collected for use.

4.2.7 Diazotized sulphanilic acid spray (Markham 1982)

An 0.3% solution of sulphanilic acid in 8% HCl (Solution A - 25 ml) was mixed with 5% sodium nitrite solution (Solution B - 1.5 ml) just prior to use. After spraying this mixture on to the chromatogram, a follow-up spray of 20% sodium carbonate (Solution C) was applied.

Solution A, per 100 ml: 0.3 g sulphanilic acid (NH₂C₆H₄SO₃H·H₂O); 8 ml conc. HCl.
Solution B, per 100 ml: 0.5 g NaNO₂.
Solution C, per 100 ml: 20 g Na₂CO₃

4.2.8 Development of methods for large scale extracts of wheat seedlings

Growth of wheat seedlings. Initially, small numbers of wheat seedlings (approximately 50) were grown on agar plates (as described in Section 2.3.3). These seedlings required careful harvesting to eliminate agar contamination. Furthermore, a very large number of seedlings was required to produce to final extract. Based on the technique described by Canter-Cremers et al. (1986), wheat seeds were subsequently grown hydroponically in sterile distilled water on metal grids in small glass containers at 25°C for 2-4 days. This technique initially yielded 100-150 seedlings, and was later scaled up to large plastic containers each yielding approximately 1000 seedlings.

Harvesting wheat seedlings. Extracts of isolated wheat roots were preferable to extracts of whole seedling, however, separating roots from the remaining plant without
damage to the root tissue was extremely labour intensive and impractical to continue with on a large scale. Thus whole seedlings were used to prepare extracts. A technique was required that would preserve the compounds in the seedlings. Commencing with the technique described in Section 4.2.1, a variety of procedures were trialed. Procedures which were evaluated included samples of wheat seedlings being:

- i) homogenised in water and then boiled;
- ii) homogenised in boiling water;
- iii) homogenised in ethanol and then boiled;
- iv) homogenised in boiling ethanol;
- v) homogenised in boiling water to which boiling ethanol (5 vols) was added and the mixture further boiled; and,
- vi) snap frozen then homogenised and boiled as in each of i) to v).

**Dichloromethane (I) extraction.** Following initial extraction of plant material in ethanol or methanol (as described in Section 4.2.1), the sample was dried under an N₂ stream, resuspended in 15% methanol/H₂O (1 ml), acidified to pH 2 using 4N HCl (40 µl), extracted with dichloromethane (2 vols), mixed, spun and separated. The lower (dichloromethane) layer contained phenolic compounds, and was dried down and resuspended in methanol. The upper, aqueous layer was acidified to pH 2 using 4N HCl (40 µl) and further extracted with dichloromethane (2 vols). The lower (dichloromethane) layer contained acids and was dried down and resuspended in methanol. The upper, aqueous, layer was retained.

**Dichloromethane (II) extraction.** Following extraction of plant material in ethanol or methanol (as described in Section 4.2.1) the sample was dried under an N₂ stream, and resuspended in H₂O containing 15% methanol and 1 mg/ml NaHCO₃ (1 ml), extracted with dichloromethane (2 vols), mixed, spun and separated. The separated layers were treated as in Dichloromethane (I).

**Ethyl acetate extraction.** Following initial extraction of plant material in ethanol or methanol (as described in Section 4.2.1) the sample was acidified to pH 3, an equal volume of ethyl acetate added, and extracted thoroughly in mixing funnel. The upper layer was retained and the aqueous lower layer was returned to the funnel for a second extraction against ethyl acetate (1 vol). The combined organic extracts were dried down rapidly under reduced pressure at no greater than 30°C and the residue redissolved in methanol.

**Methanol/Chloroform extraction.** Following initial extraction of plant material in ethanol or methanol (as described in Section 4.2.1), the filtrate was evaporated to dryness under reduced pressure, and suspected active compounds extracted from the residue into methanol (100 ml) by trituration. The dispersion was diluted with chloroform (3 vols), mixed thoroughly, allowed to stand for 2 hr and filtered. The filtrate was evaporated under reduced pressure and the residue reconstituted in methanol.
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

4.2.9 Preparative chromatography of wheat seedling extract

ASW wheat was surface sterilised by soaking in 70% ethanol for 5 min, followed by thorough washing in sterile distilled water. After imbibing for 15 min, the wheat seeds were grown hydroponically in sterile distilled water on metal grids at 25°C for 2-4 days (Canter-Cremers et al. 1986). The seedlings were drained and snap-frozen in liquid nitrogen.

Batches (500 g) of frozen whole seedlings were homogenised in boiling distilled water (100 ml). Ethanol (500 ml) was added, and the mixture was boiled for 15 min, cooled, and filtered through Whatman No. 4 filter paper. The filtrate was evaporated to dryness under reduced pressure to remove all traces of water, and suspected active compounds extracted from the finely ground residue into methanol (100 ml) (ie the residue was triturated in methanol). The dispersion was diluted with chloroform (3 vols), mixed thoroughly, allowed to stand for 2 hr and filtered. The filtrate was evaporated under reduced pressure and the residue reconstituted in methanol (5 ml).

Combined extracts from approximately 85,000 ASW seedlings were prepared as described above, preadsorbed onto silicic acid (25 g) and applied to the top of a column (200 x 30 mm) of silicic acid packed in 10% methanol/chloroform. Elution was conducted with the same solvent (450 ml), 40% methanol/chloroform (450 ml) and methanol (450 ml), and 12 fractions of 110 ml were collected (designated A1 - A12). Fractions were assayed for β-galactosidase activity. Activity was detected in fractions A2, A6, and A12 (Section 4.7).

Fraction A2 was dried down and dissolved in a small volume of methanol with water being added until the solution was 5% methanol/water. This was applied to a column (100 x 10 mm) of Matrex Silica C18 (reverse-phase adsorbent) and eluted with 45 ml of methanol/water mixtures from 5%, 10%, 20%, 40%, 60%, and 100% methanol. Seven fractions of approximately 45 ml were collected (B1 - B7). Fractions B2 and B3 (55 to 135 ml) contained inducing activity (for details see Section 4.7) and were combined, rechromatographed on a column of silicic acid, and eluted with methanol/methylene chloride (1 to 100%). Ten fractions of 25 ml were collected (C1- C10). The resultant active fraction C2 (25 - 50 ml) was evaporated carefully in a nitrogen stream and subjected to analysis by reversed-phase HPLC as described above. (See Figure 4.2 for a summary of the wheat seedling extract fractionation procedure).

RESULTS

4.3 Extracts of non-legumes activate NodD1-dependent nod genes

(This work was carried out in conjunction with G. Bender)

β-galactosidase assays to assess the induction of NGR234 NodD1-dependent nod genes
of strain ANU894(pMN40) were performed using the method described in Section 2.1.6.1. Each of the 9 plant extracts examined showed significant β-galactosidase activity when compared to the assay background of sterile distilled water. These assay results are detailed in Table 4.1. The parental strain (ANU894) showed no activity in response to the plant extracts.

For comparison, and based on the data reported in Table 4.1, where β-galactosidase activity induced in strain ANU894(pMN40) by 1 x 10^{-6} M DHF is taken to be 100%, the various plant extracts induced the following levels of β-galactosidase activity: Siratro, 75%; Trema, 69%; Parasponia, 64%; Wheat, 58%; Cotton, 48%; Sunflower, 41%; Rice, 37%; Casuarina, 22%; and Maize, 21%.

Although extracts of this wide variety of non-host plants induced β-galactosidase activity, when strains NGR234, ANU843(pMN40), or ANU851(pMN40) were inoculated onto these plants no nodules were produced (Bender et al. 1988).

4.4 Glycosides of activating flavonoids are not present in extracts of wheat, siratro and Parasponia

Extracts of samples of wheat, siratro and Parasponia, prepared as described in Section 4.2.1, were subjected to acid hydrolysis, described in Section 4.2.1.1, to release flavonoids from any glycoside substituents. β-galactosidase assays (described in Section 2.1.6.1) using strain ANU265(pMD1) (described in Section 3.2.1) showed no activity with the acid treated samples, indicating that no activating flavonoids with glycoside substituents were present in the extracts of wheat, siratro or Parasponia (data not shown).

During these experiments it became clear that insufficient Parasponia material would be available for any larger scale preparation of extracts. Neither plant nor seed stock is available commercially, necessitating continual on-site cultivation. Lack of available seed stock and plant material dictated that further analysis of root extracts of Parasponia be abandoned.

4.5 Chromatographic analysis of plant extracts

4.5.1 Thin layer and paper chromatography

Extracts of ASW wheat (imbibed seeds and germinated seedlings, both bruised and unbruised - prepared as described in Sections 4.2.1 and 4.2.2); Siratro (seedlings and roots - described in Section 4.2.1); and Parasponia (roots and root tips - described in Section 4.2.1) were purified (as described in Section 4.2.4) and subjected to thin layer chromatography (as described in Section 4.2.5) and ascending paper chromatography (as described in Section 4.2.6) beside authentic samples of flavonoid compounds. The
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

Chromatograms containing separated extracts were visualised under short and long wavelength UV light for the presence of fluorescence in various colours, indicative of many flavonoids, and for dark spots, characteristic of monocyclic phenolic compounds. Following exposure to ammonia vapour the chromatograms were again examined under UV light and modifications to any fluorescence noted. For example, light blue fluorescence under UV modified by exposure to ammonia to fluorescent yellow- or blue-green is a characteristic of flavones and flavanones lacking a free 5-hydroxyl or of flavonols lacking a free 5-hydroxyl but with a 3-hydroxyl substituted; deep purple fluorescence under UV not modified by exposure to ammonia is a characteristic of flavones or flavonols with 5-OH but with the 4'-OH absent or substituted (Mabry et al. 1970). Treatment with diazotized sulphanilic acid spray (as described in Section 4.2.7) resulting in spots of yellow, orange or red is indicative of compounds with free hydroxyl groups (Markham 1982).

These experiments indicated various polycyclic flavonoid and monocyclic phenolic compounds were present in samples of each plant extract (data not shown). Compound spots were cut from paper chromatograms, eluted, and the eluates assayed for activation of NGR234 NodD1. A wide variety of the eluted compounds showed the capacity to activate NodD1-dependent nod gene transcription in the test system ANU265(p:rvID1), as demonstrated by β-galactosidase activity (data not shown). However, these experiments did not enable the identification of separated compounds by comparison to authentic compounds using the techniques of TLC or paper chromatography described.

4.5.2 Column chromatography

Large scale extracts of siratro seeds were prepared as described in Section 4.2.3 and subjected to β-galactosidase assays (described in Section 2.1.6.1) using strain ANU265(pMD1) (described in Section 3.2.1) to assess activation of NGR234 NodD1. Significant β-galactosidase activity was detected in fractions 1, 3-6, and 8 of fractionated siratro seed extract (data not shown). Further purification of these siratro samples for HPLC routinely eliminated the activity of the fractions. It was decided to focus on the non-host plant wheat for identification of activating compounds, and no further examination of siratro extracts was undertaken.

4.6 Development of preparative scale extracts from wheat seedlings

A variety of techniques for the growth, harvesting and extraction of wheat seedlings were evaluated to arrive at a large scale method which allowed for the retention of maximal NGR234 NodD1 activation whilst removing water soluble plant compounds. These techniques are described in Section 4.2.8.

The extraction techniques took advantage of the low solubility in water of many phenolic compounds, particularly flavonoids. Standardised samples of crude wheat extract (10 ml) were subjected successively to each of the extraction techniques
described. Following each extraction, aqueous samples, TFA treated samples, chemically extracted samples and crude seedling extract were assayed for NodD-dependent nod gene induction using strain ANU265(pMD1) as described in Section 2.1.6.1. Levels of β-galactosidase activity were compared in each sample to assess the effectiveness of extraction of the activating compounds. TFA treated samples generally showed no significant activity, indicating the absence of activating flavonoids with glycoside substituents in the crude wheat extract. Comparisons between samples evaporated by different methods showed significantly less activity in those samples evaporated under an N₂ stream, than in those evaporated under reduced pressure. This difference was attributed to the increased loss of volatile phenolic compounds (able to be smelt during the evaporation processes). Evaporation under reduced pressure (using a rotavap) was thereafter routinely used in preference to a N₂ stream for drying down extracts.

The extraction techniques were used successively to arrive at a final method which could be used on a preparative scale to produce a purified wheat seedling extract suitable for analysis by HPLC.

4.7 Chromatographic analysis of wheat seedling extracts - characterisation of simple phenolic compounds

The preparation and preparative chromatography of wheat seedling extract and analysis of purified fractions by HPLC are described in Section 4.2.9 and summarised in Figure 4.2.

Compounds resulting in substantial β-galactosidase activity (assayed using reporter strain ANU265(pMD1), described in Tables 2.1 & 2.2, via the method described in Section 2.1.6.1) were found to be present in wheat seedling extract fractions A2, A6, and A12 (prepared as described in Section 4.2.9). Initial experiments indicated that DHF elutes at approximately fraction A6, and that highly hydroxylated and glycosylated flavones require 100% methanol for elution (fraction A12) (data not shown). Thus, fraction A2 appeared to contain a new class of active compound and was selected for further purification. Fractions B2 and B3 and C2 were derived from fraction A2 as described in Section 4.2.9, and showed activity in β-galactosidase assays.

Vanillin and isovanillin were characterised from fraction C2 of ASW wheat seedling extract by retention times in reversed-phase HPLC analysis and confirmed by UV spectrum analysis of the identified peaks in the stop-scan mode (as described in Section 2.2.2) (Figures 4.3 and 4.4).

Vanillin and isovanillin are structural isomers and have almost identical retention times under the HPLC conditions used. Close inspection of the UV spectra of eluates indicated a mixture of the two compounds present in ASW wheat seedlings extract. The mixture could not be further separated under the conditions available, but comparison
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

with standard preparations and their UV spectra under the same conditions indicated that the peak consisted predominantly of vanillin (Figure 4.4).

The eluate from the HPLC runs was collected and β-galactosidase assays performed on concentrated samples. The only active peak identified in fraction C2 was that of peak A (Figure 4.3), identified as vanillin/isovanillin. Other peaks present in the HPLC trace were thus not investigated further.

4.8 Vanillin, a compound activating NGR234 NodD1, is found in Australian wheat varieties

Samples of wheat varieties were processed by the same protocol described in Section 4.2.9, but on a smaller scale using approximately 2,000 seedlings of each variety, to identify an active fraction equivalent to fraction A2 of ASW. The active fractions were then analysed by reverse-phase HPLC and UV spectrum analysis as described in Section 2.2.

Whole extracts of all wheat varieties induced the nodA promoter of ANU265(pMD1). After analysis of comparable active fractions, vanillin and/or isovanillin could be unequivocally shown by HPLC to be present in samples of the following varieties: ASW, Halberd, Suneca, Rosella, Hartog, and Vulcan. In the remaining two varieties, Matong and Eradu, no clear HPLC identification of vanillin or isovanillin could be made at the levels of detection available because of unidentified overlapping peaks. Comparison of vanillin peak heights from HPLC data to known quantities of standard vanillin under identical conditions allowed approximate calculation of the amount of vanillin present in wheat variety preparations. The vanillin content was found to be between approximately 1 to 4.4 x 10^-8 moles/100g seeds. The maximal level of induction ($i_{max}$) was determined following β-galactosidase assays using increasing concentrations of extracts of each wheat variety. Table 4.2 details the characteristics of each wheat variety, the $i_{max}$, and the approximate vanillin content.

4.9 Wheat exudate co-activates nod gene transcription

In order to ascertain whether compounds which activate NGR234 NodD1 might be exuded into the rhizosphere surrounding wheat seedlings, exudate was collected from 500 seedlings of ASW (grown as described in Section 4.2.8 - Growth of wheat seedlings). Exudate was collected, strained, and directly assayed for β-galactosidase activity (as per the protocol described in Section 2.1.6.1) with reporter strain ANU265(pMD1) (Section 3.2.1). The exudate was further examined for the growth of contaminating bacteria by culture on appropriate media (Section 2.1.2) under appropriate conditions. No viable bacteria could be cultured from the exudate preparation. The exudate resulted in a moderate level of β-galactosidase activity when compared to a sterile distilled water control, demonstrating that undamaged, sterile,
wheat seedlings excrete NGR234 NodD1-activating compounds. The exudate was assayed for endogenous β-galactosidase activity using the same assay protocol with strain ANU265(pMD1) and no activity was detected.

4.10 Discussion

It is to be expected that extracts prepared from the normal host plants of *Rhizobium* strain NGR234, *Parasponia* and siratro, should activate NodD1 of this strain to transcribe NodD-dependent *nod* genes. It was interesting to find that extracts of non-host plants also contained stimulatory substances in sufficient concentration to induce *nod* gene transcription (data reported in Table 4.1). Two species with close familial associations to hosts of rhizobia are *T. aspera*, belonging to the same family as *Parasponia* and known to contain plant haemoglobin genes (Landsmann *et al.* 1986; Bogusz *et al.* 1988), and *C. cunninghamiana*, a non-legume which forms a nitrogen-fixing root nodule symbiosis with the actinomycete *Frankia*. It could easily be expected that both of these plants, non-hosts for strain NGR234, may contain stimulatory compounds in sufficient quantities to activate NGR234 NodD1. However, since wheat, rice and maize have no natural nitrogen fixing association with root-nodule bacteria, the activation of NGR234 NodD1 by extracts of these plants was less expected. This is most particularly true for monocotyledons since these plants are not only outside the host range of *Rhizobium, Bradyrhizobium* and *Azorhizobium*, but are also outside the host range of the only pathogenic member of the family Rhizobiaceae, *Agrobacterium*. Interestingly, *vir* gene activating compounds have also been found in extracts of oats and of wheat seeds (Usami *et al.* 1988). These are apparently different from acetosyringone, possibly being a phenolic structure conjugated to a hydrophobic molecule. Usami *et al.* (1988) also postulate the existence of inhibitors of *vir* gene induction in monocotyledonous tissue.

The induction of the expression of NGR234 NodD1-dependent *nod* genes by extracts of non-legumes other than *Parasponia* and siratro does not extend to nodule formation on these plants by strains NGR234 or ANU843(pMN40) (Bender *et al.* 1988). Nor does the induction of *A. tumefaciens* *vir* genes by wheat and oat extracts result in crown gall formation (Usami *et al.* 1988).

The interesting observation of activation of NGR234 NodD1 by wheat seedling extract (reported in Section 4.3, data shown in Table 4.1), and the easy availability of wheat seeds, made this monocotyledon highly suitable for further identification of stimulatory compounds. Initial experiments aimed at isolating inducing compounds from wheat extracts showed that significant losses of some inducing compounds could occur during processing for preparative chromatography. The final procedure described minimised losses of volatile, monocyclic phenolic compounds (described in Section 4.2.9).

Vanillin and isovanillin were identified in extracts of wheat seedlings, and authentic samples of these compounds in the presence of *nodD1* from strain NGR234 induced
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

expression of the \textit{nodA} promoter of \textit{R. l. bv. trifolii} strain ANU843 (ie. strain ANU265(pMD1), described in Section 3.3, Tables 3.1 and 3.2). Vanillin and isovanillin did not induce expression of the same promoter in the presence of the \textit{nodD} gene of strain ANU843. Bender et al. (1988) found that crude wheat extracts are capable of inducing \textit{nodD}-dependent \textit{nod} gene expression in conjunction with NodD1 from strain NGR234, but not NodD of strain ANU843. The findings reported in this chapter enlarge on those observations by identifying two of the activating compounds present in wheat seedling extract.

The fractionation procedure for wheat extract was optimised for isolation of simple phenolic compounds capable of inducing \textit{Rhizobium} strain NGR234 NodD1-dependent \textit{nod} genes. Other fractions of wheat extract also induced \textit{nod} gene expression in strain ANU265(pMD1) and, on the basis of the separation system used, and TLC and paper chromatography evidence, these fractions contained more complex flavonoid compounds. Since simple phenolic compounds were a newly identified group of NodD activators, the identification of these compounds was pursued, rather than identification of flavonoid or other polycyclic compounds.

A variety of simple and complex phenolic compounds have been identified previously in wheat, including tricin (Anderson 1932), vanillin glucosides, \textit{p}-hydroxybenzaldehyde, vanillin and syringaldehyde in graminaceous cell walls (Hartley and Keene, 1984); \textit{p}-coumaric acid in association with lignin and ferulic acid from non-lignified tissue (Scalbert et al. 1985); and apigenin glycosides from wheat bran (Feng et al. 1988). Of these, the aglycones syringaldehyde, vanillin and apigenin have been found to induce \textit{nodA} expression in strain ANU265(pMD1) (reported in this thesis in Chapter 3, data presented in Tables 3.1 and 3.2; Le Strange et al. 1990).

Whilst vanillin or isovanillin could not be unequivocally demonstrated in two of the wheat varieties examined due to overlapping peaks on the HPLC trace, the presence of one or both these volatile compounds could be confirmed by careful smelling of the fractionated extracts. Estimates of the vanillin content of the wheat variety extracts reported in Table 4.2 will, by their nature, understate the amount initially present in the extract since volatile compounds are partially lost during processing.

Identification of vanillin and isovanillin in extracts of a number of wheat varieties with quite different growth and yield characteristics indicated that these NGR234 NodD1 activating compounds are likely to be present in most wheat varieties, thus enhancing the potential value of the observation for application in the development of biological nitrogen fixation systems based on wheat/\textit{Rhizobium} strain NGR234 interactions.

Clearly, since simple phenolic compounds are ubiquitous in the plant kingdom, and since no anti-inducers have been identified for NGR234 NodD1, exudates from most plants releasing flavonoids or simple phenolic compounds could be expected to induce some NGR234 NodD1-dependent \textit{nod} gene activity. This was confirmed in the case of
wheat exudate, reported in Section 4.9. However, the presence of sufficient concentrations of activating compounds in the rhizosphere of most plants is unlikely, since the concentration of monocyclic phenolic compounds required for half-maximal induction ($A_{50}$) is generally above $10^{-6}$ M (Chapter 3, data presented in Table 3.2; Le Strange et al. 1990).
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.
Table 4.1 Response by *R. l. bv trifolii* strain ANU894, carrying the *nodD1* gene from *Rhizobium* strain NGR234, to seedling extracts.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>β-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANU894</td>
</tr>
<tr>
<td>None</td>
<td>22</td>
</tr>
<tr>
<td>7,4'-dihydroxyflavone (DHF)</td>
<td>509</td>
</tr>
<tr>
<td>Dicotyledons</td>
<td></td>
</tr>
<tr>
<td>Siratro</td>
<td>17</td>
</tr>
<tr>
<td><em>Paraspronia</em></td>
<td>25</td>
</tr>
<tr>
<td><em>Trema</em></td>
<td>10</td>
</tr>
<tr>
<td><em>Casuarina</em></td>
<td>26</td>
</tr>
<tr>
<td>Sunflower</td>
<td>26</td>
</tr>
<tr>
<td>Cotton</td>
<td>15</td>
</tr>
<tr>
<td>Monocotyledons</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>5</td>
</tr>
<tr>
<td>Rice</td>
<td>11</td>
</tr>
<tr>
<td>Maize</td>
<td>34</td>
</tr>
</tbody>
</table>

Note that strain ANU894 retains its original *nodD1* gene intact, but the presence of this gene was not sufficient to activate the *nodA-lacZ* transcription by extracts of the wide range of di- and monocot non-host plants.

Values are in units of β-galactosidase activity (Miller, 1972) following induction of a *nodA::MudII1734* fusion in the symbiotic plasmid of *R. l. bv trifolii* strain ANU843 (strain ANU894) carrying pMN40. Values shown are an average of two readings, with four experimental repeats. Standard deviation for all values was less than 30%.

Plant extracts were prepared as described in Section 4.2.1, β-galactosidase assays were performed as described in Section 2.1.6.1 using strain ANU894 (described in Table 2.1) containing plasmid pMN40 (described in Table 2.2 and Figure 4.1).
Compounds from non-host plants activate strain NGR234 NodD1, including simple phenolics isolated from wheat extract.

Table 4.2 Characteristics Of Australian Wheat Varieties Examined.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Characteristics</th>
<th>$i_{\text{max}}^a$</th>
<th>vanillin/ isovanillin$^b$</th>
<th>vanillin content$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASW</td>
<td>Australian Standard White</td>
<td>850</td>
<td>+</td>
<td>1.3 x 10$^{-8}$</td>
</tr>
<tr>
<td>Halberd</td>
<td>hard, dry land variety.</td>
<td>900</td>
<td>+</td>
<td>4.2 x 10$^{-8}$</td>
</tr>
<tr>
<td></td>
<td>low boron intake</td>
<td>1100</td>
<td>+</td>
<td>4.0 x 10$^{-8}$</td>
</tr>
<tr>
<td>Suneca</td>
<td>hard, high yield variety, will not germinate if wet during harvest</td>
<td>980</td>
<td>+</td>
<td>4.4 x 10$^{-8}$</td>
</tr>
<tr>
<td>Hartog</td>
<td>hard, high yield variety</td>
<td>1280</td>
<td>+</td>
<td>2.8 x 10$^{-8}$</td>
</tr>
<tr>
<td>Vulcan</td>
<td>hard, high yield variety</td>
<td>1010</td>
<td>+</td>
<td>1.0 x 10$^{-8}$</td>
</tr>
<tr>
<td>Rosella</td>
<td>soft, long season variety</td>
<td>720</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Eradu</td>
<td>soft, rapid maturing variety</td>
<td>990</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Matong</td>
<td>soft, late maturing, lower protein variety</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a $i_{\text{max}}$: maximal levels of induction of fraction A2 equivalents expressed in units of $\beta$-galactosidase activity (Miller, 1972) for comparison, DHF at 10$^{-6}$ M gives 700 units, background of distilled H$_2$O gives 110 units.

b - presence or absence of vanillin/isovanillin determined by reverse phase HPLC analysis and UV spectrum scanning as described in Section 2.2.2.

c - estimate of amount of vanillin/isovanillin in wheat variety extracts by comparison to known quantities of standard vanillin, expressed as moles per 100g seeds.

Plant extracts were prepared as described in Sections 4.2.9 and 4.8. $\beta$-galactosidase assays were performed as described in Section 2.1.6.1 using strain ANU265 (described in Table 2.1) containing plasmid pMD1 (described in Table 2.2 and Figure 3.1). $i_{\text{max}}$ was determined by the method described in Section 4.8. The technique employed for the estimation of vanillin content is described in Section 4.8.
Figure 4.1
Map of plasmid pMN40.

The location of a 2.1 kb PstI fragment carrying the *nodD1* gene from *Rhizobium* strain NGR234 cloned into the broad host range vector pSUP106 (Simon *et al.* 1983) is shown. The direction of transcription for the *nodD1* gene (crosshatched area) is indicated (arrow). The 2.1 kb PstI fragment contains an 0.8 kb segment of IS50 that is part of the Tn5 arm (heavy line), and an intergenic region of approx. 0.4 kb that contains no open reading frame (Bassam *et al.* 1986; Horvath *et al.* 1987). The location of the antibiotic resistance genes are indicated by shaded regions.

Cm<sup>R</sup> - chloramphenicol resistance gene; Tc<sup>R</sup> - tetracycline resistance gene.
Restriction enzyme sites: B = *BamHI*; E = *EcoRI*; P = *PstI*; and H = *HindIII*.

(Bender *et al.* 1988)
column of silicic acid

Tn5 arm B

0.8kb 0.4kb 0.9kb

pSUP106 E
9.9kb H

2.1kb

active fractions A 2, A 5, A 12

A 2

active fractions 0, 0.8kb, 0.4kb, 0.9kb

B 2, B 3 pooled

column of silica gel

equilibrated with CH3OH 10%

active fraction C 2

C 2 reverse phase HPLC

and UV spectrum scanning
Figure 4.2

Summary of the fractionation procedure for Australian Standard White (ASW) wheat seedling extracts.

The fractionation procedure used for ASW wheat seedling extracts is described in Section 4.2.9.
Ethanolic extract of ASW wheat seedlings

Column of silicic acid
eluted with CH$_3$OH/CHCl$_3$ : 10, 40%, & 100% CH$_3$OH
12 fractions of 110 ml collected

Active fractions A 2, A 6, A 12

A 2

Column of C$_{18}$ silica (reverse phase)
eluted with CH$_3$OH/H$_2$O : 5, 10, 20, 40, 60, 80%, & 100% CH$_3$OH
7 fractions of 40 ml collected

Active fractions B 2, B 3

B 2, B 3 pooled

Column of silicic acid
eluted with CH$_3$OH/CH$_2$Cl$_2$ : 1, 2, 4, 8%, & 100% CH$_3$OH
10 fractions of 25 ml collected

Active fraction C 2

C 2 reverse phase HPLC
and UV spectrum scanning
Figure 4.3

HPLC elution profile of fraction C2 of Australian Standard White (ASW) wheat seedling extract.

The relative retention time, occurrence of peaks detected at 254 nm, and the concentration of methanol in the eluting solvent are shown.

A: peak identified as inducing NodD1-dependent nod gene expression in strain ANU265(pMD1).

The techniques used for the HPLC analysis of ASW wheat seedling extract are described in Sections 2.2.2 and 4.7.
Figure 4.4

UV spectrum analysis of peak A of fraction C2 of ASW wheat seedling extract.

UV profile of peak A, identified in fraction C2 of ASW wheat seedling extract, recorded over the range of 225 to 400 nm in the stop-scan mode, is compared to UV profiles of authentic vanillin and isovanillin, recorded under the same conditions.

The techniques used for the HPLC analysis of ASW wheat seedling extract are described in Sections 2.2.2 and 4.7.
Peak A  Isovanillin  Vanillin
Chapter Five
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

5.1 Introduction

In (*Brady*)Rhizobium species, the *nodD* gene has a critical involvement in host specificity (Spaink *et al.* 1987b), serving to control the transcription of most *nod* genes in conjunction with phenolic compounds, typically flavonoids. Some rhizobia have multiple *nodD* alleles, each responding to a different cocktail of activating compounds (Györgypal *et al.* 1988; Hartwig *et al.* 1990; Hungria *et al.* 1992), and exhibiting different roles in host specificity.

A number of the *nodD* genes described thus far seem to have no recognisable role in nodulation (Appelbaum *et al.* 1988; Bassam *et al.* 1988; Göttfert *et al.* 1992). A key example is strain NGR234, where a mutation of the *nodD1* gene results in complete loss of the capacity to nodulate all host plants and the *nodD2* gene does not complement for the loss of *nodD1* on any plant examined (Bassam *et al.* 1988). Results from studies of the slow-growing, soybean nodulating *B. japonicum* strain USDA110 indicate that of the two *nodD* genes identified, *nodD1* is critical for optimal nodulation, whilst the *nodD2* gene has no obvious role in nodulation (Göttfert *et al.* 1992). In contrast, in the fast-growing soybean nodulating strain *R. japonicum* USDA191, two *nodD* genes were also identified and both are involved in symbiosis (Appelbaum *et al.* 1988).

Banfalvi *et al.* (1988) studied the compounds that induce *nod* gene transcription in *B. japonicum* strains and found different responses to various flavonoids. In particular, they found that genistein induced *nodYABC::lacZ* fusions most strongly, and that daidzein, apigenin and biochanin A induced the same fusions less strongly. An additional novel feature in *B. japonicum* is that *nodD* expression was positively activated by soybean exudate and various flavonoids. In contrast, *R. leguminosarum* *nodD* is constitutively expressed and regulates its own expression (Rossen *et al.* 1985; McIver *et al.* 1989), whereas in *R. meliloti* the product of *nodD1* positively activates NodD3 (Maillet *et al.* 1990).

*R. meliloti* has been shown to possess three functional *nodD* genes (Honma *et al.* 1987). In addition, *syrM* has been shown to encode a protein which also regulates *nod* genes (Reviewed in Kondorosi 1991). The multiple *nodD* genes in this species have been shown to respond differentially to plant derived flavonoid compounds (Honma *et al.*
Compounds found in alfalfa root and seed extracts activate NodD1, but only 4,4'-dihydroxy-2'-methoxychalcone activates the gene products of both nodD1 and nodD2 (Hartwig et al. 1990). Moreover, whilst R. meliloti NodD1 was found to transcriptionally activate nod genes in the presence of inducing compounds (Mulligan and Long 1985), over-expressed NodD3 requires SyrM for activity, and induces a high basal level of nod gene expression in the absence of inducers, although induction is enhanced by compounds which induce NodD1 (Mulligan and Long, 1989). Multiple nodD genes therefore define one aspect of host specificity in this species interaction with its legume co-symbionts (Honma et al. 1987; Györgypal et al. 1988).

Results from experiments with single, double and triple nodD mutants in R. meliloti indicate that at least two nodD genes are required for optimal nodulation (Honma et al. 1990). Further, Honma and colleagues (1990) demonstrated that, in alfalfa (Medicago sativa) nodulation, nodD1 is more important than nodD3, and nodD2 plays a less vital role. For the nodulation of sweet clover (Melilotus albus), the nodD1 and nodD3 genes are apparently equivalent, with nodD2 having no clear function. Similar studies were also conducted on these three nodD genes on five host plants (Györgypal et al. 1988). Of those plants assayed, Melilotus albus (Ma), Medicago sativa (Ms), Medicago quasifalcata (Mqu), Medicago truncatula (Mt), and Trigonella coerulea (Tc), only Ms nodulation was delayed by a mutation in any of the three nodD genes. Ma and Tc showed delayed nodulation where either nodD3 or nodD1, and nodD1 or nodD2 (respectively) were mutated. Finally, Mqu showed delayed nodulation only where there was a mutation in nodD1 (Györgypal et al. 1988).

R. l. bv. phaseoli also has three nodD genes, and the products of all three were found to activate the transcription of nod::lacZ fusions with each of the nine aglycones identified in black bean root and seed exudates (Hungria et al. 1992). The highest levels of induction in all cases were found in response to genistein and naringenin. Furthermore, mixtures of genistein and naringenin were found to activate the identified nod genes at a much higher level than could be accounted for by an additive effect (Hungria et al. 1992).

Multiple nodD genes have also been identified in two rhizobia capable of nodulating Parasponia - NGR234 and CP279. Of the two nodD genes identified in strain NGR234 (Rodriguez-Quinones et al. 1987), only nodD1 is functional (Bassam et al. 1988). This functional nodD gene, when transferred to other rhizobia, enables the extension of host range to other legumes and to the non-legume Parasponia (Horvath et al. 1987; Bassam et al. 1988; Bender et al. 1988). The characteristics of phenolic compounds activating NGR234 NodD1 were determined in Chapter 3 of this work, and include monocyclic phenolic compounds, such as vanillin and isovanillin, which have not been previously reported to activate NodD of rhizobia (Chapter Three; Le Strange et al. 1990).
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

Available evidence indicates that the *Parasponia* specific *Bradyrhizobium* strain CP279 contains two *nodD* genes (Bender 1987). These putative *nodD* genes were identified by hybridisation with both the 3' and the 5' regions of the *nodD1* gene of *Rhizobium* strain NGR234. Both of the identified CP279 putative *nodD* genes hybridised to the 3' region of NGR234 *nodD1*, whereas only one was found to hybridise to the 5' region of the NGR234 *nodD1* gene. Bender (1987) hypothesised that the *nodD* genes of strains NGR234 and CP279 might show strong 3' homology (unlike most *nodD* genes) since the carboxy-terminal region of NodD was thought to be involved in recognition of NodD activating compounds (Horvath et al. 1987; Burn et al. 1987; McIver et al. 1989; Spaink et al. 1989; Fisher and Long, 1993), and both strains are capable of nodulating the non-legume tree *Parasponia*.

In addition to serving as *nod*-gene inducing compounds, many plant derived phenolic compounds enhance the growth of rhizobia, usually through catabolism of such compounds. Via the catechol or protocatechuate branches of the β-ketoadipate pathway, rhizobia have been shown to use simple aromatic compounds as sole sources of carbon and energy (Chen et al. 1984; Parke and Ornston 1986). In *Rhizobium* strains the genes for this pathway are inducible, but they are generally constitutive in *Bradyrhizobium* species (Chen et al. 1984; Parke and Ornston 1986; Parke et al. 1991). Flavonoids (luteolin, quercetin, chrysin and their glucosides) have been shown to enhance the growth of *R. meliloti* in minimal medium at concentrations as low at 0.25 µM, but the compounds were apparently not metabolised (Hartwig et al. 1991). Growth enhancement is apparently by a mechanism separate to the induction of *nod* genes, since strains lacking functional copies of all three *nodD* genes also showed enhanced growth (Hartwig et al. 1991). Since many soils are carbon-limited environments, compounds resulting from the breakdown of lignin may be of some importance when rhizobia are at a distance from plant roots and their associated secretions and exudates (Chen et al. 1984).

Multiple, functional *nodD* genes have thus far been identified in a number of rhizobia including *R. meliloti* and *B. japonicum*. Of these species, *R. meliloti* has a relatively narrow host range (limited to *Medicago, Melilotus* and *Trigonella* species), where *B. japonicum* nodulates a wider variety of host genera or species. In *Rhizobium* strain NGR234 only one functional *nodD* gene has been identified, yet this strain exhibits the widest host range identified for any *Rhizobium* species (Relic et al. 1994). *Bradyrhizobium* strain CP279 is virtually *Parasponia*-specific, yet two putative *nodD* alleles have been identified (Bender 1987).

One aim of the research presented in this chapter was to confirm the presence of 2 *nodD* homologues in strain CP279, following their identification by Bender (1987). I further sought to establish the roles of multiple *nodD* genes in the host range of this strain by establishing the functionality of the identified putative NodDs through complementation assays on various plants. By comparing the suite of inducer compounds activating each of the CP279 NodDs with the compounds inducing NodD-dependent *nod* gene
transcription in the wild-type parental strain, I sought to determine the role of each NodD in the NodD-dependent induction of nod gene transcription in the wild-type strain, and to gain some insight into their roles in both nodD and nod gene regulation.

In this chapter I demonstrate that Bradyrhizobium strain CP279 possesses at least three regions homologous to NGR234 nodD1; that at least two of these putative nodD genes are functional in plant nodulation assays and that the putative nodD1 gene of strain CP279 complements nodD mutations for nodulation only on the non-legume host Parasponia, the putative nodD2 gene complements for nodulation on a broad range of plant hosts and that the putative nodD3 gene plays no apparent role in the nodulation of the plants examined. I further demonstrate that each of these putative nodD gene products co-induces nod::lacZ fusions in conjunction with a slightly different suite of phenolic compounds (when compared to each other and to the parental strain CP279). In addition, specific phenolic compounds are also shown in this chapter to affect the growth of a small test group of bradyrhizobia, and that growth enhancement is not necessarily related to any NodD activating effect of the compounds.

5.2 Experimental procedure

To confirm the number of homologues of NGR234 nodD1 in strain CP279, CP279 total DNA was digested with restriction enzymes, transferred to hybridisation membrane and probed with NGR234 nodD1 DNA. Following the identification of at least three putative nodD homologues, restricted CP279 DNA was fractionated on a sucrose gradient and the fractionated DNA probed for nodD homologues. Pools of fractions containing the hybridising DNA fragments were ligated into plasmid pJJ358, a blue/white selection cloning vector with an appropriate restriction site. The cloned, putative nodD genes were subcloned to plasmid pLAFR3 (a broad host range vector suitable for transfer to rhizobia) generating plasmids pKS1701, pKS4001 and pKS2001.

To establish the functionality of the cloned, putative, nodD genes, plasmids pKS1701, pKS4001 and pKS2001 were introduced into the nodD mutant Rhizobium strains (ANU1255 and ANU851) and assessed by plant nodulation assays for complementation of the NodD deficient host strains.

To investigate the range of phenolic compounds activating the native NodDs of various bradyrhizobia, the reporter plasmids pZB32 and pRT311 were introduced into the Bradyrhizobium strains to assay for NodD-dependent nod gene induction, indicated by β-galactosidase activity when exposed to appropriate phenolic compounds. However, each of the Bradyrhizobium strains was found to be naturally resistant to tetracycline, the antibiotic marker for both reporter plasmids. Following investigation, each of the Bradyrhizobium strains was found to be sensitive to kanamycin, and thus the NodD-inducible cassette of plasmid pZB32 was subcloned to plasmid pKT240 (with a
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

kanamycin marker). The resulting functional plasmid pLSC17 was used as the reporter plasmid in *nod* gene induction assays in the group of *Bradyrhizobium* stains.

To allow analysis of the activation of CP279 putative NodDs by phenolic compounds, reporter plasmids (pLSC17 and pRT311) were introduced into the *nod* mutant *Rhizobium* strains (ANU1255 and ANU851) containing the subcloned putative CP279 *nodD* genes (plasmids pKS1701, pKS4001 and pKS2001). Unfortunately the reporter plasmids could not be co-maintained with the cloned putative CP279 *nodD* genes in all cases.

Consistently greater cell densities of strain CP279 were observed after the induction stage of *nod* gene induction assays, following incubation with DHF. Since growth enhancement by, and metabolism of, phenolic compounds by (brady)rhizobia is well established, it was of interest to investigate this phenomenon in relation to potential NodD activating compounds. Lawns of selected strains of bradyrhizobia were grown in the presence of a variety of phenolic compounds and the effects of the compounds on bacterial growth were observed.

5.2.1 Identification of *nodD* homologues in strain CP279

Total DNA from strain CP279, prepared by G. Bender, was digested with the restriction endonucleases *BamHI, EcoRI, HindIII, PsiI* (by the method described in Section 2.4.3.3). Aliquots of each digest containing DNA fragments were separated using agarose gel electrophoresis (described in Section 2.4.4), and parallel, multiple gels were run under identical conditions. The gels were processed and the DNA transferred to Hybond-N membrane as described in Section 2.4.5.3.

Probes were prepared from plasmid pJJ252 (Figure 5.2a) by polymerase chain reaction (by the method described in Section 2.4.3.7) using a T7 universal primer, and a 22mer (sequence: GGGAATTTCAGGAGGAGGCGCATGC) coding for a region 50 bp upstream of the putative *nod* box of strain NGR234, and incorporating an *EcoRI* restriction site (as illustrated in Figure 5.2b). Annealing temperatures for the two primers were calculated to be 37°C and 47°C, and the annealing temperature used in the PCR reaction was 37°C. The resulting DNA fragment (approx. 1.2 kb) encoding the NGR234 *nodD1* gene was then digested with *EcoRI* to remove the termini, and further digested with *BamHI* to generate two fragments - an 0.75 kb 3' fragment and an 0.4 kb 5' fragment which included the *nod-box* (Figure 5.2c). These two fragments were separated by electrophoresis (as described in Section 2.4.4), and isolated onto DEAE cellulose (as described in Section 2.4.2.5). Following elution the purified DNA fragments were used to generate α<sup>32</sup>P labelled probes by the method described in Section 2.4.5.4.
5.2.2 Cloning of the putative nodD genes from strain CP279

Following further isolation of CP279 total DNA by the method described in Section 2.4.2.4, large scale BgIII digests were prepared (as described in Section 2.4.3.3) and fractionated on a sucrose gradient, as described in Section 2.4.3.4 and illustrated in Figure 5.3). Pools of fractions which contained bands hybridising to NGR234 nodD1 probes were ligated into the BgII site of vector pJJ358 by the method described in Section 2.4.3.6 and schematically illustrated in Figure 5.4, and electroporated into E. coli strain NM522 by the method described in Section 2.4.6. The cells were plated as lawns onto growth media overnight (L agar, as described in Section 2.1.2) , and then replica plated to L agar plates containing carbenicillin (100 µg/ml) and X-gal (prepared as described in 2.1.6.3). Colonies growing on selection media were then colony blotted onto Hybond-N filters by the method described in Section 2.4.5.2, and hybridised to a probe prepared from NGR234 nodD1 (the 1.95 kb fragment from plasmid pJJ252) by the method described in Section 2.4.5.4.

5.2.3 Subcloning putative CP279 nodD clones for nodD complementation assays

To enable the identified CP279 putative nodD genes to be assayed for nodD complementation on plant hosts, DNA fragments encoding the putative CP279 nodD genes (identified through mapping and hybridisation, and illustrated in Figure 5.7) were subcloned into plasmid pLAFR3 (dephosphorylated where appropriate by the method described in Section 2.4.3.5), electroporated into strain NM522 (as described in Section 2.4.6) and selected on LTc10 • Subcloning of these DNA fragments is shown schematically in Figure 5.5.

Potential subclones were selected by hybridisation of colony blots (by the methods described in Sections 2.4.5.2 & 2.4.5.5) with α32P probes prepared from appropriate nodD fragments (derived from each original clone). The resulting plasmids, and the fragments cloned into them were: pKS1701, containing a 2.1 kb PstI fragment from CP279 clone D17; pKS2001, a 2.1 kb BgII fragment from CP279 clone D20 (cloned into the BamHI site of the vector); and pKS4001 a 1.4 kb HindIII/EcoRI fragment from CP279 clone D40. The identity of the putative nodD gene in each of the subclones was confirmed by the internal restriction digest patterns identified in the original clones (for clone D17 - BamHI, for clone D20 - BamHI, HindIII, and for clone D40 - HindIII/EcoRI, BgII).

5.2.4 Subcloning the 10 kb BamHI fragment (containing a nodY::lacZ fusion, inducible by NodD activation) from pZB32 to pKT240

Plasmid pZB32 (Banfalvi et al. 1988) was digested with BamHI (as described in Section 2.4.3.3), and the 10 kb fragment containing the nodY::lacZ fusion isolated (by the
methods described in Sections 2.4.4 and 2.4.2.6). Plasmid pKT240 was digested with BamHI, and dephosphorylated with shrimp alkaline phosphatase (as described in Section 2.4.3.5). Following ligation by the method described in Section 2.4.3.6 and electroporation into strain NM522 (as described in Section 2.4.6), clones were selected on L medium (prepared as described in Section 2.1.2) containing kanamycin 100 µg/ml and ampicillin 100 µg/ml. DNA was isolated from potential clones by the alkali lysis method described in Section 2.4.2.2, and digested with BamHI. Following electrophoresis (as described in Section 2.4.4) the agarose gel was alkali blotted to Hybond-N membrane (described in Section 2.4.5.3). The membrane bound DNA was then probed (by the method described in Section 2.4.5.5) with a 32P probes prepared from both the 10 kb fragment of pZB32, and pKT240 (by the method described in Section 2.4.5.4). A number of clones were isolated by hybridisation to both radioactive probes and were reconfirmed by further hybridisation to alkaline blotted restriction digests using enzymes HindIII/PstI, and BamHI. Potential plasmid isolates were triparentally mated into strain CP279 (as described in Section 2.4.7 using helper plasmid pRK2013 (Ditta et al. 1980)) for trial nod gene induction assays and plasmid pLSC17 was selected for further use. Figure 5.6 illustrates the subcloning approach described in this section.

5.2.5 β-galactosidase assays

β-galactosidase assays to measure the level of nod gene induction under the control of NodD in the presence of selected phenolic compounds were carried out as described in Section 2.1.6. Assays for induction response to each compound were carried out in pairs and repeated once. Analysis of variance appropriate for this experimental design was undertaken with the assistance of the Statistical Consulting Unit of the Graduate School, Australian National University.

5.2.6 The effect of flavonoid compounds on the growth of Bradyrhizobia

To investigate any growth enhancement effects of phenolic compounds on bradyrhizobia, selected strains were grown overnight at 28°C as replicate lawn cultures (from liquid BMM inoculum, prepared as described in Section 2.1.2) on GMM plates (prepared as described in Section 2.1.2) before the addition of a small quantity of the test compound. Approximately 0.5 to 1 mg of crystalline compound was placed in the centre of the agar plate, and control lawns had no added crystals. After 2 days incubation at 28°C bacterial lawns were examined for differences in growth patterns between the control and treatment plates.
RESULTS

5.3 CP279 has at least three regions that show some homology to NGR234 nodD1

Hybridisation of the 3' region of NGR234 nodD1 to digested CP279 DNA (as described in Section 5.2.1, refer also to Figure 5.2) showed at least two bands of homologous DNA in each lane. The filter was also probed with the 5' region of NGR234 nodD1 and again at least two hybridisation bands were again observed in each lane. Figure 5.1A illustrates the electrophoresed, restricted CP279 DNA and Figure 5.1 B & C show the autoradiographs of CP279 DNA probed with NGR234 nodD1. Approximate sizes for each hybridising band were calculated and are shown in Table 5.1.

The presence of a single band in the BamHI digest hybridising to both 3' and 5' probes, and of two other bands each hybridising to only one probe suggested the presence of three nodD homologues. In the HindIII lane five separate bands hybridise to the two probes, and four bands in each of the EcoRI and PstI lanes. Accounting for the possible presence of restriction sites for these enzymes within the CP279 nodD DNA fragments, the patterns in these lanes support the presence of at least three nodD homologues in strain CP279, rather than the two identified by Bender (1987). Table 5.1 summarises the hybridisation patterns shown in Figure 5.1 B and C.

Three putative nodD genes were isolated from strain CP279 total DNA following the cloning strategy described in Section 5.2.2, refer also to Figures 5.3 and 5.4. The three clones had the following inserted fragments: i) clone D17, contained a BgIII fragment of approximately 2.2 kb; ii) clone D20, a BglII fragment of approximately 2.1 kb; and iii) clone D40, a BglII fragment of approximately 1.35 kb.

Clones D17, D20 and D40 (later referred to as putative nodD2, nodD1 and nodD3, respectively) were mapped using restriction digests with enzymes BamHI, BglII, EcoRI, HindIII, PstI, ScaI, and XbaI, alone and in combination. Physical maps of clones D17, D20, and D40 derived from these restriction digest studies appear in Figure 5.7. Hybridisation studies using cloned CP279 DNA fragments digested with the same restriction enzymes, and probes prepared from NGR234 nodD1 demonstrated that each cloned fragment had a different hybridisation pattern to each of the other clones. The hybridisation results are presented schematically in Figure 5.7. The differing restriction and hybridisation patterns shown in Figure 5.7 demonstrate unequivocally the molecular difference between the clones and verify the presence of three putative nodD homologues in strain CP279.

DNA from each of the three nodD homologues cloned from strain CP279 were separately hybridised back onto membranes containing restriction digests of CP279 total DNA. The hybridisation patterns showed that each clone hybridised strongly to a single
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

band in each lane, and weakly to one or more other bands in each lane. In each case the band of strongest hybridisation within each lane was different for each of the cloned *nodD* genes. The clearest hybridisation data that could be obtained showed that CP279 clone D17 hybridised to an approximately 11.5 kb band in *BamHI* digested CP279 DNA, and that clones D20 and D40 hybridised to bands of approximately 4.8 kb and 3.5 kb respectively in *PstI* digested CP279 DNA. Each of these bands is equivalent to a band identified in CP279 DNA probed with DNA from NGR234 *nodD1*, summarised in Table 5.1.

Table 5.1 Approximate sizes of the hybridising bands in each lane, in kb.

<table>
<thead>
<tr>
<th>CP279 digested with</th>
<th>Probed with 3' region of NGR234 <em>nodD1</em></th>
<th>Probed with 5' region of NGR234 <em>nodD1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BamHI</em></td>
<td>18.5 11.5</td>
<td>11.5 5.4</td>
</tr>
<tr>
<td><em>EcoRI</em></td>
<td>10.2 8.6 3.1</td>
<td>10.2 6.9</td>
</tr>
<tr>
<td><em>HindIII</em></td>
<td>5.0 1.4</td>
<td>14.5 6.5 2.9</td>
</tr>
<tr>
<td><em>PstI</em></td>
<td>3.5 2.3</td>
<td>7.8 4.8</td>
</tr>
</tbody>
</table>

5.4 Plant nodulation assays to assess complementation by the putative *nodD* genes of strain CP279

Each CP279 putative *nodD* fragment (delineated using restriction fragment length mapping and hybridisation, shown in Figure 5.7) was excised from pJJ358 and subcloned into pLAFR3 generating plasmids pKS1701, pKS2001, and pKS4001 (by the method described in Section 5.2.3, refer also to Figure 5.5). These clones were tri-parentally mated (as described in Section 2.4.7) into non-nodulating *nodD* mutants strains ANU851 and ANU1255 using the helper plasmid pRK2013 (Ditta *et al.* 1980) so that plant nodulation assays could be performed. Both strains ANU851 and ANU1255 provided a nod<sup>+</sup> background containing a Tn5 inactivated *nodD* and were ideal for these experiments.

The ability of each of the cloned CP279 putative *nodD* genes to complement *nodD* mutants for nodulation was examined in both a narrow host range background (mutant ANU851 of strain ANU843) and a broad host range background (mutant ANU1255 of strain NGR234). The transconjugants generated were inoculated onto a variety of plant species representing key hosts of the background strains: *Parasponia andersonii*; siratro; white clover; and subterranean clover. The results of these complementation assay experiments are presented in Tables 5.4 to 5.7.

Seeds were germinated as described in Sections 2.3.2 to 2.3.6. *Parasponia*, siratro and sub. clover plants were examined for nodulation using the magenta jar assay (as described in Section 2.3.8), and white clover was examined by plate assay (as described in Section 2.3.7).
Problems with the growth cabinets used for the plant assays resulted in fungal contamination of some *Parasponia*, siratro and sub. clover seedlings, having the following effects:

- The results presented in Table 5.4 show that a number of *Parasponia* seedlings inoculated with strain NGR234 were subject to fungal contamination in the magenta jars and died, and only 60% of the remaining plants nodulated, rather than the expected 100%.

- Table 5.5 presents results which show that nodulation of siratro by both strains CP283 and NGR234 was below the normal 100% rate due to fungal contamination in a number of magenta jars, affecting individual seedlings.

- A reduction in the usual number of subterranean clover plants nodulated by strain ANU843 to a rate of 90%, below the expected nodulation rate of 100%, is reported in Table 5.7 and is due to the fungal contamination of individual seedlings.

### 5.4.1 CP279 clone D17

Plasmid pKS1701 (containing CP279 clone D17) was found to complement the nodulation inability of strain ANU1255 (*nodD* mutant of strain NGR234). In the case of *Parasponia*, 75% of seedlings had nodules, and on siratro 80% of plants nodulated (shown in Tables 5.4 and 5.5). Both of these plants are normal hosts of the parent strains NGR234 and CP279. Bacteria isolated and cultured from these nodules (Section 2.3.10) showed resistance to tetracycline, confirming the presence of the introduced plasmid encoding CP279 clone D17. A small number of the nodules examined yielded no colonies and either contained no viable bacteria or bacteria at such a low level they were undetected. Thus in an ANU1255 background it can be seen that clone D17 re-established the natural host range of strain CP279.

As shown in Tables 5.6 and 5.7, plasmid pKS1701 also complemented the *nodD* mutant strain ANU851, forming nodules on 70% of white clover and 40% of subterranean clover plants. Both of these plants are homologous hosts of the ANU851 parent strain, ANU843. These transconjugants also formed nodules on 60% of siratro plants (Table 5.5), a plant outside the host range of strain ANU843.

Table 5.6 shows that nodules were found on 15% of white clover plants inoculated with ANU1255(pKLS1701), a non-host plant for either bacterial parent strain, but the bacteria isolated from the nodule could not be cultured on either tetracycline nor kanamycin media. Similarly, *Parasponia* plants inoculated with strain ANU851(pKLS1701) had nodules present on 8% of the plants but the bacteria isolated from nodules could not be cultured on media containing either tetracycline or kanamycin (Table 5.4).
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

### 5.4.2 CP279 clone D20

The results reported in Tables 5.4 - 5.7 show that plasmid pKS2001, containing CP279 clone D20, did not complement the mutated *nodD* gene of either ANU1255 nor ANU851 for nodulation on any of the plant species assayed, and thus can be considered non-functional in these experiments.

### 5.4.3 CP279 clone D40

Plasmid pKS4001, encoding CP279 clone D40, was found to complement the *nodD* mutant strain ANU851 for nodulation on *Parasponia*, where 25% of plants nodulated, as shown in Table 5.4, but not on the other plants tested in this background, nor did this plasmid complement for nodulation in the mutant ANU1255 background, as shown in Tables 5.4 - 5.7. Strain ANU843, the wild-type parent of strain ANU851, does not normally nodulate *Parasponia*. The identity of the nodulating strain (ANU851(pKS4001)) within the *Parasponia* nodules was confirmed by nodule squashes, since the isolated bacteria grew on media containing kanamycin and tetracycline. Some nodules seemed to contain no detectable bacteria.

### 5.5 NodD-dependent *nod* gene assays in bradyrhizobia using the β-galactosidase reporter plasmids pRT311 and pZB32

Plasmids pRT311 (McIver *et al.* 1989) and pZB32 (Banfalvi *et al.* 1988), both encoding tetracycline resistance, have been used to assay for NodD-dependent transcriptional activation of *nod* genes in other systems - *R. l. bv. trifolii* (McIver *et al.* 1989) and *B. japonicum* (Banfalvi *et al.* 1988). Both reporter plasmids contain an inducible *nod* gene (and promoter) fused to the *E. coli lacZ* gene. The expression of the *lacZ* gene is therefore dependent on the expression of the *nod* promoter and gene, induced by activated NodD interacting with the *nod* promoter. Plasmid pRT311 contains the *R. l. bv. trifolii nodA* promoter and gene and is illustrated in Figure 5.8 (McIver *et al.* 1989) where plasmid pZB32 contains the *nodY* promoter and gene of *B. japonicum* and is illustrated in Figure 5.6a (Banfalvi *et al.* 1988).

These plasmids were tri-parentally mated (by the method described in Section 2.4.7) into strains: CP279, CP283, ANU1182, ANU289, ANU1255, ANU843; using plasmid pRK2013 as the helper plasmid (Ditta *et al.* 1980). Selection of transconjugants proved difficult with most of the slow-growing *Bradyrhizobium* strains due to endogenous resistance to tetracycline at levels from 10 µg/ml to over 50 µg/ml (see Table 5.2). Thus transconjugants were unable to be easily selected or maintained using this antibiotic marker. This indicated that plasmids pRT311 and pZB32 were not suitable to measure *nod* gene induction in the *Bradyrhizobium* strains of interest in this work.
Bradyrhizobium strains were examined for endogenous resistance to the antibiotics ampicillin and kanamycin, antibiotic markers on plasmid vectors which would enable construction of a suitable alternative reporter plasmid. Each strain was cultured on media containing a range of concentrations of the antibiotics of interest. The results presented in Table 5.2 show that each of the strains examined were sensitive to kanamycin 100 µg/ml, and thus indicated that plasmid pKT240 (Table 2.2, Bagdasarian et al. 1983) may be suitable alternative plasmid vector.

Table 5.2 The endogenous antibiotic resistance of Bradyrhizobium strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tetracycline</th>
<th>Ampicillin</th>
<th>Kanamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP279</td>
<td>resistant at Tc50</td>
<td>sensitive at A50</td>
<td>sensitive at K100</td>
</tr>
<tr>
<td>CP283</td>
<td>resistant at Tc50</td>
<td>resistant at A200</td>
<td>sensitive at K100</td>
</tr>
<tr>
<td>ANU289</td>
<td>resistant at Tc20</td>
<td>resistant at A200</td>
<td>sensitive at K100</td>
</tr>
<tr>
<td>ANU1182</td>
<td>sensitive at Tc10</td>
<td>resistant at A200</td>
<td>sensitive at K100</td>
</tr>
</tbody>
</table>

5.6 Subcloning the 10 kb BamHI (containing a NodD-dependent nodY::lacZ fusion) from pZB32 To pKT240

The inability to use plasmids pRT311 and pZB32 to assay for nod gene induction required the construction of a new plasmid with a suitable antibiotic resistance marker. A 10 kb BamHI fragment encoding the B. japonicum nodY::lacZ fusion from plasmid pZB32 was subcloned into plasmid pKT240 (Table 2.2, Bagdasarian et al. 1983) by the procedure detailed in Section 5.2.4, and is schematically illustrated in Figure 5.6. Plasmid pKT240 encodes both kanamycin and ampicillin resistance and is known to be stably maintained in rhizobia. This procedure resulted in the isolation of a number of plasmids containing the appropriate fragment (confirmed by hybridisation as described in Section 5.2.4). Preliminary β-galactosidase assays in strain CP279 confirmed that of those plasmids isolated, the reporter plasmid designated pLSC17 was functional in assays to measure NodD-dependent nod gene induction.

5.7 Analysis of phenolics activating native NodDs in bradyrhizobia

Plasmid pLSC17 (constructed as described in Section 5.6) was tri-parentally mated using plasmid pRK2013 as the helper plasmid (Ditta et al. 1980) by the method described in Section 2.4.7 into strains CP279, CP283, and as controls into strains NGR234 and ANU843. In these experiments the native nodD genes were expected to induce the transcription of the reporter system on plasmid pLSC17. Preliminary β-galactosidase assays using construct pLSC17 were done using the standard method (Section 2.1.6.1), however, induction periods of 2 h were found to result in quite low induction of β-galactosidase activity. Longer induction periods of 8-16 h have been
used when studying nod gene activation in B. japonicum strains using the plasmid construct pZB32, from which pLSC17 was derived (Banfalvi et al. 1988; Sutherland et al. 1990). Induction periods of 8 and 16 h were trialed and both periods resulted in substantially increased β-galactosidase activity compared to 2 h inductions, and were thus adopted for subsequent assays with construct pLSC17, as described in Section 2.1.6.2.

The transconjugants containing the nodY::lacZ fusion (pLSC17) were assayed for β-galactosidase activity in the presence of groups of the following compounds: 7,4′-dihydroxyflavone (DHF), vanillin, isovanillin, coumarin, apigenin, umbelliferone, and hesperetin. The structures of these compounds are shown in Figure 5.9. These compounds were selected as they had been identified as inducers of NGR234 NodD1-dependent nod genes (reported in Chapter 3, see Section 3.3, and Tables 3.1 and 3.2), and because they represented a variety of structural characteristics typical of those which activate NGR234 NodD1 (Chapter 3, Section 3.4). For example, the presence of a variety of hydroxyl substitutions, particularly on the B-ring of flavonoids, as found in apigenin, is predictive of activity. Concentrations of the test compounds to be used in these nod gene induction assays were based on the logA_50 concentrations reported in Table 3.2.

Results of NodD-dependent nod gene induction assays in wild-type bradyrhizobia carrying the recombinant plasmid pLSC17 are presented in Figure 5.10 A-F. Levels of significance are indicated. Comparison of Figure 5.10 A and B demonstrates that plasmid pLSC17 shows induction of expression of nod genes (as indicated by β-galactosidase activity) only in the presence of a functional nodD gene, as in strain NGR234(pLSC17), and not in the absence of functional NodD, as in strain ANU1255(pLSC17).

The broad host range strain NGR234 showed significant activation of NodD1 (measured by plasmid pLSC17, and shown in Figure 5.10 A) with all of the compounds assayed in this series of experiments, except for umbelliferone, which was an activator in the test system described in Chapter 3 (ie ANU265(pMD1)). The narrow host range strain CP279 failed to induce significant expression of the reporter plasmid in the presence of hesperetin or vanillin, but was otherwise similar to strain NGR234 (shown in Figure 5.10 C & D). Strain CP283, with a broader functional host range than strain CP279, did not induce significant β-galactosidase activity in the presence of DHF nor hesperetin, showing a narrower range of interactions under these experimental conditions than either strain NGR234 or strain CP279 (reported in Figure 5.10 E & F).

A number of inconsistencies appear in the data presented in Figure 5.10, notably when comparing results in 5.10 C and 5.10 D. It is important to note that the assays reported in 5.10 C have fairly large error bars which negate any apparent, relatively low, level of
activity. In contrast, the assays reported in 5.10 D have quite small error bars which validate apparently small increases of activity over the background level.

A summary of compounds activating NodD-dependent nod-gene induction in each wild-type strain is presented in Table 5.3.

Following the induction period for the β-galactosidase assays described in this section, strain CP279 exhibited consistently greater cell densities after exposure to DHF than was seen with other test compounds, or when compared to the control with sterile distilled water. An experiment designed to further investigate this observation is discussed in Section 5.9.

5.8 Analysis of phenolics activating the cloned putative NodDs from CP279 in nodD deficient backgrounds

The β-galactosidase reporter plasmids pLSC17 and pRT311 were tri-parentally mated into transconjugant strains containing one of the three cloned CP279 nodD genes (stains ANU851(pKS1701; pKS2001; pKS4001) and ANU1255(pKS1701; pKS2001; pKS4001) by the method described in Section 2.4.7 using the helper plasmid pRK2013 (Ditta et al. 1980). The transconjugants now contained both a cloned CP279 nodD gene and a NodD-dependent β-galactosidase reporter plasmid, and assays to determine NodD activation were carried out in the presence of the same group of phenolic compounds as was used to assess NodD activation in bradyrhizobia, described above in Section 5.7. The structures of these compounds are shown in Figure 5.9.

Unfortunately the reporter plasmids could not be successfully introduced into, and maintained in, all of the test strains during the period available for the experiments. Where plasmid pLSC17 could not be maintained, results are reported for nod gene induction assays using reporter plasmid pRT311, where possible. Plasmid pLSC17 could not be maintained in strain ANU851 containing any of the CP279 cloned putative nodD genes, nor in strain ANU1255(D40). Likewise plasmid pRT311 could not be maintained in strain ANU851(D17) nor in strain ANU1255(D17).

Results of NodD-dependent nod gene induction assays with strains harbouring the cloned putative nodD genes from strain CP279 are presented in Figure 5.10 (G, H, M-P, along with the levels of significance), and described in Sections 5.8.1 and 5.8.2 below. Results for assays using each of the two reporter plasmids are discussed separately.

A summary of compounds activating NodD-dependent nod-gene induction in each transconjugant strain is included in Table 5.3.
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

### 5.8.1 The activation of CP279 putative NodDs, measured by reporter plasmid pLSC17

CP279 putative *nodD* clones D17 and D20 were assayed in the ANU1255 background containing pLSC17. The control strain (ANU1255 with no introduced *nodD* gene) showed no significant β-galactosidase activity, as shown in Figure 5.10 B.

Figure 5.10 G clearly shows that clone D17 demonstrated NodD activation in the presence of hesperetin and umbelliferone (which did not activate the parent strain CP279 as shown in Figure 5.10 B & C) and also with isovanillin and apigenin (which did). 7,4'-dihydroxyflavone and vanillin failed to activate putative NodD17 where both would induce NodD-dependent *nod* gene transcription in strain CP279 (compare Figure 5.10 C, D & G). Thus the putative NodD encoded by strain CP279 clone D17 is activated by a slightly different range of phenolic compounds compared to the parental strain CP279 when measured by plasmid pLSC17.

Clone D20 only showed additional activation by hesperetin over CP279 when compared in Figure 5.10 H & B & C, and like D17, putative NodD20 was not activated by DHF and vanillin. Thus strain CP279 putative NodD encoded by clone D20 is also activated by a slightly different range of phenolic compounds when compared to the parental strain CP279, and is different again from clone D17.

### 5.8.2 The activation of CP279 putative NodDs, measured by reporter plasmid pRT311

Significant NodD-dependent *nod* gene induction, measured by plasmid pRT311, can be seen to occur only in the presence of a functional *nodD* gene, as in the case of strains NGR234 and ANU843 (illustrated in Figure 5.10 I & J), and not in the absence of such a gene, as shown for strains ANU1255 and ANU851, illustrated in Figure 5.10 K & L).

The putative *nodD* gene of strain CP279 encoded by clones D20 and D40 were assayed for their ability to induce NodD-dependent *nod* gene expression in strains ANU851 and ANU1255 also containing plasmid pRT311 The results are shown in Figure 5.10 M, N, O & P. A more limited range of compounds were assayed using pRT311 than with pLSC17 (described in Section 5.8.1).

Figure 5.10 N shows that clone D20 in the ANU851 background was activated only by DHF, an identical pattern to the host parent strain ANU843 shown in Figure 5.10 J. In the ANU1255 background, clone D20 was activated by coumarin in addition to those compounds which activate the host parent strain NGR234 (both DHF and vanillin, illustrated in Figure 5.10 M & I). Thus the CP279 putative NodD encoded by clone D20 is activated by coumarin where strain CP279(pLSC17) was not (shown in Figure 5.10 C & D).
Clone D40 was activated by DHF and coumarin in both host backgrounds and by vanillin in ANU851, illustrated in Figure 5.10 O & P. As for clone D20, the putative NodD encoded by clone D40 is activated by coumarin where strain CP279(pLSC17) was not.

Flavonoid compounds induce β-galactosidase activity from the NodD-dependent nod gene construct pRT311 in the presence of both of the putative CP279 NodDs (D20 and D40) assayed in these experiments, indicating that both are likely to be complete copies of the relevant nodD homologues.

5.9 Bacterial growth enhancement by phenolic compounds

Growth enhancement of Bradyrhizobium strain CP279 was noted during NodD-dependent nod gene induction assays (performed as described in Section 2.1.6.2), following induction with DHF, when compared with the bacterial control incubated with sterile distilled water (as noted in Section 5.7). Bradyrhizobium strains CP279, CP283 and ANU289 were grown in the presence of a selection of phenolic compounds in order to establish specific evidence of any growth effect of the compounds on each of the particular strains. Each test compound was selected on the basis of known activation of NodDs of these bradyrhizobia (as reported in Section 5.7), or of demonstrated growth enhancement effects on other rhizobia (reported in the relevant literature). Compounds examined for growth enhancing effects by the method described in Section 5.2.6 were: acetylsalicylic acid; benzoic acid; coumarin; daidzein; genistein; 2,4,4-trihydroxychalcone; DHF; and dimethoxyflavone. The results of the growth experiments are presented in Table 5.8.

The results reported for strain CP279 in Table 5.8 show growth inhibition at high concentrations of benzoic acid and acetylsalicylic acid. That is, in the area immediately surrounding the crystals there was a cleared zone where no bacteria grew. Bacterial growth was enhanced at a slightly lower concentration of acetylsalicylic acid, shown by a zone of dense growth at a further distance from the crystals. 2,4,4-trihydroxychalcone, DHF and genistein each enhanced the growth of strain CP279 over the bacterial lawn when compared to the control lawn, and no inhibition zone was observed around the crystals. As Table 5.8 shows, the remaining test compounds had no apparent effect on the growth of strain CP279.

The growth of strain CP283 was inhibited by a high concentration of benzoic acid and acetylsalicylic acid, indicated by cleared zones close to the crystals, but no zone of growth enhancement was observed at lower concentrations of either compound. The remaining test compounds had no apparent effect on the growth of strain CP283, as shown in Table 5.8.
The growth of strain ANU289 was enhanced by the addition of genistein and dimethoxyflavone, shown in Table 5.8, but was apparently unaffected by the remaining test compounds. The difference between this strain and CP283 is interesting since strain ANU289 is a streptomycin resistant derivative of strain CP283, and possibly reflects differing transport mechanisms for these compounds between the two strains.

5.10 Discussion

In his PhD thesis of 1987, Bender reported two genes in *Bradyrhizobium* strain CP279 homologous to the *nodD* gene of *Rhizobium* strain NGR234. In this study it was demonstrated that CP279 contains at least three putative *nodD* homologues. Three clones were identified and analysed and in each case a gene of approximately 1 kb was delineated by hybridisation to the *nodD*1 gene of strain NGR234. This size is similar to that found for the *nodD* genes of other rhizobia (Horvath *et al.* 1987; Appelbaum *et al.* 1988).

In plant nodulation assays discussed in Section 5.4.1, strain CP279 clone D17 complemented the mutated *nodD* gene of strain ANU1255 for nodulation of *Parasponia* and siratro, re-establishing the wild-type host range of the parental strain CP279. Interestingly, the wild-type strain CP279 only nodulates siratro at a low frequency, whereas the test strains containing the CP279 D17 fragment nodulated siratro quite efficiently. Clone D17 also complemented strain ANU1255 for white clover nodulation at a low rate, despite this plant falling outside the host range of either parental strain.

In conjunction with strain ANU851, CP279 clone D17 complemented the inactivated *nodD* gene for nodulation on both white clover and subterranean clover, normal hosts of the recipient wild-type strain ANU843, and on *Parasponia* and siratro, both hosts of strain CP279 but not of ANU843. This complementation confirms that under the control of the appropriate NodD, the *nod* genes of strain ANU843 (the parent of strain ANU851) elaborate the appropriate Nod factors to initiate and achieve nodulation of *Parasponia*, as previously demonstrated by Bender *et al.* 1988 and McIver *et al.* 1989.

The bacteria isolated and cultured from the nodules formed by clone D17 in ANU851 on *Parasponia* and in ANU1255 on white clover were not found to possess the appropriate antibiotic resistance markers. However, the nodules may have been the result of a strain derived from double reciprocal cross-over events, rather than contaminating rhizobia. Such an event, between the resident, mutated, *nodD* gene of the recipient strain, and the introduced D17 *nodD* clone, would result in a strain possessing a hybrid *nodD* gene and a plasmid encoding both antibiotic resistance markers. The resulting strain could then lose the recombined plasmid (with both markers) since no selective pressure was present during the plant assays. Investigation of this possibility was not undertaken, but would be achieved by visualising the plasmids harboured by the strain, using the method of Plazinski and Rolfe, 1985. The identity of the nodule bacteria as the transconjugant
inoculating strain was not confirmed in these two cases, but neither was the possibility of the strains being the result of double reciprocal cross-over events followed up. Thus these results, whilst interesting, would need further work before any conclusions could be drawn.

As reported in Section 5.4.3, CP279 clone D40 complemented strain ANU851 but not strain ANU1255 for nodulation of *Parasponia*, although nodulation occurred at a low frequency. McIver *et al.* 1989 reported that a mutant (C58), of *R. l. bv. trifolii* strain ANU843, with point mutations in the *nodD* gene was able to successfully nodulate *Parasponia*. This result indicated that strain ANU843 possesses nodulation and host range determinants to enable nodulation of the non-legume host. Therefore, under the influence of the appropriate transcriptional activator (in this case the CP279 clone D40) nodulation of *Parasponia* by strain ANU851 might be expected. This putative *nodD* allele would be expected to also complement strain ANU1255 on this homologous host since strain NGR234 possesses nodulation and host range determinants to nodulate *Parasponia*. As it is known that *nod* gene promoters vary slightly in their ability to be activated (Spaink *et al.* 1989), it is possible that clone D40 of strain CP279 is unable to induce transcription of all the *nod* gene operons in strain ANU1255 necessary for production of appropriate Nod factors for the successful nodulation of *Parasponia*.

The experiments reported in Section 5.4.3 indicate that the CP279 putative *nodD* gene encoded by clone D40 confers a narrow host range including only *Parasponia*. Since this host range most closely reflects that of the parent strain I have designated this putative *nodD* gene CP279 *nodD1*.

CP279 Clone D17 in the transconjugant test strains reported in Section 5.4.1 confers a much wider host range than the wild-type parent strain CP279 and will be designated CP279 *nodD2*. The results of the experiments reported in this chapter also demonstrate that the putative *nodD1* and *nodD2* genes cloned from strain CP279 are likely to be complete genes since they are both able induce *nod* gene expression in trans and to complement for inactivated *nodD* gene in plant nodulation assays.

As discussed in Section 5.4.2, CP279 clone D20 showed no capacity to complement for inactivated *nodD* genes in the plant assay strains. On the basis of plant nodulation experiments alone, clone D20 cannot be said to encode a complete functional putative *nodD* gene. However, evidence obtained in NodD-dependent *nod* gene induction assays discussed in Sections 5.8.1 and 5.8.2 demonstrate that this putative NodD is functional since NodD-dependent *nod* gene induction of both plasmids pLSC17 and pRT311 occurs. Thus sufficient evidence exists to refer to this clone as a putative *nodD* gene (CP279 *nodD3*), although the gene copy may be incomplete in the clone D20.

The putative *nodD* gene encoded by CP279 clone D20 may play a role in the nodulation of other plant species, although some species of rhizobia have been found to possess a *nodD* gene for which no role has yet been identified (Horvath *et al.* 1987; Bassam *et al.* 1988; Göttfert *et al.* 1992). There are yet other *nodD* genes which show only minimal or
no involvement in nodulation phenotypes, for example, the nodD2 of R. meliloti on alfalfa (Honma et al. 1987). One may speculate that these genes do not play any role in nodulation but may instead regulate other promoters and gene operons. Recent work has shown that the expression of a number of nol genes (nolBTU and nolX) can be enhanced by flavonoid signal molecules, but no nod-box promoters have been identified for these genes (Meinhardt et al. 1993). One may speculate that the nodD genes with no identified roles in the transcriptional induction of the nod genes (for example, nodD2 of strain NGR234 (Horvath et al. 1987)) may play some role in the induction of these more recently identified nol genes, or perhaps, of other genes which have yet to be identified. Of course the possibility remains that these genes have no definite role in nodulation.

A number of control strains inoculated onto Parasponia, siratro and sub. clover were affected by fungal contamination, and the rate of nodulation was below what would normally be expected in each case (reported in Tables 5.4 - 5.7). However, no control strain which would normally nodulate a plant host failed to do so. Thus, for the transconjugant strains containing the putative CP279 nodD clones, nodulation rates may have been higher had there been no fungal contamination and some of the other transconjugant strains may have formed some nodules had no fungal contamination been present. Some pre-nodulation stages may have been successful (e.g. root hair curling on siratro and clovers) but were not assessed in these experiments.

By separating the CP279 putative nodD2 gene from the other putative nodD genes present in the parental strain and introducing it into another strain of rhizobia, the range of plants nodulated by the transconjugant strains is broadened considerably over that of strain CP279. Clearly the nodD2 gene of strain CP279 can function to activate the expression of the nodulation and host range genes in R. l. bv. trifolii strain ANU843 and thus extend the host range of this strain.

As discussed in Sections 5.7 and 5.8, the control strains used in the NodD-dependent nod gene induction assays clearly confirm that both reported plasmids only exhibit significant β-galactosidase activity in the presence of functional nodD genes.

When the cloned CP279 putative nodD genes were individually examined for induction of transcription of the NodD-dependent nodY::lacZ fusion derived from plasmid pZB32 (Banfalvi et al. 1988), discussed in Section 5.8, each of the three clones were shown to be functional. Furthermore, the results of the experiments presented in Section 5.8.1 indicate that both clones D17 and D40 are likely to contain complete copies of nodD, since both can be activated by phenolic and flavonoid compounds and both complement for nodulation on certain plant hosts. These experiments also confirm that each clone encodes a different protein since the products are activated by slightly different groups of phenolic compounds and complement for nodulation on different plants. In particular, when clone D17 was assayed against a range of phenolic compounds it was found to be activated by compounds with similar substitution patterns to those compounds which
activate NodD1 of the broad host range strain NGR234 (Chapter 3; Le Strange et al. 1990). Compounds which are inhibitors (or anti-inducers) of NodD-dependent nod gene expression in the narrow host range Rhizobium strains include coumarins, isoflavones and flavonols (Redmond et al. 1986; Firmin et al. 1986; Djordjevic et al. 1987). Of these types of compounds, umbelliferone was shown to induce NodD-dependent nod gene transcription by clone D17. Coumarin was shown to be an activator for clones D20 and D40 but was not assayed against D17. Clones D20 and D40 were not assayed against umbelliferone. No isoflavones or flavonols were included in this study.

Different strains often show different background levels of β-galactosidase activity in the presence of the same reporter plasmid. Relatively low levels of β-galactosidase activity were induced using plasmid pLSC17 to assay NodD-dependent nod gene induction. This also seems to be a feature of the parent reporter plasmid pZB32. When used to assess the activation of nodD gene products of B. japonicum strains by phenolic compounds, pZB32 assays required increased sensitivity in the chromophore (Banfalvi et al. 1988). Chlorophenyl red β-D-galactopyranoside (CPRG) was used by Banfalvi et al. (1988) in place of 2-nitrophenyl-β-D-galactopyranoside (ONPG) to achieve increased sensitivity. Unfortunately, CPRG was unavailable in our laboratory at the time the assays were performed. The longer induction periods required for NodD-dependent nod gene induction in bradyrhizobia is interesting since this induction occurs rapidly in various Rhizobium strains (Redmond et al. 1986 and others). Given their slower generation times, it could be expected that an uptake mechanism for phenolic compounds in Bradyrhizobium strains would be slower than for rhizobia. Alternatively these molecules may require bacterial processing before serving as activating compounds. An example of such processing, C-ring fission of flavonoids, was recently demonstrated in B. japonicum (Rao and Cooper 1994).

All the phenolic compounds examined for induction of NodD-dependent nod gene induction in strain CP279 are activators of NGR234 NodD1 - reported in Chapter 3, using strain ANU265(pMD1), see section 3.3. A summary of the activation profiles of the CP279 putative nodD clones and other bradyrhizobia is presented in Table 5.3.

Direct comparison of assay results for strain CP279 and its cloned putative nodD genes is difficult since different reporter plasmids were used. Plasmid pRT311 was unsuitable for use in CP279 due to endogenous tetracycline resistance, and pLSC17 could not be maintained in some of the transconjugant assay strains.

Furthermore, comparison between assays performed using plasmid pLSC17 and those using pRT311 must be somewhat cautious since the constructs relied on different NodD-dependent nod-gene cassettes (in the former case nodY from B. japonicum, in the latter nodA from R. l. bv. trifolii). Although there is evidence of extremely strong homology between the nod box elements of the nod gene promoters of the different species of rhizobia, which argues for extremely similar induction of transcription of the
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

### Table 5.3 A  Summary of NodD activation

<table>
<thead>
<tr>
<th>Figure</th>
<th>assay strain details</th>
<th>β-gal activity induced by</th>
<th>Not induced by</th>
<th>not assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NGR234(pLSC17)</td>
<td>dhf1 van1 van2 isov1 hes1 api2</td>
<td>umb</td>
<td>cou</td>
</tr>
<tr>
<td>B</td>
<td>ANU1255(pLSC17)</td>
<td>NONE</td>
<td>dhf van isov umb hes api</td>
<td>cou</td>
</tr>
<tr>
<td>C</td>
<td>CP279(pLSC17)</td>
<td>isov1 api2</td>
<td>dhf van umb hes</td>
<td>cou</td>
</tr>
<tr>
<td>D</td>
<td>CP279(pLSC17)</td>
<td>dhf3 dhf4 van3</td>
<td>cou</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CP283(pLSC17)</td>
<td>van4</td>
<td>dhf cou</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CP283(pLSC17)</td>
<td>isov1 api2</td>
<td>dhf van umb hes</td>
<td>cou</td>
</tr>
<tr>
<td>G</td>
<td>ANU1255(D17)(pLSC17)</td>
<td>isov1 umb2 hes2 api1 api2</td>
<td>dhf van</td>
<td>cou</td>
</tr>
<tr>
<td>H</td>
<td>ANU1255(D20)(pLSC17)</td>
<td>isov1 hes2 api1 api2</td>
<td>dhf van umb</td>
<td>cou</td>
</tr>
<tr>
<td>I</td>
<td>NGR234(pRT311)</td>
<td>dhf3 dhf4 van3 van4</td>
<td>cou</td>
<td>as above</td>
</tr>
<tr>
<td>J</td>
<td>ANU843(pRT311)</td>
<td>dhf4</td>
<td>cou van</td>
<td>as above</td>
</tr>
<tr>
<td>K</td>
<td>ANU1255(pRT311)</td>
<td>NONE</td>
<td>dhf cou van</td>
<td>isov umb hes api</td>
</tr>
<tr>
<td>L</td>
<td>ANU851(pRT311)</td>
<td>NONE</td>
<td>dhf cou van</td>
<td>as above</td>
</tr>
<tr>
<td>M</td>
<td>ANU1255(D20)(pRT311)</td>
<td>dhf3 dhf4 cou1 van3 van4</td>
<td>as above</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>ANU851(D20)(pRT311)</td>
<td>dhf4</td>
<td>cou van</td>
<td>as above</td>
</tr>
<tr>
<td>O</td>
<td>ANU1255(D40)(pRT311)</td>
<td>dhf4 cou1</td>
<td>van</td>
<td>as above</td>
</tr>
<tr>
<td>P</td>
<td>ANU851(D40)(pRT311)</td>
<td>dhf3 dhf4 cou1 van4</td>
<td>as above</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.3 B  Phenolic compounds used in nod-gene induction assays

<table>
<thead>
<tr>
<th>abbreviation</th>
<th>compound</th>
<th>concentration</th>
<th>abbreviation</th>
<th>compound</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>api1</td>
<td>apigenin</td>
<td>1.00 x 10^-6</td>
<td>hes2</td>
<td>hesperetin</td>
<td>5.00 x 10^-5</td>
</tr>
<tr>
<td>api2</td>
<td>&quot;</td>
<td>1.00 x 10^-7</td>
<td>isov1</td>
<td>isovanillin</td>
<td>1.00 x 10^-4</td>
</tr>
<tr>
<td>cou1</td>
<td>coumarin</td>
<td>1.00 x 10^-5</td>
<td>umb1</td>
<td>umbelliferone</td>
<td>1.00 x 10^-5</td>
</tr>
<tr>
<td>dhf1</td>
<td>7,4′-dihydroxyflavone</td>
<td>5.00 x 10^-7</td>
<td>umb2</td>
<td>&quot;</td>
<td>5.00 x 10^-4</td>
</tr>
<tr>
<td>dhf2</td>
<td>&quot;</td>
<td>5.00 x 10^-6</td>
<td>van1</td>
<td>vanillin</td>
<td>1.00 x 10^-6</td>
</tr>
<tr>
<td>dhf3</td>
<td>&quot;</td>
<td>1.00 x 10^-7</td>
<td>van2</td>
<td>&quot;</td>
<td>5.00 x 10^-4</td>
</tr>
<tr>
<td>dhf4</td>
<td>&quot;</td>
<td>1.00 x 10^-6</td>
<td>van3</td>
<td>&quot;</td>
<td>1.00 x 10^-5</td>
</tr>
<tr>
<td>hes1</td>
<td>hesperetin</td>
<td>1.00 x 10^-7</td>
<td>van4</td>
<td>&quot;</td>
<td>1.00 x 10^-4</td>
</tr>
</tbody>
</table>

The structures of some compounds referred to in Table 5.3 are presented in Figure 5.9.

**nod operons** (Rostas et al. 1986; Spink et al. 1987a; Wang and Stacey 1991), there is also some evidence of differential transcription of nod operons (McKay and Djordjevic 1993). In addition, the nodD mutants used as background strains for these assays may have different transport affinities or mechanisms for phenolic compounds compared to the parent strain CP279, and each transconjugant strain may thus require specific concentrations of phenolic compounds to be present before significant (putative) NodD activation occurs. For example clone D20, assayed in ANU1255 was activated by vanillin and DHF in assays with pRT311, but by neither of these with pLSC17 (at slightly higher concentrations), yet both induce the β-galactosidase activity in CP279. The compounds isovanillin, umbelliferone, hesperetin and apigenin were not used in pRT311 assays due to the time period available in which to complete these experiments.

The structures of some compounds referred to in Table 5.3 are presented in Figure 5.9. The statistical approach used to analyse the assay data does not rely on multiples of background activity to determine if activity is significant, nor on the absolute level of induction seen in controls and test samples. Thus it is inappropriate to discuss the relative 'strength' of induction. Some assays show a wider variability between replicates, thereby increasing the size of the standard error of the difference (sed) and reducing the likelihood of significant activity.
It is interesting to note the different activation profiles of clone D20 when assayed with each of the two plasmids (see Table 5.3 H & M), specifically, the non activation of clone D20 by DHF and vanillin when assayed with pLSC17. It serves to highlight the caution required when making comparisons between assays using the two different reporter plasmids, and supports the observation that pRT311 is the more sensitive of the two reporter plasmids used in these experiments.

Bearing these caveats regarding direct comparison of β-galactosidase assay results in mind, some comparative statements may be useful. When assayed with plasmid pLSC17 in ANU1255, clones D17 and D20 were both activated by more phenolic compounds (D17 and D20 by hesperetin, and D17 by umbelliferone) than the parent strain CP279. However, neither showed significant activation by vanillin where CP279 was clearly activated by this compound. When assayed with plasmid pRT311, the product of clone D40 was activated by coumarin in both backgrounds and vanillin in strain ANU851, where putative NodD20 was activated by either compound only in the ANU1255 background.

Despite the problems of comparison detailed above, the cocktail of compounds which co-induce NodD-dependent nod gene expression in the wild-type strain CP279 appears to be narrower in the experiments reported in this chapter than the group of compounds shown to activate the individual putative nodD clones from that strain. These data point to the interesting prospect that in the wild-type strain potentially all three putative nodD genes of strain CP279 function in a co-ordinated manner to regulate the induction of NodD-dependent nod genes.

The regulation of nodD genes in B. japonicum differs from that of most Rhizobium species (excepting R. meliloti) (Mulligan and Long 1989; Long 1989) in that nodD transcription can be enhanced by the same inducer compounds co-activating the nodYABC operon (Banfalvi et al. 1988). It is currently unknown if the nodD genes of strain CP279 are similarly regulated.

Of the other bradyrhizobia examined for NodD-dependent transcriptional activation, strain CP283 showed significant activation by the compounds vanillin, isovanillin and apigenin, but the range was slightly narrower than for strain CP279, which was in turn narrower than for strain NGR234 (as shown by pLSC17 assays). This belies the relative broadness of the host range of the three strains, where CP279 has the narrowest host range. The nod gene induction obtained in strain ANU843(pRT311) was consistent with previous studies (Redmond et al. 1986; Djordjevic et al. 1987; McIver et al. 1989).

A smaller range of phenolic compounds was examined for co-induction with CP279 nodD genes than was the case with strain NGR234 nodD1 (discussed in Chapter Three), but the results presented in this chapter demonstrate that a variety of flavonoid structures will induce NodD-dependent nod gene transcription in the wild-type Bradyrhizobium strain CP279 assayed with plasmid pLSC17. Moreover, the simple phenolic compounds
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

Isovanillin and vanillin are also NodD activators in strain CP279, demonstrating that the *nodD* gene products of this strain, as a group, are capable of ‘recognising’ a wide variety of inducing compounds.

Umbelliferone did not elicit any significant induction of expression in strain NGR234(pLSC17) (Figure 5.10 A). However, when assayed using plasmid pMD1 umbelliferone was shown to be a NodD1 activator (see Chapter Three, Table 3.1). These results further support the proposition that plasmid pLSC17 is a relatively insensitive reporter plasmid in the test system reported in these experiments.

The hypothesis that the capacity of NodD to interact with a wide variety of plant derived compounds, including simple phenolic compounds, is the initial determinant of broad host range is supported by the results reported in this chapter since CP279 clone D17 (encoding the putative CP279 *nodD2*) recognises a relatively broad range of phenolic structures and confers nodulation on each of the plant hosts examined. Furthermore, the activation of NodD by monocyclic phenolic compounds has been shown here to be a common factor of strains able to nodulate the non-legume *Parasponia*. However, regulation of *nodD* gene expression or of NodD activity clearly influences the nodulation phenotype expressed by strains of rhizobia. Regulation of *nodD* expression may be achieved by the separate autoregulation of each of the putative *nodDs*, or by the co-ordinated regulation of the NodDs, and may also be influenced by phenolic compounds. Regulation of NodD activity could be achieved through modification of the conformation of NodD, altering the activation capacity of the protein. Other effects on nodulation related to NodD activation could involve alteration of the amounts and types of Nod metabolites (CLOS) produced via activated *nodD* products inducing transcription of various *nod* gene operons to different extents, as was recently reported by McKay and Djordjevic, (1993).

Whilst the direct interaction of NodD with intact flavonoid molecules has yet to be proved, recent evidence shows various rhizobia are able to degrade flavonoid structures via C-ring fission (Rao and Cooper 1994). Indeed the products from flavonoid degradation have been shown to be incorporated into Nod factors elaborated by rhizobia (Cooper *et al.* 1995), at flavonoid concentrations which activate NodD.

Aromatic and flavonoid compounds have been shown by previous workers to variously enhance and inhibit bacterial cell growth in rhizobia (Chen *et al.* 1984; Hartwig *et al.* 1991). In the soil, these compounds, some of which can be found in plant root exudates and others as the result of lignin breakdown, can constitute an important carbon and energy source for a wide variety of soil bacteria. Indeed the family Fabales have relatively high rates of root exudation. For example, the roots of 9 day old black seeded beans release genistein, eriodictoyl and naringenin at rates of 42, 281 and 337 nmoles per plant per day (Hungria *et al.* 1991), and a 10 day-old *Cicer arietinum* (chick pea)
seedling can excrete 80 µg amino acids and 30 µg sugar and uronic acids per day, supporting the growth of about $3 \times 10^8$ cells of *B. japonicum* (Werner 1992, p 75).

In the simple experiments reported in this chapter, monocyclic phenolic compounds had significant affects on the growth of some bradyrhizobia, as did a number of the polycyclic, flavonoid compounds. Of the small range of compounds examined for growth enhancing capacity, those that activate NodD1 of strain NGR234 (Table 3.1) also enhanced growth of strain CP279, but not of strain CP283. 7,4'-dihydroxyflavone enhanced the growth of strain CP279 and was an inducer of nodulation gene expression in this strain shown in Figure 5.10 C. The different effects of the compounds on strains CP283 and ANU289 are interesting since the latter is a streptomycin resistant mutant of the former. Strain ANU289 was not subject to the inhibiting effects of acetylsalicylic and benzoic acids that affected strain CP283 but its growth was enhanced by genistein and dimethoxyflavone where that of CP283 was not. Differing permeability of the cell outer membrane of each of the two strains would appear to be an effect co-existent with the resistance to streptomycin of strain ANU289, and is a common mechanism of antibiotic resistance in gram negative strains (Davis 1980). Interestingly, quercetin was shown to increase the growth rate of *R. meliloti* but did not induce transcription of NodD1- or NodD2-dependent *nod* gene fusions (Hartwig et al. 1991).

Since isoflavonoids and flavonoids play an important role in plant defence against some pathogenic bacteria and fungi, particularly in the *Leguminoseae* (Koes et al. 1994), it makes some sense for rhizobia to have adapted to the presence of this type of compound in the rhizosphere of legume hosts. Evidence also shows that in many cases rhizobia can use such compounds as sources of carbon and energy.

Chen and co-workers (1984) showed that strains of *R. l. bv. trifolii* and *bv. viciae* are able to catabolize various simple aromatic compounds and to grow rapidly on 3mM 4-hydroxybenzoate and protocatechu ate (3,4-dihydroxybenzoate), key metabolites in convergent branches leading to the β-ketoadipate metabolic pathway. Derivatives of catechol and protocatechuate pathway intermediates were also investigated and both strains were found to grow well on 3mM 3,4,5-trihydroxybenzoate, to grow more slowly on 3,5-dihydroxybenzoate, 4-hydroxy-3-methoxybenzoate, 4-hydroxy-3-methoxyphenyl propenoate (ferulate) and 4-hydroxy-3-methoxyphenyl propanoate (dihydroferulate). *R. l. bv. trifolii* also grew on benzoate, 1,2-dihydroxybenzene (catechol), 2-hydroxybenzoate (salicylate), and 4-methylbenzoate (p-toluate) (Chen et al. 1984). Clearly, in enhancing growth of these rhizobia, hydroxy substitutions at position 3 and 4 are of most importance, particularly when positioned opposite an electron-withdrawing function. These structural features are also important in the NGR234 NodD1-dependent activation of *nod* genes detailed in Section 3.4 (Le Strange et al. 1990). Bradyrhizobia also catabolize aromatic compounds, and have been shown to utilise a wider variety of aromatic compounds than the faster growing rhizobia (Parker et al. 1977).
The role of Bradyrhizobium strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

A 5,7-dihydroxy substitution pattern of flavonoids was shown to be important for growth enhancement of R. meliloti and R. l. bv. trifolii, with 25µM chrysin, luteolin and quercetin promoting growth but flavone, 5-hydroxyflavone and 7-hydroxyflavone having no impact (Hartwig et al. 1991). This effect was thought to be independent of nodD, since a R. meliloti strain mutated in all three nodD genes also showed an enhanced growth response, and that quercetin increased growth but did not induce nodC::lacZ fusions (Hartwig et al. 1991). Interestingly, no evidence was found that luteolin was metabolised by R. meliloti. Luteolin, quercetin and their glycosides all promoted bacterial growth rate, and growth enhancement was most marked when the bacteria were grown on minimal medium (Hartwig et al. 1991). It is not established by the experiments reported in Section 5.9 whether the Bradyrhizobium strains showing growth enhancement in response to the test compounds in fact metabolised the compounds.

Ecochemical zones around roots, established as a result of the release of compounds from plant roots and seeds, including a variety of flavonoids, can therefore be seen to influence the bacterial population in the emerging plants’ rhizosphere (Djordjevic et al. 1987). For example, Redmond and colleagues (1986) established that sufficient concentrations of activating compounds were exuded from the roots of white clover and alfalfa seedlings to induce NodD-dependent nod gene transcription, resulting in blue zones around the roots of the seedlings on X-gal medium (Redmond et al. 1986). It is unclear whether sufficient concentrations of growth enhancing compounds would be present in the rhizosphere of the normal host plants of the Bradyrhizobium strains investigated in Section 5.9 to result in increased growth. It would thus be of interest to modify the experiments reported in Section 5.9 by placing seedlings of various plant species onto lawns of the same bradyrhizobia, and thereby examine any growth effect on the rhizobia of compounds released by the seedlings into their rhizosphere.

Available evidence in relation to rhizobial metabolism of monocyclic aromatic compounds indicates an active uptake mechanism (Chen et al. 1984), but no such mechanism has been identified for flavonoids. However, evidence from Schlaman et al. 1989 indicated localisation of NodD at the cell membrane, and thus flavonoids may not need to be transported into the cytoplasm for NodD activation to occur. The outer membrane of gram negative bacteria behaves as a coarse molecular sieve, allowing diffusion of molecules up to molecular weight 800 (Levie and Davis 1980). Since most flavonoids are of the order 250 M.W., simple diffusion through the outer membrane to the cytoplasmic membrane may be possible. Nevertheless the possibility remains that the interaction of NodD and activating compounds occurs within the cell, necessitating an active transport mechanism for flavonoid compounds. If this is the case, then the use of Rhizobium strains as the nodD mutant background for nod gene induction assays may not accurately reflect the possible activation of the putative cloned CP279 NodDs due to different flavonoid uptake characteristics of the cell membranes of the different genera.
Table 5.4 The nodulation of *Parasponia* by CP279 putative *nodD* clones.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% nodulated plants</th>
<th>analysis of bacteria from nodule squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENTAL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU851 - <em>nodD</em> mutant of strain ANU843</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS1701)</td>
<td>8</td>
<td>x</td>
</tr>
<tr>
<td>ANU851(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS4001)</td>
<td>25</td>
<td>√</td>
</tr>
<tr>
<td>ANU1255 - <em>nodD</em> mutant of strain NGR234</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS1701)</td>
<td>75</td>
<td>√</td>
</tr>
<tr>
<td>ANU1255(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CONTROL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP279</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CP283</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NGR234</td>
<td>60*</td>
<td></td>
</tr>
</tbody>
</table>

**Legend**

- √ bacteria isolated from nodule grew on tetracycline and kanamycin supplemented media
- x bacteria isolated from nodule FAILED to grow on tetracycline and kanamycin supplemented media
- * lower than normal rate of nodulation due to fungal contamination (usually nodulates at 100%).

*Parasponia* seeds were germinated as described in Section 2.3.2. The magenta jar assay was performed as described in Section 2.3.8, with 5 plants per replicate, 2-3 replicates per experiment and 1 experimental repeat.

Each nodulated plant had between 10 and 15 nodules.

Nodule squashes were performed on selected, excised nodules as described in Section 2.3.10, and the isolated bacteria examined for growth on appropriate antibiotic (Tc and Km) supplemented media.
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

Table 5.5 The nodulation of siratro by CP279 putative *nodD* clones.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% nodulated plants</th>
<th>analysis of bacteria from nodule squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENTAL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU851 - <em>nodD</em> mutant of strain ANU843</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>ANU851(pKS1701)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255 - <em>nodD</em> mutant of strain NGR234</td>
<td>0</td>
<td>√</td>
</tr>
<tr>
<td>ANU1255(pKS1701)</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CONTROL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP279</td>
<td>0</td>
<td>C</td>
</tr>
<tr>
<td>CP283</td>
<td>50*</td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NGR234</td>
<td>20*</td>
<td></td>
</tr>
</tbody>
</table>

Legend

√ bacteria isolated from nodule grew on tetracycline and kanamycin supplemented media

ND nodule squash not carried out.

C bacteria isolated from nodule grew only on media appropriate for this control strain.

* lower than normal rate of nodulation due to fungal contamination (usually nodulates at 100%).

Siratro seeds were germinated as described in Section 2.3.4. The magenta jar assay was performed as described in Section 2.3.8, with 5 plants per replicate, 4 replicates per experiment and 3 experimental repeats.

Each nodulated plant had between 5 and 10 nodules.

Nodule squashes were performed on selected, excised nodules as described in Section 2.3.10, and the isolated bacteria examined for growth on appropriate antibiotic (Tc and Km) supplemented media.
Table 5.6 The nodulation of white clover by CP279 putative \textit{nodD} clones.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% nodulated plants</th>
<th>analysis of bacteria from nodule squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENTAL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU851 - \textit{nodD} mutant of strain ANU843</td>
<td>0</td>
<td>(\checkmark)</td>
</tr>
<tr>
<td>ANU851(pKS1701)</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255 - \textit{nodD} mutant of strain NGR234</td>
<td>15</td>
<td>x</td>
</tr>
<tr>
<td>ANU1255(pKS1701)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CONTROL STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP279</td>
<td>0</td>
<td>C</td>
</tr>
<tr>
<td>CP283</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NGR234</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Legend

\(\checkmark\) bacteria isolated from nodule grew on tetracycline and kanamycin supplemented media

x bacteria isolated from nodule FAILED to grow on tetracycline and kanamycin supplemented media.

C bacteria isolated from nodule grew only on media appropriate for this control strain.

White clover seeds were germinated as described in Section 2.3.5. The plate assay was performed as described in Section 2.3.7, with 5 plants per replicate, 4 replicates per experiment and 2 experimental repeats.

Each nodulated plant had between 5 and 10 nodules.

Nodule squashes were performed on selected, excised nodules as described in Section 2.3.10, and the isolated bacteria examined for growth on appropriate antibiotic (Tc and Km) supplemented media.
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

Table 5.7 The nodulation of subterranean clover by CP279 putative *nodD* clones.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% nodulated plants</th>
<th>analysis of bacteria from nodule squash</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EXPERIMENTAL STRAINS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU851 - <em>nodD</em> mutant of strain ANU843</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS1701)</td>
<td>40</td>
<td>√</td>
</tr>
<tr>
<td>ANU851(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU851(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255 - <em>nodD</em> mutant of strain NGR234</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS1701)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS2001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU1255(pKS4001)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>CONTROL STRAINS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP279</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CP283</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C</td>
</tr>
<tr>
<td>NGR234</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Legend**

√ bacteria isolated from nodule grew on tetracycline and kanamycin supplemented media.

C bacteria isolated from nodule grew only on media appropriate for this control strain.

<sup>a</sup> 2 replicates only. Some plants were affected by fungal contamination and reduced the nodulation rate to 90%. Nodulation would normally be 100%.

Subterranean clover seeds were germinated as described in Section 2.3.6. The magenta jar assay was performed as described in Section 2.3.8, with 5 plants per replicate, 4 replicates for the experiment. When carried out on plates (plate assay, Section 2.3.7) NO nodules were formed by transconjugant strains.

Nodule squashes were performed on selected, excised nodules as described in Section 2.3.10, and the isolated bacteria examined for growth on appropriate antibiotic (Tc and Km) supplemented media.
Table 5.8  The effect of phenolic compounds on growth of Bradyrhizobia

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CP279</th>
<th>CP283</th>
<th>ANU289</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lawn (no compound added)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>5 mm CZ</td>
<td>3 mm CZ</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7-10 mm GEZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>3 mm CZ</td>
<td>3 mm CZ</td>
<td>+</td>
</tr>
<tr>
<td>coumarin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>244 trihydroxylchalcone</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7,4'-dihydroxyflavone</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>daidzein (4',7 diOH isoflavone)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>genistein</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>dimethoxyflavone</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Legend

+ Level of bacterial growth on unsupplemented media.
++ Bacterial growth significantly heavier than on unsupplemented media over entire plate surface.
CZ Cleared Zone - no bacteria grew within this zone. Growth on remainder of plate + unless otherwise indicated. The size of the cleared zone is indicated and represents the radius measured from the crystals in the centre of the plate.
GEZ Growth enhancement zone - Bacterial growth significantly heavier than on unsupplemented media, in a zone radiating form the compound crystals. Growth on remainder of plate + unless otherwise indicated. The size of the zone is indicated and represents the radius measured from the crystals in the centre of the plate.

Replicate lawns of each strain on GMM plates (as described in Section 2.1.2) were prepared from log phase cultures in BMM liquid media (also described in Section 2.1.2). Lawns were grown overnight at 28°C, then crystals were added to the centre of the plate. Growth effects were recorded after 2 days growth at 28°C.
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

This page is intended to be left blank.
Figure 5.1

Hybridisation of NGR234 \textit{nodD1} probes to digests of strain CP279 total DNA.

A) Total DNA from strain CP279 was digested with the restriction enzymes \textit{BamHI} (lane 2); \textit{EcoRI} (lane 3); \textit{HindIII} (lane 4) and \textit{PstI} (lane 5).

Lane 1 is lambda DNA digested with \textit{HindIII} and \textit{EcoRI} as a size standard.

DNA fragments were transferred to Hybond-N membranes and hybridised to $[^{32}\text{P}]$-dATP labelled probes prepared from B) a 0.4 kb \textit{EcoRI - BamHI} fragment carrying the 5' region of the \textit{nodD1} gene of strain NGR234; C) a 0.75 kb \textit{BamHI-EcoRI} - fragment carrying the 3' region of the \textit{nodD1} gene of strain NGR234.

The approach adopted for the hybridisations is described in Section 5.2.1.
Figure 5.2

The generation of $\alpha^{32}$P probes fragments from NGR234 nodD1 by the polymerase chain reaction (PCR).

A: Map of plasmid pJJ252, showing the location of a 1.9 kb PstI fragment carrying the nodD1 gene of strain NGR234 (crosshatched area) inserted into plasmid pBluescript SK'. The direction of transcription of the nodD1 gene, and the primer sites for the T7 universal primer and the 22mer, are indicated (arrows). Other sites in the multi-linker not shown. B - BamHI; E = EcoRI; P = PstI; and S = SmaI.

B: The sequence of the 22mer primer for PCR amplification of the NGR234 nodD1 gene. The 22mer consists of a 16mer homologous to the NGR234 DNA sequence outside of the coding region, and bases added to the 5' end to code for and EcoRI site.

C: Map of the DNA fragment generated by PCR, showing: the coding region for the NGR234 nodD1 gene (crosshatched area); the nod-box. The direction of transcription of the nodD1 gene, and the primer sites for the T7 universal primer and the 22mer, are indicated (arrows). Also shown are the introduced restriction site EcoRI (under the 22mer arrow), and the existing restriction sites BamHI, PstI and EcoRI.

Sections a) and c) are not to scale.

The approach adopted for the generation of the probe fragments is described in Section 5.2.1.
**Figure 2.**

**a.**

![Diagram of nodD1 region](image)

**b.**

16mer homologous region

\[
\begin{align*}
C & \quad G & \quad T & \quad A & \quad C & \quad G & \quad C & \quad G & \quad G & \quad A & \quad G & \quad G & \quad A & \quad C & \quad T & \quad T \\
A & \quad A & \quad G & \quad G & \quad G
\end{align*}
\]

EcoR1 site

**c.**

![Diagram of EcoR1 sites](image)
Figure 5.3

The fractionation of strain CP279 DNA.

CP279 DNA, digested with *Bgl*II and fractionated by density gradient centrifugation through a sucrose gradient. Lane numbers indicate the collected fraction. The unnumbered lane on the far right is \( \lambda \) DNA size markers, size in kb indicated.

The approach adopted for the sucrose gradient fraction of CP279 DNA is described in Section 5.2.2.
Figure 5.4

Cloning of the CP279 DNA BgII fragments into plasmid pJJ358.

Plasmid pJJ358, showing the multiple cloning site introduced into plasmid Bluescript SK−, and indicating the BgII site into which CP279 BgII fragments were ligated. Also shown by solid bars are the promoterless lacZ gene (arrow indicates the direction of transcription), the ampicillin resistance gene, and the internal ScaI restriction site.

The approach adopted for the cloning of CP279 BgII fragments into pJJ358 is described in Section 5.2.2.
AmpR

pBluescript
SK+

2.95kb

SacI

AmpR

SacI

pBluescript
SK+

2.95kb

HindIII

Xbal

BglII

PstI

BamHI

EcoRI

CP279

DNA Fragments

ATPase

ATPase

ATPase

ATPase

ATPase
Figure 5.5

The subcloning of CP279 putative nodD genes into plasmid pLAFR3.

Shown are: the insertion sites of the three cloned CP279 nodD genes; the multiple cloning site of plasmid pLAFR3; the promoterless lacZ gene and its direction of transcription (arrow); and some of the pLAFR3 internal restriction sites.

The approach adopted for the subcloning of the CP279 putative nod genes into plasmid pLAFR3 is described in Section 5.2.3.
Diagram of restriction sites in various plasmids:

- **pLAFR3**: 22.4kb
  - Restriction sites: Scal, BglII, BgIII
  - lacZ

- **pKS4001**: HindIII/EcoRI insert
  - Restriction sites: EcoRI, SacII, KpHl, BamHI, Sall

- **pKS2001**: BglII insert
  - Restriction sites: BglII, Sall, PstI, SphI, HindIII

- **pKS1701**: PstI insert
  - Restriction sites: PstI, SphI, HindIII
Figure 5.6

Construction of plasmid pLSC17, showing the cloning of the *nodY::*lacZ* fusion from plasmid pZB32 to plasmid pKT240.

a) Map of plasmid pZB32, adapted from Banfalvi *et al.* 1988. Shown are the *nodY* gene (shaded area, arrow indicates direction of transcription), positioned to drive transcription of the *lacZ* gene (solid arrow), the *nod*-box site (solid bar); and the extent of the *BamHI* fragment cloned into plasmid pKT240.

b) Map of plasmid pKT240, showing the site of insertion of the *nodY::*lacZ* fusion from plasmid pZB32.

Restriction sites: B = *BamHI*, E = *EcoRI*, H = *HindIII*, P = *PstI*. Abbreviations: Te\(^R\) - tetracycline resistance, Km\(^R\) - kanamycin resistance, Amp\(^R\) - ampicillin resistance.

The approach adopted for subcloning the *nodY::*lacZ* fusion into plasmid pKT240 is described in Section 5.2.4.
Figure 6.2

(a) Map of pZB32 gene map

- pZB32: 31.5kb
- Tc^r
- B: Bgl II
- P: Pst I
- lacZ
- nodY

(b) Map of pKT240

- pKT240: 12.9kb
- Amp^r
- Km^r
- E: Eco RI
- H: Hind III
Figure 5.7

Restriction maps of the three cloned CP279 putative nodD genes.

Clones were generated from CP279 DNA digested with BgIII, fractionated on a sucrose gradient, and cloned into plasmid pJJ358. Maps were constructed from multiple restriction digests, and hybridisation of the resulting fragments to radiolabelled probe prepared from the nodD1 gene of strain NGR234. Each map indicates the size of the cloned fragment, restriction sites of the cloned genes, the sizes of the fragments generated by restriction digests, and the degree of hybridisation to the NGR234 nodD1 probe. The bar underneath indicates degree of hybridisation - the heavily shaded bar indicates strong hybridization to the NGR234 nodD1 probe, the crosshatched bar indicates weak homology to the NGR234 nodD1 probe, and the unshaded bar indicate no hybridization to the NGR234 nodD1 probe.

Size indications are approximate only. Restriction sites internal to the cloned fragment are shown above the line, those of the multiple cloning site of plasmid pJJ358 are below the line.

Restriction sites: B = BamHI, Bg = BgIII, E = EcoRI, H = HindIII, P = PstI, X = XbaI.

a) Map of CP279 clone nodD17
b) Map of CP279 clone nodD20
c) Map of CP279 clone nodD40
Figure 5.8

Physical map of plasmid pRT311.

Shown are the 0.7 kb BamHI fragment, containing the nodD/nodA intergenic region (shaded region) and the nodA promoter (vertical bar) positioned to drive the transcription of the lacZ gene of plasmid pMP220 (solid segment of circle) (McIver et al. 1989)

Plasmid pRT311 was used to measure NodD-dependent nod gene induction in the assays described in Section 5.8.2.
multiple cloning site

LacZ + nodA114

B

pMP220
10.5kb

B
Figure 5.9

The structures of phenolic compounds examined for activation of the NodDs of bradyrhizobia.

Vanillin, isovanillin - the structure of vanillin is shown, isovanillin has the two substituents reversed.

These compounds were used to assess NodD activation in the NodD-dependent nod gene induction assays described in Sections 5.7 and 5.8.
OH
H
I
C = O
OCH₃
OH
vanillin
isovanillin
coumarin
OH
OH
0, 0
coumarin
OH
OH
0
0
0
0
7, 4'-dihydroxyflavone
umbelliferone
OH
OH
0
0
OH
0
0
apigenin
hesperetin
NodD-dependent \textit{nod} gene induction assays measuring the activation of bradyrhizobia NodDs

Reporter plasmid pLSC17 in strain

A) NGR234(pLSC17)  
B) ANU1255(pLSC17)  
C) CP279(pLSC17)  
D) CP279(pLSC17)  
E) CP283(pLSC17)  
F) CP283(pLSC17)  
G) ANU1255(nodD17)(pLSC17)  
H) ANU1255(nodD20)(pLSC17)  

Reporter plasmid pRT311 in strain

I) NGR234(pRT311)  
J) ANU843(pRT311)  
K) ANU1255(pRT311)  
L) ANU851(pRT311)  
M) ANU1255(nodD20)(pRT311)  
N) ANU851(nodD20)(pRT311)  
O) ANU1255(nodD40)(pRT311)  
P) ANU851(nodD40)(pRT311)

NodD-dependent \textit{nod} gene induction assays to measure the level of induction of expression of an introduced $\beta$-galactosidase gene under the control of NodD in the presence of selected compounds were carried out as described in Sections 2.1.6.1 and 2.1.6.2, using sterile distilled water for the compound-free background control.

Assays for induction response to each compound were carried out in pairs and repeated once with distilled water as the background control. Analysis of variance appropriate for this experimental design was undertaken with the assistance of the Statistical Consulting Unit of the Graduate School, Australian National University.

Significance (p value) and SED (standard error of the difference) are indicated. Error bars show 2 x SED.

Outlying values (with large residuals) were removed from analyses where necessary and are indicated.

In a number of assays there was evidence that the between tube variation within treatment pairs was greater than the variation between the means of the replicates. In these cases p values have been adjusted accordingly.

### Phenolic compounds used in \textit{nod}-gene induction assays

<table>
<thead>
<tr>
<th>abbreviation</th>
<th>compound</th>
<th>concentration</th>
<th>abbreviation</th>
<th>compound</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dh2o</td>
<td>distilled water</td>
<td></td>
<td>hes2</td>
<td>hesperetin</td>
<td>5.00 x 10^{5}</td>
</tr>
<tr>
<td>api1</td>
<td>apigenin</td>
<td>1.00 x 10^{8}</td>
<td>isvo1</td>
<td>isovanillin</td>
<td>1.00 x 10^{4}</td>
</tr>
<tr>
<td>api2</td>
<td>&quot;</td>
<td>1.00 x 10^{7}</td>
<td>umb1</td>
<td>umbelliferone</td>
<td>1.00 x 10^{5}</td>
</tr>
<tr>
<td>cou1</td>
<td>coumarin</td>
<td>1.00 x 10^{5}</td>
<td>umb2</td>
<td>&quot;</td>
<td>5.00 x 10^{4}</td>
</tr>
<tr>
<td>dhf1</td>
<td>7,4'-dihydroxyflavone</td>
<td>5.00 x 10^{7}</td>
<td>van1</td>
<td>vanillin</td>
<td>1.00 x 10^{6}</td>
</tr>
<tr>
<td>dhf2</td>
<td>&quot;</td>
<td>5.00 x 10^{6}</td>
<td>van2</td>
<td>&quot;</td>
<td>5.00 x 10^{4}</td>
</tr>
<tr>
<td>dhf3</td>
<td>&quot;</td>
<td>1.00 x 10^{6}</td>
<td>van3</td>
<td>&quot;</td>
<td>1.00 x 10^{5}</td>
</tr>
<tr>
<td>dhf4</td>
<td>&quot;</td>
<td>1.00 x 10^{6}</td>
<td>van4</td>
<td>&quot;</td>
<td>1.00 x 10^{4}</td>
</tr>
<tr>
<td>hes1</td>
<td>hesperetin</td>
<td>1.00 x 10^{7}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

A) NGR234(plSC17)  \( p < 0.001 \)

one outlier removed  \( \text{s.e.d} = 6.56 \)

B) ANU1255(plSC17)  \( p = 0.117 \) (NS)

\( \text{s.e.d} = 1.64 \)

C) CP279(plSC17)  \( p = 0.002 \)

\( \text{s.e.d} = 6.64 \)

D) CP279(plSC17)  \( p < 0.001 \)

one outlier removed  \( \text{s.e.d} = 1.94 \)

E) CP283(plSC17)  \( p = 0.004 \)

\( \text{s.e.d} = 3.78 \)
F) CP283(pLSC17) \( p < 0.001 \)  
\( \text{sed} = 0.56 \)

G) ANU1255(nodD17)(pLSC17) \( p < 0.001 \)  
one outlier removed  
\( \text{sed} = 1.14 \)

H) ANU1255(nodD20)(pLSC17) \( p < 0.001 \)  
one outlier removed  
\( \text{sed} = 3.72 \)
The role of *Bradyrhizobium* strain CP279 putative NodDs in the nodulation of plant hosts, and their activation by phenolic compounds.

**I) NGR234(pRT311)**  
$p < 0.001$  
one outlier removed  
sed = 6.02

**J) ANU843(pRT311)**  
$p < 0.001$  
one outlier removed  
sed = 2.35

**K) ANU1255(pRT311)**  
$p = 0.003$  
one outlier removed  
sed = 1.001

**L) ANU851(pRT311)**  
$p = 0.131$ (NS)  
sed = 0.64

**M) ANU1255(nodD20)(pRT311)**  
$p = 0.004$  
one outlier removed  
sed = 2.56

**N) ANU851(nodD20)(pRT311)**  
$p < 0.001$  
one outlier removed  
sed = 0.93

**O) ANU1255(nodD40)(pRT311)**  
$p < 0.001$  
one outlier removed  
sed = 2.31

**P) ANU851(nodD40)(pRT311)**  
$p < 0.001$  
one outlier removed  
sed = 1.67
Chapter Three

- A wide variety of phenolic compounds, both mono- and poly-cyclic, activate NGR234 NodD1 to induce nod gene expression, as reported in Section 3.3, Tables 3.1 and 3.3;
- compounds with particular substitutions are more likely to be activating compounds (described in Section 3.4 and illustrated in Figure 3.2), for example:
  - the presence of a hydroxy group para to an electron withdrawing group (e.g. a carbonyl function) increases activity, as in 7-hydroxyflavone,
  - the presence of a cluster of oxygen functions increases activity, as in pyrogallol,
  - hydroxylation of the B ring increases the potency of flavonoids, as in 7,4’-dihydroxyflavone (DHF);
- simple phenolics activating NGR234 NodD1 include intermediates in the phenyl propanoid pathway and lignin breakdown products and are illustrated in Figure 3.2.

Chapter Four

- Extracts of non-host, non-legume plants activate NGR234 NodD1, as reported in Table 4.1 and Section 4.3;
- the simple phenolic compounds vanillin and isovanillin, which activate NGR234 NodD1, were isolated from wheat seedling extracts in Section 4.7;
- vanillin and isovanillin were identified in 5 of 7 Australian wheat varieties examined in Section 4.8, and could be detected by smell, but not demonstrated in HPLC analysis of the other two varieties;
- exudate from wheat seedlings was also show to have nod gene inducing activity, reported in Section 4.9.

Chapter Five

- Three putative nodD genes were identified by hybridisation and cloned from Bradyrhizobium strain CP279, described in Section 5.3;
- nodD complementation/nodulation assays described in Section 5.4 and reported in Tables 5.4 - 5.7 showed that
  - CP279 putative nodD1 complements only for Parasponia nodulation in strain ANU851, conferring a narrow host range closely reflecting that of the parental strain,
  - CP279 putative nodD2 confers a wider host range than the parental strain and can restore natural host range in both recipient strains ANU1255 and ANU851, and
  - CP279 putative nodD3 does not complement for nodulation of any plant examined in this research, in either background strain;
  - NodD-dependent nod gene induction assays described in Section 5.7 and illustrated in Figure 5.10 showed that the native NodDs of strain CP279 were activated by a variety of phenolic compounds, including flavonoids and monocyclic phenolics;
  - NodD-dependent nod gene induction assays with each of the cloned CP279 putative nodD genes, illustrated in Figure 5.10 and described in Section 5.8, showed that
    - CP279 putative NodD2 and D3 assayed with pLSC 17 were both activated by more phenolic compounds (D2 and D3 by hesperetin, and D2 by umbelliferone) than the parent strain CP279, but neither showed significant activation by vanillin or DHF where CP279 was activated by these compounds.
    - CP279 putative NodD1 and D3 assayed with pRT311 were activated by DHF, coumarin and vanillin, depending on the background strain;
- specific phenolic compounds enhance the growth of the Bradyrhizobium strains discussed in Section 5.9 and shown in Table 5.8, but do not necessarily activate NodD to induce nod gene transcription.
6.1 Introduction

The major aims of this study were to establish if the prime determinant of broad host range in rhizobia is the capacity of the nodD gene product to be activated by a wide variety of plant-derived compounds, and to determine if the activation of NodD is critical in the nodulation process of the non-legume tree Parasponia, as it has already been shown to be for rhizobia in establishing symbioses with legumes. The principal results presented in this thesis are summarised in Table 6.1.

During the germination of seeds and the early growth of seedlings, plants influence the bacterial population of their rhizosphere through the activity of compounds released into the soil from seeds and through roots (Djordjevic et al. 1987; Hartwig et al. 1991; Werner 1992, p75). Legumes enrich their rhizosphere for Rhizobium and Bradyrhizobium spp. and other bacteria through the release of compounds for which these bacteria are chemotactic (for example, cinnamic and coumaric acids) (Parke et al. 1985; Kape et al. 1991). Phenolic compounds, particularly flavonoids, which co-induce nod gene induction (e.g. luteolin) have also been shown to elicit a chemotactic response from rhizobia at concentrations as low as 10^{-9} M (Caetano-Anolles et al. 1988a). The fact that legumes influence their rhizosphere, resulting in a favourable environment for the proliferation of rhizobia, indicates an extensive evolutionary interaction between these organisms. Phenolic molecules are released from roots and can enhance (or inhibit) bacterial growth, sometimes serving as sources of carbon and energy (Hartwig et al. 1991). The same compounds also serve to co-induce or inhibit, in conjunction with NodD, the transcription of nod genes, depending on the rhizobia involved (Mulligan and Long 1985; Innes et al. 1985; Rossen et al. 1985; Shearman et al. 1986; Redmond et al. 1986; Firmin et al. 1986; Spaink et al. 1987b; Bassam et al. 1988; Spaink et al. 1989).

Nod factors, chito-lipo-oligosaccharide molecules (CLOS), are produced by rhizobia as a result of nod gene transcription and serve to initiate the infection process by inducing root hair curling and cortical cell division (Lerouge et al. 1990; Spaink et al. 1991).

It has been hypothesised that broad host range capacity may represent a less specific form of Rhizobium-plant symbiosis (Le Strange et al. 1990), since those rhizobia with broad host range tend to have a lower nodulation rate on some of their plant hosts than do the narrow host range rhizobia. Kape et al. (1991) further suggested that nod gene induction by simple phenolic compounds may represent a stage of incomplete adaptation to the presence of the less common components of root exudates of specific legumes. Whilst evidence supports these propositions, it remains clear that there is some form of advantage to rhizobia of extended host range, since, if it were not so,
evolutionary selective pressures would have worked against the continued survival of broad host range rhizobia.

The nodD gene product, NodD, is a positive transcriptional regulator. NodD is thought to bind to the nod gene promoter region at the nod-box in an inactive conformation (Hong et al. 1987; Fisher et al. 1988; Fisher and Long 1989; Kondorosi et al. 1989; Schlaman et al. 1989). Following interaction with flavonoid compounds (the mechanism of which is as yet undefined), NodD becomes activated and the transcription of nod gene operons occurs. In some rhizobia, certain compounds appear to competitively inhibit inducing compounds from activating NodD. NodD has recently been shown to regulate noU of R.fredii but no nod-box sequence has been found, indicating that the transcriptional activation of noU occurs via a different mechanism than that of other nod genes (Boundy-Mills et al. 1994).

6.2 Compounds and plant extracts activating NGR234 NodD1

It had previously been established that the product of the nodD1 gene of NGR234 was activated by a wide range of flavonoid compounds, and that this range was far more extensive than for the narrow host range R. l. bv trifolii strain ANU843 (Bassam et al. 1988). Prior to this work, the relatively narrow range of flavonoids identified as NodD activating compounds had led to the delineation of specific substitution patterns of the compounds which either led to or inhibited NodD activation (Peters et al. 1986; Djordjevic et al. 1987; Kosslak et al. 1987; Götterfert et al. 1988). Whilst the same substitution patterns of flavonoid compounds were shown in Chapter Three of this study to activate NGR234 NodD1 (for example, hydroxylation of the flavonoid skeleton at the 7 and 4' positions), additional substitution patterns were identified in other activating compounds. Furthermore, related substitution patterns on a range of defined plant-derived monocyclic phenolic compounds were also shown to activate NGR234 NodD1. These substitution patterns include the presence of a cluster of oxygen functions, hydroxylation of the B-ring of flavones, and the presence of an hydroxy group para to an electron withdrawing function. These results are discussed in Chapter Three, Sections 3.3, 3.4 and 3.5, and illustrated in Figure 3.3.

Some of the compounds which had previously been identified as inhibitors of NodD activation in narrow host range rhizobia were also shown in Chapter Three to activate NodD1 of NGR234. Many of the simple phenolic compounds activating NGR234 NodD1 are produced in response to plant wounding, such as acetosyringone. As part of plant defence responses, cell walls are reinforced by increased callose and lignin deposition, and a variety of simple phenolic compounds serve as precursors of lignin production (Ride 1986). The local synthesis of phytoalexins is another major plant defence response, and in legumes the isoflavonoid compounds form part of this group of compounds. Flavonoid compounds are also produced in response to certain stress situations, including ozone wounding and UV exposure and are therefore plant defence
compounds in certain circumstances (Hurwitz et al. 1979; Maxwell and Phillips 1990). In addition, isoflavonoid phytoalexins are produced in association with necrosis of plant tissue (VanEtten and Pueppke 1976). Certain plant defence response compounds and their intermediates have now been shown to activate NGR234 NodD1. This activation has important implications for the nodulation of other non-legumes since such compounds are produced in most plant families.

Compounds present in extracts of non-host plants were also shown to activate the expression of NGR234 NodD1-dependent nod genes in Chapter Four, Section 4.3. Given the variety of compounds of different types which have been identified as activators of NGR234 NodD1, and the absence of identified inhibitors, it would be expected that extracts or exudates of many plants would induce the expression of NodD-dependent nod genes under the control of NGR234 NodD1. Although strain NGR234 has a very broad host range, predominantly on tropical legumes, not all infection events result in nodules, and not all nodule associations result in nitrogen fixation. The variety of compounds and plant extracts activating NGR234 NodD1 identified in this work in Chapters Three and Four support the hypothesis that, at least in strain NGR234, efficiency of nodulation and nitrogen fixation is sacrificed at the expense of broad host range capacity, which is initially demonstrated by NodD1 activation.

Although experiments to determine the identity of Parasponia derived compounds responsible for NGR234 NodD1 activation were unable to be completed due to a lack of available plant material (referred to in Chapter Four, Section 4.4), the suite of inducing compounds isolated from Parasponia is likely to include both monocyclic and polycyclic phenolic compounds, since a wide variety of plant compounds activate NGR234 NodD1. Infection of Parasponia roots to form nodules involves ‘crack entry’ or entry through bacterium-induced wound-like sites (Bender et al. 1987), and thus compounds likely to be present would include lignin intermediates and phytoalexins. Flavonoid constituents of Parasponia and the closely related Trema include glycoflavones but not flavonols (Giannasi 1978). The glycoflavones present in Parasponia include the C-glycosides of apigenin, luteolin and chrysoeriol (Giannasi 1978; Giannasi and Niklas 1977). As aglycones, apigenin and luteolin are highly active co-inducers of NGR234 NodD (Chapter Three; Bassam et al. 1988; Bender et al. 1988). It remains to be determined if NGR234 NodD1 is activated directly by the C-glycosides of inducing flavonoids or if this bacterial strain NodD1 is activated by the C-glycosides of inducing flavonoids or if this bacterial strain hydrolyses these to the active aglycones.

The potential importance of monocyclic phenolic compounds to the induction of NodD-dependent nod genes in the rhizosphere has been demonstrated by the isolation of vanillin and isovanillin from wheat, a non-host plant of rhizobia and bradyrhizobia, reported in Chapter Four, Sections 4.7 and 4.8. Purified samples of these compounds were shown to activate NodD1 of NGR234 in Chapter Three, Section 3.3. Monocyclic phenolic compounds are likely to be found in the rhizosphere of legumes and non-legumes, particularly where roots have been subject to damage and at sites of lignin
deposition (Stachel et al. 1985b; Koes et al. 1994). Moreover, the monocyclic phenolic compounds caffeic acid, p-coumaric acid, ferulic acid, coniferyl alcohol and chlorogenic acid were all found to be co-inducers of nod gene activity in conjunction with the nodD gene of B. japonicum strain 110spc4 (Kape et al. 1991).

6.3 The putative nodD genes of CP279 and the nodulation of Parasponia

Most Bradyrhizobium species show a relatively broad host range when compared to Rhizobium species, yet a particular exception is strain CP279, which is generally referred to as a Parasponia-specific strain, although it also nodulates a few tropical legumes with varying efficiency. Work presented in Chapter Five addressed the role of the CP279 native nodD genes and putative nodD clones in the host range of this species.

Transconjugant strains containing cloned putative nodD genes from CP279 were shown in Chapter Five, Section 5.4 to control different host range responses. The conclusions drawn from these experiments were that the clone designated as CP279 putative nodD3 has no apparent role in host specificity, the clone designated putative nodD1 imparts a marked Parasponia specificity, and the clone designated putative nodD2 imparts a broad host range capacity. The CP279 putative nodD1 gene was able to complement the inactivated nodD gene of strain ANU843 only for Parasponia nodulation but not on other test plants. Paradoxically, this clone did not complement the nodD1 mutant of strain NGR234. In contrast, the putative nodD2 gene was able to complement the inactivated nodD1 gene of strain NGR234 for nodulation of Parasponia and siratro, and the inactivated nodD gene of R. l. bv. trifolii strain ANU843 for nodulation of white clover and subterranean clover. The CP279 putative nodD3 gene did not complement the inactivated nodD genes of the recipient strains on any of the test plants (Parasponia, siratro, white clover and subterranean clover) (Chapter Five). The parental strain CP279 exhibits a similar host range to strain ANU851 containing the cloned CP279 putative nodD1 gene, whilst CP279 has a narrower host range than that shown by both test strains containing the putative nodD2 gene.

Given that strain CP279 exhibits a narrow host range, one might expect that only a narrow range of flavonoids would activate the NodDs of CP279, as occurs in other narrow host range strains, for example, R. l. bv trifolii. In contrast, products of the native CP279 NodDs were shown in Chapter Five, Section 5.7, to be activated by a variety of flavonoids and monocyclic phenolic compounds with differing chemical substituents, giving a response pattern more consistent with that of the broad host range strain NGR234 NodD1, determined in Chapter Three. In addition, the products of the three putative nodD genes cloned from strain CP279 demonstrated different responses to phenolic compounds, reported in Section 5.8. For example, DHF and vanillin significantly activated putative NodD1 and NodD3, but not NodD2, isovanillin activated
NodD1 and NodD2, hesperetin and apigenin activated NodD2 and NodD3, and coumarin activated NodD1 and NodD3.

These results suggest that within the wild-type host strain CP279 the putative nodD genes cloned in this study are subject to regulation which narrows the host range phenotype of the strain. Alternatively, the NodD-controlled expression of the nod genes in the parental strain may produce a cocktail of Nod factors which are fairly specific for Parasponia nodulation. While nodD is not involved directly in Nod factor production, it may regulate various nod gene and/or hsn operons, resulting in increased or decreased total amounts of Nod factors produced. With the introduction of the putative CP279 nodD genes into nodD mutants, nodulation efficiency is affected in the following ways:

(i) in the ANU843 nodD mutant background, nodulation of clover plants is reduced from 100% to 40-70% with CP279 putative nodD2, and is eliminated with the presence of putative nodD1; and

(ii) on Parasponia, complementation by putative nodD1 gives 25% nodulation in the ANU843 nodD mutant background; and by putative nodD2 gives 75% nodulation in the NGR234 nodD mutant background.

Differential transcription of hsn operons, altering the balance of the Nod factor cocktail produced by rhizobia, has been demonstrated under nodulation adverse conditions in R. l. bv. trifolii (McKay and Djordjevic 1993), and similarly, very low production of Nod factors was seen in strain TOM of R. l. bv. viciae when compared to other R. l. bv. viciae strains (Firmin et al. 1993).

One possible explanation, then, for the nodulation of Parasponia by the ANU843 nodD mutant strain ANU851 is that the products of the introduced putative CP279 nodD1 and nodD2 genes fail to activate transcription of the ANU843 hsn operons encoding host specific decorations to the Nod factors produced, thus altering the cocktail of Nod factors produced compared to the parental strain ANU843. Altering the amount or balance of Nod factors produced could also explain the reduction of nodulation efficiency on clovers by strain ANU851 containing the CP279 nodD1 gene. The Nod factors produced by the Parasponia nodulating strain NGR234 have many substitutions to both the reducing and non-reducing ends of the chitin backbone and this strain is able to nodulate Parasponia. In contrast strain ANU843 produces relatively simple Nod factors with few substitutions to the chitin backbone, but with a wide diversity in the fatty acid chain at the non-reducing end of the CLOS. For example C16, 18, 20, and 22 fatty acids with one, two, or three double bonds are produced, resulting in over 60 different CLOS species (Spaink et al. 1995). Yet this strain is able to (poorly) nodulate Parasponia when the mutant nodD gene C58, from strain ANU843, is present (McIver et al. 1989). It would be most interesting to define the substitutions present on the CP279 Nod factors as this may give more concrete indications of the structure of any Parasponia-specific Nod factor(s).
NodD is required for the three early stages of Parasponia infection (Morrison et al. 1984; Bender et al. 1987b), shown schematically in Figure 6.1, while hsn genes are required for the induction of cell division in the first and third stages of Parasponia infection, and nodABC genes are required for the formation of the prenodule (2nd stage), and the induction of the second cycle of cell division in the third stage (Bender et al. 1987b). Nod Factors are therefore clearly involved in certain stages of the initiation of infection in Parasponia (Morrison et al. 1984; Bender et al. 1987a and 1987b), although differential transcription of nod and hsn operons also seem to operate.

McIver et al. (1989) demonstrated that mutants with one or two point mutations in the nodD gene of R. l. bv trifolii strain ANU843 were altered in their NodD activation by inducer molecules, and this effect facilitated the extension of the host range of these mutants to Parasponia. Strain ANU843 apparently produces appropriate Nod factors for Parasponia infection when nod gene transcription is activated by an appropriate NodD, such as in the case of the mutated ANU843 nodD genes (McIver et al. 1989). Both the putative nodD1 and nodD2 genes from strain CP279 were also able to extend the host range of strain ANU843 to the non-legume Parasponia (Chapter Five). Each of these two putative CP279 nodD genes is therefore clearly able to initiate the transcription of the appropriate ANU843 nod (and possibly hsn) gene operons to produce effective Nod factors (and/or other required signal molecules) for Parasponia infection. Results of complementation studies in strain ANU851 indicate also that the CP279 putative nodD1 gene appears to be principally responsible for the Parasponia-specificity, or narrow host range, of strain CP279, whereas the expression of the putative nodD2 gene seems to enable nodulation of a broader range of plants in the background of strain ANU843.

The root hairs of the non-nodulated test plants were not examined for deformation, curling, infection threads, or induction of cortical cell division, so it is unknown precisely to which stage infection was established, if any. Experiments to determine the extent of any interaction between the test plants and rhizobia carrying the cloned putative CP279 nodD genes which failed to produce nodules would indicate any production of suitable Nod factors by the nod genes of strain ANU843 or strain NGR234 under the control of the putative CP279 nodD genes.

6.4 The putative nodD genes of CP279 and the activation of NodD

When examined in the wild-type strain for the ability to induce nod gene transcription in response to phenolic compounds, the resident nodD genes of strain CP279 exhibited the capacity to interact with a variety of both polycyclic flavonoid and monocyclic phenolic compounds. When examined individually in nodD mutant rhizobia, each of the putative CP279 nodD genes were shown in Chapter Five, Section 5.8 to have slightly different activation patterns, compared to each other and the parental strain. For example, the putative NodD1 and NodD2 induced nod gene transcription in conjunction with
isovanillin (as did the parent strain), the putative NodD1 and NodD3 induced nod gene transcription in conjunction with coumarin where the wild-type strain did not, and NodD1 and NodD2 induced nod gene transcription with hespererit, where the parent strain did not. Apigenin co-induces in conjunction with both the putative nodD2 and nodD3 gene products of strain CP279, as well as in the wild-type strain, and its C-glycoside would be expected to be found in Parasponia exudates based on the distribution of flavonol aglycones and glycoflavones in the Ulmaceae (Giannasi 1978).

Interestingly, the ANU843 nodD mutant able to nodulate Parasponia (C58) reported by McIver et al. (1989) was activated by the coumarin-type compound umbelliferone. CP279 putative NodD2 is activated significantly by umbelliferone where putative NodD3 and the parental strain are not (Chapter Five, Sections 5.7 and 5.8). No implication for any relationship between coumarin-type compound recognition and Parasponia nodulation can thus be drawn since the parental strain CP279 was not activated significantly by either umbelliferone or coumarin in the experiments reported in Chapter Five, although only two coumarin type compounds were assessed.

Phenolic compounds have been shown to both enhance and inhibit the growth of rhizobia (through both catabolic and non-catabolic mechanisms). Of the compounds shown to enhance growth of Bradyrhizobium strain CP279 reported in this study in Chapter Five, Section 5.9, DHF was also shown to activate CP279 native NodDs (discussed in Section 5.7, and shown in Figure 5.10 and Table 5.3). Acetylsalicylic acid was found to inhibit growth of strain CP279 at high concentrations and to enhance the growth of this strain at lower concentrations. Interesting differences were also noted in Section 5.9 between strain CP283 and strain ANU289 (a streptomycin derivative of CP283), where growth of CP283 was inhibited by acetylsalicylic and benzoic acids, yet neither compound affected the growth of ANU289. Furthermore, genistein and dimethoxyflavone both enhanced the growth of ANU289, but had no effect on CP283. These differences support the conclusion that differing cell permeability characteristics of various (brady)rhizobia can result in quite significant differences in the effects of mono- and poly-cyclic phenolic compounds on bacterial growth and are likely to contribute to the different characteristics wild-type rhizobia may exhibit in assays to assess NodD activation.

### Regulation of nodD genes

The results of the NodD-dependent nod gene induction assays in which the cloned CP279 putative nodD gene products were activated by a wider range of compounds than the parental strain (reported in Chapter Five, Section 5.8), suggest that the group of putative NodDs of strain CP279 are activated by a wider range of phenolic compounds than is exhibited by the wild-type strain. Logically, this would argue that strain CP279 might display a broader host range phenotype than is actually the case. One explanation for the difference between the demonstrated and expected phenotypes could be that the
wild-type NodDs are regulated in such a way that only putative NodD1 is responsible for the NodD-dependent induction of nod genes in the native strain, resulting in the narrow the host range phenotype of strain CP279. NodD regulation could be achieved by the action of repressors, for example from other nodDs or syrM-like genes. An alternative explanation for the narrow functional host range of strain CP279 is that the Nod factors produced by this strain have an unusual specificity for Parasponia. Yet another factor to consider is that there is as yet no evidence for or against possible inhibitors of NodD activation in strain CP279 since no experiments utilising mixtures of phenolic compounds were included in the work reported in Chapter Five.

The regulation of nodD genes in various rhizobia has received increasing attention. In addition to autoregulation of nodD by the NodD protein which is seen in some rhizobia, a repressor of nod gene transcription, NolR, has been identified in many R. meliloti strains (Kondorosi et al. 1989; Kondorosi 1991). NolR binds to promoters of nodD1 and nodD2 and is thought to be involved in regulation of nodD transcription (Kondorosi 1991). The transcription of nodD is also reduced in bacteroids (Sharma and Singer 1990; Schlaman et al. 1991), and this reduction may be caused by a bacteroid-specific transcriptional repressor protein (Schlaman et al. 1992a). Moreover the binding of NodD to nod-boxes is ineffective in bacteroids (Schlaman et al. 1992a).

A model has recently been proposed which relates the activation state of NodD to the degree of bend mediated upon the DNA (bound at the nod-box), facilitating RNA polymerase binding and nod gene transcription (Fisher and Long 1993), illustrated in Figure 1.5. A similar mechanism of target operon transcriptional activation has also been proposed for other members of the LysR family (Schell 1993).

Specific domains have been identified in LysR family members which are involved in co-inducer recognition (two domains toward the C-terminus), DNA binding (one domain at the amino-terminus) and a further domain at the C-terminus involved in both co-inducer recognition and DNA binding (reviewed in Schell 1993). However, direct interaction between NodD and co-inducing compounds has yet to be demonstrated.

Ammonia has been shown to be involved in the regulation of some nod gene operons, and of nodD in some rhizobia. The mechanisms involved vary, but include nodD1 involvement in B. japonicum (Wang and Stacey 1990), and the involvement of ntrC, ntrA, syrM and nodD3 in R. meliloti (Dusha et al. 1989; Dusha and Kondorosi 1993). However, the levels of nitrate required to repress nod gene activity via ntrC are quite high, and are unlikely to be significant at physiological levels (Dusha and Kondorosi 1993).

In the family Rhizobiaceae there is at least one other example of phenolic-induced plant infection genes. A. tumefaciens has a two component regulatory system that is responsible for induction of the vir genes, which mediate the transfer of the oncogenic Ti DNA to the nuclei of infected cells (Ream 1989). Induction of these genes is
elevated by phenolic compounds and potentiated by monosaccharides and acid pH, all encountered at plant wound sites (Stachel et al. 1986; Cangelosi et al. 1990; reviewed by Winans 1992). Transcription of vir genes is regulated by VirA and VirG, while stimulation by monosaccharides requires ChvE, which is thought to interact with the periplasmic domain of VirA (Cangelosi et al. 1990), further activating VirA (Beirjersbergen and Hooykaas 1993). VirA has been shown to contain domains for sensing monosaccharides (periplasmic domain), and for sensing phenolic compounds and acidity (linker domain) (Chang and Winans 1992). After sensing stimulatory compounds VirA autophosphorylates and transfers this phosphoryl group to VirG (Jin et al. 1990a, 1990b). VirG binds to vir binding sites, preceding vir promoters (Jin et al. 1990c). No evidence has yet been presented that VirA binds directly to inducing phenolic compounds. Compounds serving as inducers to the virA/virG regulatory system include acetosyringone, produced in increased amounts at wound sites, and lignin precursors (Stachel et al. 1985a and 1985b). There is no evidence that a phosphorlyation reaction mediates NodD regulation.

Other regulatory systems also influence nodulation. In addition to NolR (Kondorosi et al. 1989), Göttfert et al. (1990) identified the nodV and nodW genes of B. japonicum, members of a postulated two component regulatory system similar to that of A. tumefaciens virA and virG. NodV is the predicted sensor, and NodW the predicted regulator. NodW has been shown to function in co-ordination with NodD to regulate the expression of the common nod genes in conjunction with flavonoid inducers in B. japonicum (Sanjuan et al. 1994). Both genes are also involved in the regulation of nolY and nolZ, which were recently identified in B. japonicum and for which no function has yet been identified, although nolY does not have a role in the production of CLOS (Dockendorff et al. 1994).

Luteolin is released by alfalfa seeds, and in addition to being a key activator of NodD in R. meliloti, also enhances the growth of this species (Hartwig et al. 1991). Flavonoid compounds which activate NodD are absorbed by the rhizobial cells preferentially to flavonoids which are not NodD activators (luteolin vs naringenin and quercetin in R. meliloti), and are absorbed at a higher level than in other Gram-negative bacteria (Hubac et al. 1993). Mutations in the nodD gene reduced the absorption of luteolin, implicating NodD in the absorption of flavonoids into R. meliloti, and there is further evidence that sites other than NodD may be involved in luteolin binding (Hubac et al. 1993). However, the growth enhancing effects of luteolin on R. meliloti are also to be seen when all three nodD genes are non-functional, arguing for a separate flavonoid binding mechanism (Hartwig et al. 1991), although no other mechanism has yet been delineated. Thus NodD appears to play a role in the absorption of phenolic compounds which activate it, but other mechanisms for flavonoid absorption must also be present in rhizobia.
6.6 Conclusions

The specificity of general signalling mechanisms can be achieved by differential effects of signal molecules on different bacterial species or strains. Compounds which serve as chemoattractants to one species fail to attract other species, and compounds which enhance growth or serve as sources of carbon and energy in certain species fail to do so in other species, or may even inhibit their growth. However, the first key step in a successful infection event in the legume/rhizobia interaction is the differential recognition of phenolic co-inducers, generally flavonoid compounds, through activation of Nod.D. NodDs of most narrow host range rhizobia are quite specifically activated by a limited variety of polycyclic phenolic compounds present in the rhizosphere of host plants. In contrast, broad host range (brady)rhizobia are activated by a much wider spectrum of phenolic compounds, suggesting the hypothesis that the activation of NodD by a wide variety of compounds is a key molecular determinant of broad host range.

Data presented in this thesis are both consistent and inconsistent with this hypothesis. In Chapter Three, the data showed that the capacity of *nodD* gene products to recognise a wide variety of plant compounds represents the first level of host specificity in rhizobia, particularly for broad host range rhizobia. Other factors also influence this first level of specificity, including *nodD* regulation, either autoregulation, or regulation by exogenous factors such as ammonia (Dusha and Kondorosi 1993). The second level of host specificity is the elaboration of a spectrum of Nod factors as a result of transcriptional activation of the *nod* genes by NodD. Together, these two determinants of host specificity control the capacity of rhizobia to recognise signals from, and send signals to, potential host plants and together can be considered the prime determinants of host specificity.

Both of the strains examined in detail in this study are able to nodulate the non-legume *Parasponia*. *Rhizobium* strain NGR234 is a broad host range fast-growing strain which forms ineffective nodules on *Parasponia*. *Bradyrhizobium* strain CP279 is slow-growing strain whose host range is essentially limited to *Parasponia*. Both strains were found to recognise a wider range of flavonoid compounds than narrow host range Rhizobium strains and were also able to recognise monocyclic phenolic compounds such as vanillin, which other rhizobia are unable to do (for example *R. l. bv. trifolii*). These data are inconsistent with the hypothesis outlined above since the capacity to recognise a wide range of inducer compounds does not correlate with the narrow host range phenotype of the wild type strain CP279. The three putative *nodD* genes showed different recognition patterns for a variety of phenolic compounds in NodD-dependent *nod* gene induction assays. When the three putative *nodD* genes of this strain were examined for *nodD* complementation in plant assays, the CP279 putative *nodD1* gene restored the host range in *nodD* mutants, the putative *nodD2* gene was *Parasponia*-specific, and the putative *nodD3* gene failed to complement for nodulation on any of the test plants. The nodulation phenotype of strain CP279 can therefore be seen to be more
restricted than the characteristics of the separated putative nodD genes might suggest, possibly due to complex regulation (or autoregulation) of the native nodD genes. Furthermore, the successful nodulation of Parasponia by the two native strains studied in this thesis - Bradyrhizobium strain CP279 and Rhizobium strain NGR234 - would appear to be favoured by the capacity of the NodDs of the wild-type strains to be activated by a wide variety of phenolic compounds.

The delineation of the molecular structure of the Nod factors elaborated by rhizobia was reported by various groups during the latter stages of the research reported in this thesis. The information revealed about the variety of structures and their effects on host plants makes it clear that the nature of the CLOS molecules produced by a strain plays a significant role in the successful development of nodules and is therefore a second major determinant of host range in rhizobia.

### 6.7 Future directions

When considering the requirements for successful nodulation by rhizobia of non-legume plants other than Parasponia, the basic requirements would appear to be that:

i) the bacterium be able to ‘recognise’ the plant and induce nod-gene expression (that is, have a NodD protein capable of being activated by a broad spectrum of plant compounds);

ii) the bacterium must produce appropriate signal molecules (Nod factors) to induce cortical cell division (which, on the basis of current information, is responsible for altering the phytohormone balance in the root); and

iii) the bacterium must be able to infect the plant root, probably via ‘crack entry’ as occurs in Parasponia nodulation, without eliciting a full scale defence response from the plant.

The strains studied in this work are both able to provide models for these functions. The NodDs of the wild-type strains NGR234 and CP279 are both activated by monocyclic and polycyclic phenolic compounds with a variety of structural features. Both strains apparently also induce nod gene operon transcription which results in Nod factors able to initiate infection of Parasponia roots. Finally, the development of nodules demonstrates these strains successfully evade the host plant defence responses. The data presented in this thesis point the way to future research which will help to define the molecular requirements for nodulation of Parasponia. Attempting to understand the role of Nod factors in the nodulation of Parasponia appears to be the next logical step in delineating the nodulation of this non-legume by rhizobia.

Future experiments could also help to unravel the further requirements for the successful nodulation of other non-legumes, particularly those of economic importance such as cereal crops. It is also important to remember that the extension of nodulation by
rhizobia to other non-legumes could have negative agronomic implications (as discussed in Chapter 1, Section 1.1) which may outweigh the benefits of reduced reliance on applied nitrogen fertilisers.

Future experimental directions could include the following.

- Further examination of the nodulation phenotypes of the three putative nodD genes identified in strain CP279: In NGR234 and ANU843 backgrounds, what other plants (legumes and non-legumes) can be nodulated? Is the CP279 putative nodD3 gene functional with respect to nodulation phenotype on any plant?

- Sequencing of the putative CP279 nodD genes - particularly nodD2 since this is the first Parasponia specific nodD gene identified. Are each of the cloned CP279 nodD genes complete?

- Isolation of CP279 nodD mutants (particularly multiple nodD mutants), and an examination of the native CP279 nodD genes individually and in combination in the CP279 background (particularly with nodD::lacZ fusions): How are the nodD alleles regulated to achieve the narrow host range of the wild type strain? Does the suite of compounds activating each (putative) NodD change when assayed in the CP279 background, for example, is coumarin an activator?

- Isolation of the CP279 Nod factors. Is there a CLOS substitution pattern which confers Parasponia-specificity to the Nod factors of CP279? How does the cocktail of Nod factors produced change when each of the CP279 nodD genes is mutationally inactivated? How do the Nod factors of CP279 compare to the Nod factors of other strains capable of Parasponia nodulation, particularly of NGR234 and other bradyrhizobia? Is there evidence of convergent evolution?

- Do Nod factors influence the nodulation of Parasponia in the same way that they do in legumes?

- Are there two sets of signals involved in the nodulation of Parasponia roots, as implied by the model illustrated in Figure 6.1, possibly resulting from the differential activation of subsets of nod genes giving rise to Nod factors with a variety of decorations?

- What are the differences between the Nod factors produced by strain ANU851 containing each of the CP279 putative NodDs and the Nod factors of the native strain CP279, and what does this indicate about the regulation of the native NodDs?

- Did double reciprocal cross-over events occur in the nodules found on Parasponia inoculated with transconjugant strain ANU851(pKS1701), and on white clover inoculated with transconjugant strain ANU1255(pKS1701)?
General Discussion

- Does the mixture of Nod factors produced by strains NGR234 and CP279 elicit a response from other non-legumes? Does that of CP279 elicit any pre-nodulation response from legume species?
- Are there homologues of \( R. \) meliloti syrM in the Parasponia nodulating strains? If so how do these genes affect the regulation of the \( nodD \) genes?
- Are any of the \( hsn \) operons in strains ANU843 and NGR234 not induced by activated CP279 putative NodD1 and NodD2?

6.8 Publications arising from the research reported in this thesis

The following publications have arisen from this research:

Bender, G.L., Nayudu, M., Le Strange, K.K. and Rolfe, B.G. 1988. The \( nodD1 \) gene from Rhizobium strain NGR234 is a key determinant in the extension of host range to the non-legume Parasponia. Mol. Plant-Microbe Interact. 1:259-266.

Figure 6.1

Model of the infection of *Parasponia andersonii* roots, and some of the *Rhizobium* genes known to be involved.

IT - infection thread; En - endoderm; P - pericycle
This page is intended to be left blank.
Known *Rhizobium* genes required

\[ \text{nod} \ D \ hsn \]

\[ \text{Erosion of epidermal cells} \]

\[ \text{First cell division signal} \]

\[ \text{Subepidermal cells induced to divide} \]

\[ \text{Prenodule} \]

\[ \text{structure formed of dividing cells} \]

\[ \text{nod} \ D \ nod \ ABC \]

\[ \text{Second cell division signal required} \]

\[ \text{nod} \ D \ nod \ ABC \ hsn \]
References


References


Burn, J., Rossen, L. and Johnston, A.W.B. 1987. Four classes of mutations in the \textit{nodD} gene of \textit{Rhizobium leguminosarum} biovar \textit{viciae} that affect it's ability to autoregulate and/or activate other \textit{nod} genes in the presence of flavonoid inducers. Genes Dev. 1:456-464.


References


References


References


Published Papers
The **nodD1** Gene from *Rhizobium* Strain NGR234
Is a Key Determinant in the Extension of Host Range
to the Nonlegume *Parasponia*

Gregory L. Bender, Murali Nayudu, Kathryn K. Le Strange, and Barry G. Rolfe

Plant Molecular Biology, Research School of Biological Sciences, Australian National University. Box 475 P.O., Canberra City, A.C.T. 2601.

Received 21 June 1988. Accepted 15 September 1988.

The narrow host range of *Rhizobium leguminosarum* biovar *trifolii* strain ANU843 can be extended from clovers to the nonlegume *Parasponia* by the addition of the **nodD1** gene from *Rhizobium* strain NGR234. The presence of the **nodD1** gene from NGR234 enabled induction of a **nod**::MudH11734 fusion in strain ANU843 by seedling extracts from *Parasponia* and other nonlegumes, including *Trema* (a tree genus closely related to *Parasponia*), *Casuarina* (a genus that forms symbiotic relationships with the actinomycete *Frankia*) and the cereals wheat, rice, and maize. There was also an increase in the range of flavonoids causing induction, suggesting that the **nodD1** gene from the broad host range strain NGR234 is nonspecific in action rather than host specific, as found for the **nodD** genes from narrow host range species. Although the presence of the **nodD1** gene from NGR234 enabled the induction of **nod** genes by a range of nonlegume extracts, no nodules were elicited on any nonlegumes other than *Parasponia*.

When available nitrogen is limited, some dicotyledonous plants can form a symbiosis with nitrogen-fixing prokaryotes as an alternative means of obtaining nitrogen. Members of the legume family specifically form a symbiosis with *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* bacteria, whereas nonlegumes interact with actinomycetes of the genus *Frankia*. *Parasponia*, a woody member of the elm family confined to the Malay archipelago, is the only nonlegume known to form a symbiosis with *Rhizobium* or *Bradyrhizobium* strains (Trinick 1973; Akkermans et al. 1978).

The infection of *Parasponia* differs in some ways from that of legumes (Lancelle and Torrey 1984; Bender et al. 1987a). Root hair curling and the initiation of infection threads within curled hairs, commonly found in legumes, have not been observed in *Parasponia*. The first event in the infection of *Parasponia* appears to be the initiation of cell division within a zone of the root that is highly susceptible to infection (Bender et al. 1987a). Cell division produces a break in the epidermis through which bacteria gain entry into the root. Infection threads are then initiated from intercellular colonies within the cortex. Subsequent stages of *Parasponia* nodule development, and final nodule structure, resemble those found in the symbiosis between other nonlegumes and *Frankia* (Lancelle and Torrey 1985). Unlike legume nodules, a *Parasponia* nodule resembles a swollen lateral root with an apical meristem and central vascular bundle.

The activation of *Rhizobium* nodulation (**nod**) genes, and hence the initiation of a legume symbiosis, requires both root exudate and the constitutive expression of the regulatory **nodD** gene (Innes et al. 1985; Mulligarran and Long 1985; Rossen et al. 1985). Hong et al. (1987) have shown that the **nodD** gene product binds directly to a conserved promoter sequence called the **nod-box** (Rostas et al. 1986) located upstream from **nod** genes. The inducing compounds found in legume exudates or extracts have been identified as flavones, flavanones, or closely related compounds that are thought to interact with **nodD** to induce expression of **nod** genes (Peters et al. 1986; Redmond et al. 1986; Firmin et al. 1986; Kossiak et al. 1987; Rolfe 1988).

*Rhizobium* strain NGR234 has a broad host range among tropical legumes and can form *Parasponia* nodules that do not fix nitrogen (Trinick and Galbraith 1980). Nodulation genes have been localized on a large symbiotic (Sym) plasmid in this strain (Morrison et al. 1983; Pankhurst et al. 1983). Removal of the Sym plasmid results in the loss of nodulation ability (Morrison et al. 1983), whereas transfer of this plasmid to other *Rhizobium* species extends their host range to include plants nodulated by strain NGR234 (Morrison et al. 1984; Broughton et al. 1984). Lewin et al. (1987) have located three host-specific nodulation (Hsn) regions on the Sym plasmid of strain NGR234. HsnI is located next to **nodD**, HsnII is linked to the nitrogenase structural genes **nifH**/**DK**, and HsnIII to **nodC**. All three Hsn regions, when transferred to the appropriate recipients, confer the ability to curl the root hairs of *Macropodium atri purpureum* (siratro). However, only HsnI enables the formation of nodules on siratro and also specifies the nodulation of *Vigna unguiculata* (cowpea), *Glycine max* (soybean) and *Psophocarpus tetragonolobus*. HsnII is able to complement a mutation in the *Medicago sativa* (alfalfa)-specific **nodH** gene of *R. meliloti*. Bachem et al. (1986) have isolated an 11.4-kb fragment from the Sym plasmid of strain MPIK3030 (a derivative of NGR234) that is specific for siratro nodulation. Genes on this fragment did not complement mutations in either conserved or host-specific nodulation genes in *R. meliloti*.

Although the **nodD1** gene from strain NGR234 is required for the nodulation of all hosts, including *Parasponia* (Bender et al. 1987b), there is evidence that this conserved gene also has an important role in determining
broad host range (Horvath et al. 1987; Bassam et al. 1988). In this paper we show that *R. leguminosarum* biovar *trifolii* strain ANU843, carrying a 2.1-kb *PstI* fragment spanning the *nodDI* gene from strain NGR234, can nodulate *Parasponia*, a nonlegume host where the mode of infection differs markedly from that of the homologous host clover. Gene expression studies that use a *nodA*:MudII1734 fusion suggest that the nodulation of *Parasponia* by strain ANU843 requires the induction of nodulation genes by *Parasponia* seedling extract. In addition, the presence of the NGR234 *nodDI* gene enabled the induction of ANU843 nodulation genes by seedling extracts from a wide range of other nonlegumes, including cereals.

**MATERIALS AND METHODS**

**Bacterial strains used.** All bacterial strains and plasmids, their characteristics and the source from which they were obtained, are listed in Table 1.

**Media.** BMM media for *Rhizobium* has been described (Bergersen 1961). GMM minimal media for *Rhizobium* strains. Herridge plant nutrient solution has been described (Bender and Rolfe 1985). BM1 plates containing tetracycline (4 µg/µL) were also added for the culture of *Rhizobium* strains via patch mating (Sinclair and Holloway 1982). GMM minimal media for *Plasmids.* Yeast extract replaced with monosodium glutamate (0.5 g/L). The vitamins biotin (25 µg/µL) and thiamine (100 µg/µL) were also added for the culture of *R. l. bv. trifolii* strains. Herridge plant nutrient solution has been described (Delves et al. 1986).

**Bacterial genetics.** Plasmids were constructed as described (Nayudu and Rolfe 1987; Bassam et al. 1988) and transferred from *E. coli* strain HB101 to *R. l. bv. trifolii* strains via patch mating (Sinclair and Holloway 1982). GMM plates containing tetracycline (4 µg/µL) were used for the selection of *R. l. bv. trifolii* transconjugants carrying the plasmid pMN40.

**Seed germination.** *Parasponia andersonii* and *Trema aspera* seeds were germinated as described (Bender and Rolfe 1985). Seeds of *Tritium repens* (white clover) and *T. subterraneum* (sub clover) were germinated as described by Rolfe et al. (1980).

**RESULTS**

Bassam et al. (1986) reported that a 6.7-kb *HindIII* fragment from strain NGR234 carries the *nodDI* gene and two loci involved in the host-specific nodulation of siratro but not the other hosts of NGR234. Mutational analysis of the 6.7-kb fragment showed that only the two *Hsn* loci and not the *nodD* gene were involved in extension of the host range of *R. l. bv. trifolii* strain ANU843 to siratro. Subsequent analysis of a 17-kb *XhoI* fragment (Bassam et al. 1988), which includes the 6.7-kb fragment, has shown that a mapping error was made and that the *nodDI* gene is a key element in determining the broad host range of NGR234. This is consistent with Horvath et al. (1987), who have shown that mutations in the *nodDI* gene of strain MPIK3030 (a derivative of NGR234) prevent host range extension. In addition, the use of the Magenta jar plant seedling extract. The agar plate plant assays for clover (Rolfe et al. 1980), siratro (Cen et al. 1982) and *Parasponia* (Bender and Rolfe 1985) have been described. A modified Leonard jar plant-assay system designed by E. Appelbaum of Agrigenetics Inc., Madison, WI was also used for nodulation assays. We have termed this system a "Magenta jar." *Rhizobium* inoculum for Magenta jars was taken from selective plates and suspended in Herridge solution to a density of 10^6–10^7 cells per milliliter. One ml of inoculum was applied to each of five seedlings transplanted to a Magenta jar. The acetylene reduction assay, used to measure the nitrogen- fixation capacity of root nodules, has been described (Bender and Rolfe 1985). Bacteria were isolated from nodules as described by Gresshoff et al. (1977).

**β-galactosidase assays.** Freshly germinated seedlings were submerged in a minimum volume of 30% ethanol in a screw-topped vial. Nitrogen gas was bubbled through the solution for 5 min, and the vial was sealed and placed in darkness for 2 hr. The solution was then passed through filter paper to remove suspended matter. One-ml aliquots of the filtrate were immediately dried overnight under vacuum. The dried extract was then resuspended in 1.2 ml of water by vigorous shaking. Log-phase *Rhizobium* cells (0.4 ml of a BMM liquid culture at an absorbance of 0.20–0.25, wavelength 600 nm) were added without shaking and left at 28 °C for 3 hr. *β*-galactosidase activity was then assayed as in Miller (1972), with 4 hr allowed for color development.

**Microscopy.** Plant tissue for light and electron microscopy was prepared and examined as described (Bender et al. 1987b).

**References**

Bassam et al. (1986) reported that a 6.7-kb *HindIII* fragment from strain NGR234 carries the *nodDI* gene and two loci involved in the host-specific nodulation of siratro but not the other hosts of NGR234. Mutational analysis of the 6.7-kb fragment showed that only the two *Hsn* loci and not the *nodD* gene were involved in extension of the host range of *R. l. bv. trifolii* strain ANU843 to siratro. Subsequent analysis of a 17-kb *XhoI* fragment (Bassam et al. 1988), which includes the 6.7-kb fragment, has shown that a mapping error was made and that the *nodDI* gene is a key element in determining the broad host range of NGR234. This is consistent with Horvath et al. (1987), who have shown that mutations in the *nodDI* gene of strain MPIK3030 (a derivative of NGR234) prevent host range extension. In addition, the use of the Magenta jar plant seedling extract. The agar plate plant assays for clover (Rolfe et al. 1980), siratro (Cen et al. 1982) and *Parasponia* (Bender and Rolfe 1985) have been described. A modified Leonard jar plant-assay system designed by E. Appelbaum of Agrigenetics Inc., Madison, WI was also used for nodulation assays. We have termed this system a "Magenta jar." *Rhizobium* inoculum for Magenta jars was taken from selective plates and suspended in Herridge solution to a density of 10^6–10^7 cells per milliliter. One ml of inoculum was applied to each of five seedlings transplanted to a Magenta jar. The acetylene reduction assay, used to measure the nitrogen- fixation capacity of root nodules, has been described (Bender and Rolfe 1985). Bacteria were isolated from nodules as described by Gresshoff et al. (1977).

**β-galactosidase assays.** Freshly germinated seedlings were submerged in a minimum volume of 30% ethanol in a screw-topped vial. Nitrogen gas was bubbled through the solution for 5 min, and the vial was sealed and placed in darkness for 2 hr. The solution was then passed through filter paper to remove suspended matter. One-ml aliquots of the filtrate were immediately dried overnight under vacuum. The dried extract was then resuspended in 1.2 ml of water by vigorous shaking. Log-phase *Rhizobium* cells (0.4 ml of a BMM liquid culture at an absorbance of 0.20–0.25, wavelength 600 nm) were added without shaking and left at 28 °C for 3 hr. *β*-galactosidase activity was then assayed as in Miller (1972), with 4 hr allowed for color development.

**Microscopy.** Plant tissue for light and electron microscopy was prepared and examined as described (Bender et al. 1987b).
Cloning of the nodD1 gene from Rhizobium strain NGR234. To test the observation further by Bassam et al. (1988) that the nodD gene is an essential determinant for the broad host range of NGR234, we cloned the smallest possible DNA fragment containing the NGR234 nodD1 gene into the broad host range vector pSUP106 (Fig. 1). A 2.1-kb PstI fragment was cloned from the NGR234 Tn5 mutant number 7 (Bassam et al. 1986) into the plasmid pSUP106 by replacing the small PstI fragment carrying the cos site on pSUP106. This plasmid construct (pMN40) contains a 0.8-kb segment of IS50 which is part of a Tn5 arm and about 0.4 kb of an intergenic region (Bassam et al. 1986) which separates Tn5 from the nodD1 gene. DNA sequence analysis of the equivalent 0.4-kb region upstream from the nodD1 gene in strain MPIK3030 (Horvath et al. 1987) did not reveal an open reading frame, suggesting that the nodD1 gene is the only nod gene present on pMN40. The 2.1-kb fragment contains a functional copy of the NGR234 nodD1 gene, as shown by the ability of a nodD1 mutant of NGR234 (ANU1255) carrying plasmid pMN40, to nodulate siratro and Parasponia.

Extension of Rhizobium host range to Parasponia. Plasmid pMN40 was transferred to R. l. bv. trifolii strain ANU843 selecting for the Te' marker of plasmid pSUP106. Strain ANU843(pMN40) could still nodulate closer, but its host range was now extended to include Parasponia and the tropical legume siratro (Table 2). All Parasponia plants grown in Magenta jars were nodulated with an average of three nodules per plant for strain ANU843(pMN40) and five per plant for strain NGR234. In contrast, when using the agar plate assay, strain ANU843(pMN40) did not nodulate Parasponia, and NGR234 nodulated only 10% of plants. Parasponia nodules formed by strain ANU843(pMN40) were similar in external appearance to the nodules formed by strain NGR234 (Fig. 2). Sections of ANU843(pMN40) nodules revealed a smaller infected zone with bacteria encased in thin-walled peribacteroid-like membranes called threads (Trinick 1979), as opposed to infection threads, which have thicker walls. The poor growth of Parasponia plants nodulated by either strain ANU843(pMN40) or strain NGR234 and the absence of acetylene reduction by nodules indicated that no nitrogen was fixed by resident bacteria.

All siratro plants were nodulated by either strain ANU843(pMN40) or NGR234 when using agar plate assays. Siratro nodules induced by ANU843(pMN40) were similar in external appearance to those formed by strain NGR234. However, there were no infection threads or plant cells occupied by bacteroids. It is interesting to note that strain ANU851 (a nodD mutant of strain ANU843) carrying pMN40 formed nodules that were infected with bacteroids (Fig 3). In comparison with nodules formed by NGR234, nodule development for ANU851(pMN40) was incomplete with a smaller infection zone, and the nodules produced did not fix nitrogen, unlike those of the parent strain NGR234. No difference was observed in the internal structure of Parasponia nodules formed by strains ANU843(pMN40) and ANU851(pMN40). Bacteria isolated from Parasponia, siratro, and closer nodules retained the appropriate antibiotic-resistance markers, and pMN40 DNA could be visualized in strains ANU843(pMN40) and ANU851(pMN40) by using a modified Eckhardt method.

Table 2. Plant response to R. l. bv. trifolii strain ANU843 carrying the nodD1 gene from strain NGR234

<table>
<thead>
<tr>
<th>Plant spp.</th>
<th>NGR234</th>
<th>ANU843</th>
<th>ANU843(pMN40)</th>
<th>ANU851</th>
<th>ANU851(pMN40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifolium repens</td>
<td>Nod'</td>
<td>Nod'Fix</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
</tr>
<tr>
<td>(white clover)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. subterraneum</td>
<td>Nod'</td>
<td>Nod'Fix</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
</tr>
<tr>
<td>(sub clover)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroptilium</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
</tr>
<tr>
<td>airopurpureum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(siratro)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasponia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magenta jas</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
<td>Nod'</td>
<td>Nod'Fix</td>
</tr>
<tr>
<td>Agar plates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Strain NGR234 nodulated only 10% of Parasponia plants when using an agar plate assay, in contrast to 100% of plants when using Magenta jars. Nod', nodules formed on all inoculated plants; Nod', no plant response observed; Fix', the presence of nitrogen fixation as evidenced by an obvious benefit to plant growth, compared with controls and acetylene reduction by root nodules; Fix', poor plant growth, with pale green to yellow leaves and no detectable acetylene reduction. Two experimental repeats were used each, with 10 plants per strain. Agar plate assays only were used for closer and siratro.
Fig. 2. Response by *Parasponia* to *R. l. trifolii* strain ANU843 carrying the *nodD1* gene from strain NGR234. Nodules (N) formed by strains NGR234 (a) and ANU843(pMN40) (b) on *Parasponia* roots (R). Sections of *Parasponia* roots and attached nodules formed by strains NGR234 (c) and ANU843(pMN40) (d) showing zones of bacterial infection (BZ). VB, nodule vascular bundle; bars = 1 mm. TEM sections taken through the infection zones showing strain NGR234 (e; bar = 1 µm) and ANU843(pMN40) (f; bar = 0.5 µm) bacteria in the bacteroid (B) surrounded by a thread (T) membrane. P, β-hydroxybutyrate/granules.
Fig. 3. Response by *Macropstium atropurpureum* to *R. l. trifoli* strain ANU851 carrying the *nodDI* gene from strain NGR234. Nodules formed by strains NGR234 (a; bar = 1 mm) and ANU851(pMN40) (b; bar = 0.5 mm) showing zones of bacterial infection (BZ); VB, nodule vascular bundle; R, root. TEM micrographs of the infection zones showing bacteroids of strains NGR234 (c) and ANU851(pMN40) (d); B, bacteroid; P, β-hydroxybutyrate granule. Bars = 0.5 µm.
1._"ompou nds

(Plazinski et al. 1985a). When retested these bacteria could still nodulate the same hosts.

Induction of R. l. bv. trifoli i strain ANU843 nodulation genes by nonlegume extract. Plasmid pMN40 was transferred into strain ANU894, which contains a transcriptional fusion of the E. coli lac genes (MudIII1734) with the nodA gene of the nodABC operon on the resident Sym plasmid of strain ANU843. This construction was used to assay the induction of the nodABC operon by purified plant signal compounds and extracts from seedlings of Parasponia and various other plants (Table 3). The addition of the NGR234 nodD1 gene increased the range of extracts that could induce nod gene expression in strain ANU894. Extract from Parasponia induced activity, as did extract from siratro. This was expected because both of these plants are nodulated by strains NGR234 and ANU843(pMN40), but not by strain ANU843. A surprising result was that stimulatory substances were also found in extracts from plants nodulated by neither strain. Extract from seedlings of *Trema aspera*, a species found in the same family as *Parasponia* and known to contain plant hemoglobin genes (Landsmann et al. 1986; Bogusz et al. 1988), essential for a Rhizobium symbiosis, induced nod gene activity. Extracts from *C. cunningharniana*, which forms a nitrogen-fixing root nodule symbiosis with the actinomycete *Frankia*, induced activity, as did extracts from sunflower and cotton and the cereals wheat, rice, and maize (Table 3). The presence of the nodD1 gene from NGR234 also increased the range of purified flavonoid compounds that induced expression of the nodABC operon (Table 3). These compounds included umbelliferone, which is normally inhibitory to the induction of nod genes in strain ANU843 (Djordjevic et al. 1987). The expression of the nodABC operon was increased up to 40-fold by the presence of the NGR234 nodD1 gene with the additional compounds tested. It appears that the NGR234 nodD1 gene enables nod gene induction by plant signals in a nonspecific manner.

The induction of the nodD1::MudIII1734 fusion by Parasponia extract is consistent with the observation that the nodD1 gene from strain NGR234 is also required for Parasponia nodulation by strain ANU843. Although the nod genes of ANU843 can be induced by signal compounds from nonlegumes other than Parasponia, the inoculation of Trema, Casuarina, sunflower, cotton, wheat, rice, and maize with strains NGR234, ANU843(pMN40), and ANU851(pMN40) did not produce nodules on these hosts.

**DISCUSSION**

It has been reported previously that transfer of the nodD1 gene from *Rhizobium* strain NGR234 to *R. meliloti* (Horvath et al. 1987) and *R. l. bv. trifoli i* (Bassam et al. 1988) extended the host range of the recipients to new legume hosts such as siratro. This paper extends these observations to a nonlegume. Specifically, the NGR234 nodD1 gene enables *R. l. bv. trifoli i* strain ANU843 to nodulate Parasponia, which has a rather different pathway of infection and nodule development to that of legumes (Lancelle and Torrey 1984, 1985; Bender et al. 1987a). This suggests that the different pathways of infection are largely due to a difference in plant response between legumes and Parasponia and not to major differences in the bacterial genome. It is possible that the induction of conserved nodulation genes, present in most *Rhzobium* species, is sufficient for the nodulation of this host. The NGR234 nodD1 gene also enables strain ANU843 to respond to a broadened spectrum of purified flavonoid inducers and extracts from other nonlegumes. The induction by monocotyledons was unexpected because these plants are not only outside the known host range of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, but are also outside the host range of the pathogenic member of the Rhizobiaceae, *Agrobacterium*. It is interesting to note that extracts from seedlings of wheat and oats can induce vir genes in *A. tumefaciens* (Usami et al. 1988).

The induction of nod genes in strain ANU843 by *Parasponia* extract was correlated with the ability of ANU843(pMN40) to nodulate *Parasponia*. However, the induction of nod genes by extracts from other nonlegumes was not correlated with nodule formation. Similarly, the induction of vir genes by wheat and oat extracts does not lead to crown gall formation (Usami et al. 1988). Previous work in our lab has shown that strain ANU845 (A Sym derivative of ANU843), carrying nodD, nodF, and nodABC genes from ANU843 on a multicopy vector, was able to curl the root hairs of rice and maize plants (Plazinski et al. 1985b). In light of the present work, this is unexpected, because the nodD gene from ANU843 does not enable the induction of nodulation genes in the presence of extract from these plants. The response observed may be due to the high copy number of nod genes, because no effect was observed when a low copy number vector was used, or perhaps it is due to a cloning artifact. The resolution of this question is the focus of a series of experiments currently in progress.

---

**Table 3. Response by R. l. bv. trifoli i strain ANU894, carrying the nodD1 gene from strain NGR234, to flavonoids and seedling extracts**

<table>
<thead>
<tr>
<th>Inducer</th>
<th>ANU894</th>
<th>ANU843 (pMN40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flavones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4-dihydroxyflavone</td>
<td>509</td>
<td>636</td>
</tr>
<tr>
<td>Luteolin</td>
<td>123</td>
<td>470</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>14</td>
<td>490</td>
</tr>
<tr>
<td><strong>Flavanones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4-dihydroxyflavanone</td>
<td>310</td>
<td>400</td>
</tr>
<tr>
<td>Naringenin</td>
<td>122</td>
<td>93</td>
</tr>
<tr>
<td><strong>Isoflavones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>10</td>
<td>440</td>
</tr>
<tr>
<td>Daidzein</td>
<td>9</td>
<td>450</td>
</tr>
<tr>
<td><strong>Coumarin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coumestrol</td>
<td>9</td>
<td>450</td>
</tr>
<tr>
<td><strong>Diocysteolans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>10</td>
<td>140</td>
</tr>
<tr>
<td><strong>Monocotyledons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siratro</td>
<td>17</td>
<td>474</td>
</tr>
<tr>
<td><em>Parasponia</em></td>
<td>25</td>
<td>410</td>
</tr>
<tr>
<td>Trema</td>
<td>10</td>
<td>440</td>
</tr>
<tr>
<td>Casuarina</td>
<td>26</td>
<td>140</td>
</tr>
<tr>
<td>Sunflower</td>
<td>26</td>
<td>261</td>
</tr>
<tr>
<td>Cotton</td>
<td>15</td>
<td>310</td>
</tr>
<tr>
<td><em>Wheat</em></td>
<td>5</td>
<td>371</td>
</tr>
<tr>
<td><em>Maize</em></td>
<td>11</td>
<td>237</td>
</tr>
<tr>
<td><em>Rice</em></td>
<td>24</td>
<td>134</td>
</tr>
</tbody>
</table>

*Values are in units of β-galactosidase activity (Miller 1972) following induction of a nodA::MudIII1734 fusion in the symbiotic plasmid of R. l. bv. trifoli i strain ANU843 (strain ANU849 carrying pMN40). A concentration of 1 X 10^6 M was used for each compound, except for umbelliferone, which was used at 1 X 10^7 M. Values shown are the average of two readings with four experimental repeats. Standard deviation for all values was less than 30%.

264 Molecular Plant-Microbe Interactions
Although the broad host range function of the nodD1 gene is clearly evident in strain NGR234, the nodD genes from narrow host range strains are much more specific in action. Spank et al. (1987) have shown that all three nodD genes from R. meliloti, R. leguminosarum, and R. I. bv. trifolii can interact with Trifolium repens (white clover) root exudate, but only the R. I. bv. trifolii nodD gene can respond to exudate from T. pratense (red clover). Transfer of the R. I. bv. trifolii nodD gene to R. meliloti and R. leguminosarum also enabled the induction of nod::lacZ fusions in these strains by red clover root exudate. Thus, the nodD gene from R. I. bv. trifolii determines red clover host specificity. Honma and Ausubel (1987) and Gyorgypal et al. (1988) have shown that broad host range in R. meliloti is determined by three copies of the nodD gene. The three nodD genes are allelic forms with divergent flavonoid specificity and activate nodulation genes in response to exudates from different hosts (Gyorgypal et al. 1988).

It is interesting to speculate that Rhizobium have evolved different strategies to nodulate a broader range of hosts. R. meliloti has acquired additional nodD alleles with divergent C-terminal regions (Gyorgypal et al. 1988) to interact with signals from different hosts, whereas strain NGR234 has retained a single functional nodD gene with alterations in the C-terminal region (Horvath et al. 1987) that enable nonspecific interaction. These considerations and our own observations support the conclusion by Spank et al. (1987) and Horvath et al. (1987) that the nodD gene should no longer be recognized as a common nod gene but as a gene that plays a key role in determining host range. The NGR234 nodD1 gene is unusual in that it does not determine host specificity but rather nonspecificity for host interaction.

Bradyrhizobium strains ANU289 and Rp501 have a broad host range similar to that of NGR234, and both can form nitrogen-fixing nodules on Parasponia. Mutation of the nodD gene adjacent to the nodABC operon in strain ANU289 (Scott et al. 1987) does not affect nodulation on either Parasponia or siratro, which suggests that this strain has another copy or copies of the nodD gene capable of interacting with legume and/or Parasponia plant signals. This assumption may be correct, as additional copies of the nodD gene have recently been located in strain ANU289 (K. Scott, personal communication). A nodD gene that has no apparent function in siratro or Parasponia nodulation is also found next to the nodABC operon in Bradyrhizobium strain Rp501 (Marvel et al. 1987)

The nodD1 gene from strain NGR234, in the presence of extracts from a range of nonlegumes, did not enable nodulation of any nonlegume other than Parasponia. Similarly, interaction with alfalfa signal by the nodD1 gene from strain NGR234 was not sufficient for alfalfa nodulation (Horvath et al. 1987). Only the transfer of R. meliloti host-specific nodulation genes could accomplish this (Horvath et al. 1986; Putnoky and Kondorosi 1986). Hence, the presence of a nodD gene capable of interacting with a signal from a particular legume host does not guarantee nodulation of that host. Nodulation may also require the presence of particular host-specific genes. The existence of Parasponia-specific DNA regions has been shown for strain NGR234 (Bender et al. 1987b) and Bradyrhizobium strains ANU289 (Scott et al. 1987) and Rp501 (Marvel et al. 1987). The NGR234 nodD1 gene, when transferred to R. I. bv. trifolii strains ANU843 and ANU851, enabled nodules to form with a less well-developed structure than those of NGR234. This suggests that ANU843 lacks genes needed for Parasponia nodule development. Transfer of an 18-kb DNA fragment, containing a nonfunctional nodD gene, the nodABC operon, and a presumptive Parasponia-specific region from strain ANU289 to strain ANU843, extended the host range from clover to Parasponia but not siratro (Scott et al. 1987). Similarly, a Tn5 insertion in a presumptive Parasponia-specific region adjacent to nodC in Bradyrhizobium strain Rp501 abolished Parasponia but not siratro nodulation (Marvel et al. 1987).


The \textit{Rhizobium} Strain NGR234 \textit{nodD1} Gene Product Responds to Activation by the Simple Phenolic Compounds Vanillin and Isovanillin Present in Wheat Seeding Extracts

Kathryn K. Le Strange, Gregory L. Bender, Michael A. Djordjevic, Barry G. Rolfe, and John W. Redmond

Plant-Microbe Interaction Group, Research School of Biological Sciences, Australian National University, Canberra, ACT, 2601; and School of Chemistry, Macquarie University, North Ryde, NSW, 2109, Australia.

Received 18 September 1989. Accepted 16 January 1990.

Vanillin and isovanillin are present in extracts of wheat seedlings and interact with the nodulation (\textit{nod}) gene \textit{nodD1} from \textit{Rhizobium} strain NGR234 to induce expression of \textit{R. leguminosarum} bv. \textit{trifolii nod} genes. Seven varieties of Australian wheat were examined. Vanillin, isovanillin, or both were present in five of the varieties tested. Assays of a wide range of authentic flavonoid and other phenolic compounds for transcriptional induction of the same \textit{nodA}:\textit{lacZ} fusion revealed that a hydroxyl group para to an electron-withdrawing group and/or the presence of a cluster of oxygen functions are the prime structural requisites for transcriptional activation of \textit{nodD1}-activated \textit{nod} genes.

Additional keywords: \textit{nodD}, \textit{nod} gene induction, phenolic inducers, wheat extract.

Extensive studies have shown that the nodulation (\textit{nod}) gene \textit{nodD} is a crucial gene involved in the earliest stages of host recognition between various rhizobia and legumes (Spank et al. 1987b). The product of \textit{nodD} is believed to be a transcriptional activator protein (Henikoff et al. 1988) that binds to the promoters of inducible \textit{nod} genes (Hong et al. 1987; Fisher et al. 1988) in a region closely corresponding to the \textit{nod}-box (Rostas et al. 1986; Long 1989). Plant-synthesized compounds, in concert with the \textit{nodD} gene product, activate inducible \textit{nod} genes, which then initiate the early processes involved in nodulating a plant host (Mulligan and Long 1985; Innes et al. 1985; Rossen et al. 1985; Shearman et al. 1986; Spank et al. 1987b; Bassam et al. 1988; Spank et al. 1989).

Flavonoid compounds that interact with \textit{nodD} have been identified in many narrow host range \textit{Rhizobium}-legume symbioses. Examples include luteolin, isolated from alfalfa (Peters et al. 1986), and 7,4'-dihydroxyflavone (DHF), isolated from clover (Redmond et al. 1986). Anti-inducers such as coumarin, flavonol, and some isoflavonones (Redmond et al. 1986; Firmin et al. 1986; Djordjevic et al. 1987) appear to competitively inhibit \textit{nodD}-dependent induction (Djordjevic et al. 1987). In contrast, the iso-flavone daidzein has been isolated from soybean and shown to induce \textit{nodD}-dependent \textit{nod} genes of \textit{Bradyrhizobium japonicum} (Buchanan) Jordan (Kossak et al. 1987).

\textit{Rhizobium} strain NGR234 (Trinick 1980) is a broad host range \textit{Rhizobium} and is one of the few \textit{Rhizobium} strains capable of forming nodules with a nonlegume host, the woody tree \textit{Parasponia} from the Ulmaceae family (Trinick and Galbraith 1980). Molecular characterization of the \textit{nodD1} gene of strain NGR234 shows it to be highly conserved at the DNA sequence level with the \textit{nodD} genes of other rhizobia (J. J. Weinman, unpublished data). Bendet et al. (1988) prepared a range of plant extracts from various legume hosts, from \textit{Parasponia}, and from nonhost plants such as the nonlegumes \textit{Trema} (a close relative of \textit{Parasponia}), \textit{Casuarina} (which forms symbioses with \textit{Frankia}), and the cereals rice, maize, and wheat. These extracts are capable of inducing transcriptional activation of \textit{nod} genes in the presence of the \textit{nodD1} gene from strain NGR234. Despite the high level of molecular conservation with other \textit{nodD} alleles, the \textit{nodD1} gene of strain NGR234 is less specific in host recognition than the \textit{nodD} genes from more widely characterized, narrow host range rhizobia (Bender et al. 1988; Bassam et al. 1988).

Flavonoid compounds are widespread throughout plant families. A broad range of these compounds induce \textit{nodD}-dependent activation in strain NGR234, including some which have been identified as anti-inducers in other rhizobia (Bassam et al. 1988). While flavonoids and isoflavonoids induce expression of \textit{Rhizobium nod} genes (Djordjevic et al. 1987; Horvath et al. 1987) and simple phenolic compounds induce expression of \textit{Agrobacterium tumefaciens} (Smith and Townsend) \textit{Con} \textit{vir} genes (Stachel et al. 1985a, 1985b), simple phenolic compounds have not been previously shown to induce \textit{Rhizobium nod} genes.

To determine the characteristics of compounds capable of inducing \textit{nod} gene transcription in conjunction with the \textit{nodD1} gene from \textit{Rhizobium} strain NGR234, a large number of authentic plant-derived phenolic compounds were assayed, and the common structural features of the inducing compounds were analyzed. Unlike \textit{nodD} alleles from other \textit{Rhizobium} strains, simple compounds also activated \textit{nod} gene transcription. Simple compounds were identified from fractionated wheat seedling extracts that similarly activated \textit{nod} gene transcription.
MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are detailed in Table 1.

Plant materials. The principal wheat (Triticum aestivum L.) used in this investigation was Australian Standard White (ASW), the chief grade of Australian export wheat containing a mixture of hard wheat varieties. Seven varieties of wheat, grown under differing Australian conditions, were examined for the presence of inducing compounds following the identification of such compounds in extracts of ASW. Varieties were obtained as a gift from the CSIRO Bread Research Institute, North Ryde, Australia.

Media. BMM (Bergersen 1961), GMM, and GMY (Bender et al. 1988) media for the growth of Rhizobium have been described elsewhere. The vitamins thiamine (100 µg/L) and biotin (25 µg/L) were added for the culture of R. leguminosarum bv. trifolii Jordan strains. The antibiotic tetracycline was used for the selection and maintenance of Rhizobium strains harboring plasmid pMD1 in solid media at a concentration of 4 µg/ml.

Construction of pMD1. A 0.7-kilobase (kb) BamHI DNA fragment containing the nodD/nodA intergenic region of R. l. bv. trifolii ANU843 was cloned into the pBS+ vector obtained from Stratagene (La Jolla, CA). This DNA fragment was derived from ANU843 containing an insertion of MudII1734 in the 5' end of nodA and included 116 base pairs (bp) of the MudII1734 DNA (McIver et al. 1989). Using a PstI site located 207 bp 3' to the initiation codon for nodD, and lying within the nodD coding region of this fragment (Schofield and Watson 1986), and the PstI site from the polylinker of the pBS+ vector, a 0.7-kb PstI fragment was excised and cloned (see Fig. 1) into the PstI site of the plasmid pMP220 (Spänik et al. 1987a). This resulted in the fusion of the nodA promoter with the promoterless lacZ gene of pMP220.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGR234</td>
<td>Wild-type, fast-growing, broad host range Rhizobium strain infecting tropical legumes</td>
<td>Trinick 1980</td>
</tr>
<tr>
<td>ANU265</td>
<td>Sym plasmid-cured derivative of strain NGR234; Sm', Sp', and Nod⁺</td>
<td>Morrison et al. 1983</td>
</tr>
<tr>
<td>ANU843</td>
<td>Wild-type R. leguminosarum bv. trifolii strain</td>
<td>Rolle et al. 1980</td>
</tr>
<tr>
<td>pMP220</td>
<td>Broad host range IncPl promoter cloning vector, stably maintained in Rhizobium strain ANU265; Tc⁺, promoterless Escherichia coli lacZ gene</td>
<td>Spänik et al. 1987a</td>
</tr>
<tr>
<td>pMD1</td>
<td>pMP220 carrying a 2.9-kb EcoRI fragment containing the NGR234 nodD1 gene and a 0.7-kb PstI fragment containing the nodA promoter of R. l. bv. trifolii ANU843</td>
<td>This study</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for mobilization into rhizobia, unable to be maintained in Rhizobium</td>
<td>Ditta et al. 1980</td>
</tr>
</tbody>
</table>

* nod⁺, the inability to form nodules on the hosts of strain NGR234; Sm', resistance to streptomycin; Sp', resistance to spectinomycin; Tc⁺, resistance to tetracycline; and kb, kilobase.

Fig. 1. Map of plasmid pMD1. The locations of the 2.9-kilobase (kb) EcoRI fragment containing the nodD1 gene from Rhizobium strain NGR234 (Bassam et al. 1988) and the 0.7-kb PstI fragment containing the nodA promoter region from strain ANU843 (McIver et al. 1989) inserted into the multicloning site of plasmid pMP220 (Spänik et al. 1987a) are shown. The nodA promoter region is positioned to drive transcription of the promoterless lacZ gene in the vector (arrow). The nodA promoter region contains 207 base pairs (bp) of the 5' region of the ANU843 nodD gene (stippled area); the nodD/nodA intergenic region (crosshatched area); the nod-box (black vertical bar); the nodA coding region (unshaded area); and 116 bp of MudII1734 DNA (horizontal shaded area) inserted in the 5' end of the nodD coding region. Plasmid pMP220 is not drawn to the same scale as the inserted fragments. Other sites in the multicloning site are not shown. E = EcoRI; P = PstI; and H = HindIII.
Amicon Corporation (Danvers, MA), and silicic acid (Keiselgel 60, 70–230 mesh) was from E. Merck (Darmstadt, Federal Republic of Germany).

Reverse-phase high performance liquid chromatography (HPLC) was conducted using an RP-8 column (MPL analytical cartridge, 100 × 4.6 mm, 5 μm) from Brownlee Labs (Santa Clara, CA), eluted with a gradient generated from solvent A (0.2% trifluoroacetic acid in water) and solvent B (methanol), and monitored by a Waters 490 multiwavelength detector; data were acquired with a Shimadzu Chromatopak CR-4. Peaks were characterized by retention time and ultraviolet (UV) profiles in the stop-scan mode.

Isolation and characterization of simple phenolic compounds from wheat seedlings. Wheat (ASW) was sterilized by soaking in 70% ethanol for 5 min, followed by thorough washing in sterile distilled water. After imbibing for 15 min, the wheat seeds were grown hydroponically in sterile distilled water on metal grids at 25°C for 2–4 days (Canter-Cremers et al. 1986). The seedlings were drained and snap-frozen in liquid nitrogen.

Batches (500 g) of frozen seedlings were homogenized in boiling distilled water (100 ml). Ethanol (500 ml) was added, and the mixture was boiled for 15 min, cooled, and filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, U.K.). The filtrate was evaporated to dryness under reduced pressure, and the residue was triturated thoroughly in methanol (100 ml). The dispersion was diluted with chloroform (3 volumes), mixed thoroughly, allowed to stand for 2 hr, and filtered. The filtrate was evaporated under reduced pressure, and the residue was reconstituted in methanol.

Combined extracts from approximately 85,000 ASW seedlings were preadsorbed onto silicic acid (25 g) and applied to the top of a column (200 × 30 mm) of silicic acid packed in 10% methanol/chloroform. Elution was conducted with the same solvent (450 ml), 40% methanol/chloroform (450 ml) and methanol (450 ml), and 12 fractions of 110 ml were collected. Fractions were assayed for NGR234 nodD1-dependent nod-gene-inducing activity using strain ANU265(pM D1) in β-galactosidase assays as described above. Activity was detected in fractions A2, A6, and A12. Earlier experiments (data not shown) indicated that DHF elutes in an appropriately fraction A6 and that highly hydroxylated and glycosylated flavones require methanol for elution (fraction A12). Thus, fraction A2 appeared to contain a new class of active compound and was selected for further study.

Fraction A2 was dried down and dissolved in a small volume of methanol with water being added until the solution was 5% methanol/water. This was then applied to a column (100 × 10 mm) of Matrex Silica C18 (reverse-phase material) and eluted with methanol/water mixtures from 5 to 100% methanol. Seven fractions of approximately 45 ml were collected. Fractions B2 and B3 contained inducing activity (collected from 55 to 135 ml) and were combined, rechromatographed on a column of silicic acid, and eluted with methanol/methylene chloride (1 to 100%). Ten fractions of 25 ml were collected. The resultant active fraction C2 (25 to 50 ml) was evaporated carefully in a nitrogen stream and subjected to analysis by reverse-phase HPLC as described above. (See Fig. 2 for a summary of the wheat seedling extract fractionation procedure.)

Samples of different wheat varieties were processed on a smaller scale than ASW wheat (approximately 2,000 seedlings of each). An active fraction equivalent to fraction A2 of ASW was identified from extracts of each variety, and these fractions were then analyzed by reverse-phase HPLC and UV spectrum analysis as described above.

RESULTS

Phenolic compounds inducing nodD-dependent transcription. Some 70 phenolic compounds were examined for their capacity to induce the nodA: lacZ fusion of the nodD1-dependent construct pMD1 in Rhizobium strain ANU265. Assays were conducted over a range of concentrations from 10^{-3} to 10^{-7} M (Table 2). Inhibition of bacterial cell growth was detected for a number of compounds and is also indicated in Table 2. The concentration at which half-maximal induction occurs (A_{50}) was determined for approximately 20 representative active compounds, and the results are shown in Table 3. The comparison of A_{50} gives a clearer picture of potent and weak inducers than does the comparison of I_{max}.

The most potent inducers were daidzein, genistein, DHF, and apigenin, all with A_{50} values of 10^{-8} or lower (Table 3). Other compounds able to induce activity at low concentrations (A_{50} below 10^{-6}) included coumestrol, formononetin, biochanin A, quercetin, kaempferol, naringenin, hesperitin, and 7-hydroxyflavone. Those inducing at higher concentrations (A_{50} above 10^{-5}) included vanillin, isovanillin, syringaldehyde, 7-hydroxychromone, and umbelliferone. Those unable to induce detectable nodA
expression included 3,4-dimethoxybenzoic acid, 3,4-dimethoxybenzaldehyde, 4-methoxyacetophenone, o-hydroxyacetophenone, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and flavone (Table 2).

The key features of compounds able to induce expression of nodD1-dependent nod genes in strain NGR234 were determined by analysis of the characteristics of potent inducers, weak inducers, and noninducing compounds. These are presented in Figure 3 and can be summarized as follows:

1) The presence of an hydroxyl (—OH) group para to an electron-withdrawing function increases biological activity, for example —OH para to a carbonyl function as in 4-hydroxybenzoic acid or in 7-hydroxylavone.

2) The presence of a cluster of oxygen functions increases biological activity, for example pyrogallol.

3) Hydroxylation of the B-ring increases the potency of flavones (that is, decreases A90).

Chromatographic analysis of wheat seedling extracts. Vanillin and isovanillin were characterized from fraction C2 of ASW wheat seedling extract by retention times in reverse-phase HPLC analysis and confirmed by UV spectrum analysis of the identified peaks in the stop-scan mode (Fig. 4).

Vanillin and isovanillin are structural isomers and have almost identical retention times under the HPLC conditions used. Close inspection of the UV spectra of eluates indicated a mixture of the two compounds present in ASW wheat seedling extract. The mixture could not be further separated under the conditions available, but comparison with standard preparations and their UV spectra under the same conditions indicated that the peak consisted predominately of vanillin (data not shown).

Whole extracts of all varieties induced the nodA promoter of ANU265(pMD1). After analysis of comparable active fractions, vanillin and/or isovanillin could be unequivocally shown in the following varieties: ASW, Halberd, Suneca, Rosella, Hartog, and Vulcan. In the remaining two varieties, Matong and Eradu, no clear identification of vanillin or isovanillin could be made at the levels of detection available because of unidentified overlapping peaks. Comparison of vanillin peak heights from HPLC data to known quantities of standard vanillin under identical conditions allowed approximate calculation of the amount of vanillin present in wheat variety preparations. Table 4 gives details of varietal characteristics, Imax, and vanillin concentration.

Table 2. Phenolic compounds assessed for biological activity

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Active compounds (range 10^-7 to 10^-8 M)</th>
<th>Inactive compounds (range 10^-7 to 10^-6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>4-OH benzoic acid (A), 2,3-diOH benzoic acid (B, I), 2,4-diOH benzoic acid (B, I), 2,4-diOH benzoic acid (B, I), 3,4-diOH benzoic acid (A, I), pyrogallol acid (B), acetyl salicylic acid (A)</td>
<td>Benzoic acid (I), salicylic acid (I), 3,OH benzoic acid, 3,5-diOH benzoic acid, 3,4-diOMe benzoic acid, cinnamic acid (I), 2-coumaric acid, 3-coumaric acid (I), acetyl salicylic acid (A), AC3, diOMe benzoic acid (I), salicylic acid (I)</td>
</tr>
<tr>
<td>Phenols</td>
<td>Catechol (C), resorcinol (A), vanillin (C, I), isovanillin (C), quinol (A)</td>
<td>Anisole</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Benzaldehyde (A), 3,OH benzaldehyde (B), 3,4-dOH benzaldehyde (C), 3,4-dOMe benzaldehyde (A), syringaldehyde (C)</td>
<td>Salicylaldehyde, 2,4-diOMe benzaldehyde, cinnamaldehyde (I), piperonal</td>
</tr>
<tr>
<td>Ketones</td>
<td>3-OH acetoephone (C), 4-OH acetoephone (C), 2,4'-diOH acetoephone (C), 2,4-dihydroxyacetophenone (B, 2,3,4-triOH acetoephone (C), 4-OH,3'-OMe acetoephone (B), 4-OH,3'-OMe acetoephone (C), 4-OH,3'-OMe acetoephone (C), 2-OH,4-OMe acetoephone (A), acetylsyringone (B), 2,4,4-triOH chalcone (B)</td>
<td>Acetophenone, 2-OH acetophenone, 4-OMe acetophenone</td>
</tr>
<tr>
<td>Chromone</td>
<td>7-OH chromone (A)</td>
<td>Fraxetin</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Coumarin (A), umbelliferone (C)</td>
<td>Bayin, taxifolin, naringis, 4'-OH flavanone</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Coumarin (A), umbelliferone (C)</td>
<td>Flavanone, 3-OH flavanone, 7,4'-dihydroxyflavone, 5-OH,7'-dihydroxyflavone, diosmin</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>Daidzein (C), biochanin A (C), formononetin (C), genistein (C)</td>
<td>Quercetin (B), kaempferol (C)</td>
</tr>
<tr>
<td>Flavonol</td>
<td>Quercetin (B), kaempferol (C)</td>
<td>Flavone, 7,4-dihydroxyflavone (A), hesperetin (C), naringenin (C)</td>
</tr>
<tr>
<td>Flavonol</td>
<td>Quercetin (B), kaempferol (C)</td>
<td>Flavone, 7,4-dihydroxyflavone (C), morin (C), 7-OH flavone (C), chrys (C), apigen (C)</td>
</tr>
</tbody>
</table>

DISCUSSION

This study reports the first case of simple plant phenolic compounds contributing to transcriptional activation of the nodD-dependent nod genes of Rhizobium. Vanillin and isovanillin were identified in extracts of wheat seedlings, and authentic samples of these compounds in the presence of nodD1 from strain NGR234 induced expression of the nodA promoter of R. i. trifolii ANU843. Vanillin and isovanillin did not induce expression of the same promoter in the presence of the nodD gene of strain ANU843. These findings extend the work of Bender et al. (1988) who found that wheat extracts are capable of inducing nodD-dependent nod gene expression only with the nodD1 gene from strain NGR234.

The fractionation procedure for wheat extract was optimized for extraction of simple phenolic compounds capable of inducing Rhizobium nodD-dependent nod genes. Other fractions of wheat extract also induced nodD-dependent expression in strain ANU265(pMD1) and, on the basis of
Table 3. Relative induction levels of compounds inducing *Rhizobium* strain NGR234 nodD1-dependent nod gene expression (indicated by β-galactosidase activity)

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Compound</th>
<th>log $A_{50*}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>4-Hydroxybenzoic acid</td>
<td>$-4.3$</td>
</tr>
<tr>
<td>Phenols</td>
<td>Vanillin</td>
<td>$-6.3$</td>
</tr>
<tr>
<td></td>
<td>Isovanillin</td>
<td>$-5.5$</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>Syringaldehyde</td>
<td>$-5.3$</td>
</tr>
<tr>
<td>Ketone</td>
<td>Acetophenone</td>
<td>$-4.3$</td>
</tr>
<tr>
<td>Chromone</td>
<td>7-Hydroxychromone</td>
<td>$-3.5$</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Umbelliferone</td>
<td>$-5.2$</td>
</tr>
<tr>
<td>Coumestan</td>
<td>Coumestrol</td>
<td>$-7.3$</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>Daidzein</td>
<td>$-8$</td>
</tr>
<tr>
<td></td>
<td>Genistin</td>
<td>$-8$</td>
</tr>
<tr>
<td></td>
<td>Formononetin</td>
<td>$-6.3$</td>
</tr>
<tr>
<td></td>
<td>Biochanin A</td>
<td>$-6.3$</td>
</tr>
<tr>
<td>Flavanol</td>
<td>Quercetin</td>
<td>$-7$</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>$-7.5$</td>
</tr>
<tr>
<td>Flavanone</td>
<td>Naringenin</td>
<td>$-7.3$</td>
</tr>
<tr>
<td></td>
<td>Hesperetin</td>
<td>$-7$</td>
</tr>
<tr>
<td>Flavone</td>
<td>7-Hydroxyflavone</td>
<td>$-7.3$</td>
</tr>
<tr>
<td></td>
<td>7,4'-Dihydroxyflavone</td>
<td>$-8$</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>$-8.3$</td>
</tr>
</tbody>
</table>

$A_{50*}$, concentration at which induction is half-maximal for each compound.

![Chemical structures](image)

**Fig. 3.** Representative substances that induce *Rhizobium* strain NGR234 nodD1-dependent β-galactosidase activity in strain ANU265(pMD1). Modifications to the basic structures are listed numerically as follows, with the effect on nodA induction also being provided ($A_{50*}$ = concentration at which induction is half-maximal): at circle position (1), removal of the OH group increases $A_{50*}$; at circle position (2), the OH group is essential for activity; at circle position (3), an OH group is necessary in at least one of the positions; at circle position (4), both functional groups are required for activity, but may be interchanged; at circle position (5), an OH group at this site decreases $A_{50*}$; at circle position (6), reduction of the double bond does not significantly affect activity; at circle position (7), an OH group increases $A_{50*}$; at circle position (8), an OH at this site marginally lowers $A_{50*}$; and at circle position (9), a methoxy group at this site increases $A_{50*}$.

the separation system used, these probably contained more complex flavonoid compounds.

A variety of simple and complex phenolic compounds have been identified previously in wheat including tricin (Anderson 1932), vanillin glucosides, p-hydroxybenzaldehyde, vanillin, and syringaldehyde in graminaceous cell walls (Hartley and Keene 1984), p-coumaric acid in association with lignin and ferulic acid from nonlignified tissue (Scalbert et al. 1985), and apigenin glucosides from wheat bran (Feng et al. 1988). Of these, the aglycons syringaldehyde, vanillin, and apigenin have been found to induce nodA expression in strain ANU265(pMD1) (Tables 2 and 3). Neither vanillin nor isovanillin has been specifically reported to occur in host plants of NGR234. Analysis of root extracts of *Parasponia* was initiated but abandoned due to lack of available plant material.

Plant substances capable of inducing optimal expression of nodD-dependent nod genes in other rhizobia have specific structural characteristics, such as hydroxylation of the flavonoid skeleton at the 7 and 4' positions of both flavones and flavanones (Firmin et al. 1986; Redmond et al. 1986; Kapulnik et al. 1987; Gyorgypal et al. 1988). Induction of the nod genes of *R. leguminosarum* biovars also occurs with compounds hydroxylated at the 3' position, and activity is further enhanced if a methoxy group is present at the 4' position. Any substitution at the 3 position results in inactive compounds (Firmin et al. 1986; Zaat et al. 1987).

Induction in *R. i. bv. trifolii* and *R. meliloti* Dangeard is also observed with compounds hydroxylated at either or both the 5 and 3' positions (Peters et al. 1986). Antagonists of activation in these species are usually isoflavonoids (Djordjevic et al. 1987). In contrast *B. japonicum*, a slow-
growing species, is activated by flavonoids similar to those described above and also by isoflavonoid compounds conforming to the same general parameters; however, compounds hydroxylated at either or both the 3 and 5' positions are inactive (Kossak et al. 1987; Gottfert et al. 1988).

These characteristics specify a subset of compounds capable of inducing nod genes in the presence of the nodD1 gene from Rhizobium strain NGR234. However, the range of compounds capable of interaction with this nodD1 gene extends to include a greater variety of complex, multiringed phenolic compounds, in addition to the simple phenolic compounds reported here.

The nodD1 gene from strain NGR234 may thus represent a less specialized nodD allele, perhaps retaining some aspects of an ancestral plant recognition gene. Alternatively, the NGR234 nodD1 gene may be a more versatile gene, incorporating the highly specialized interactive capacity of the narrow host range rhizobia along with the capacity to respond to simple phenolic compounds.

Several recent findings indicate the second alternative as being unlikely. Chemically induced single base substitution mutations to R. l. trifolii nodD result in the extension of host range to Parasponia, the nonlegume host of strain NGR234 (McVern et al. 1989). Clearly R. l. trifolii has most of the genes required to infect this illegitimate host, since only a point mutation of the nodD gene confers extended nodulation host range. Furthermore, a significant relationship at the genetic level has recently been established between the nodD genes of several Rhizobium species and the nahR gene of Pseudomonas putida (Treviran) Migula (Schell and Sukordhaman 1989). NahR is a transcriptional activator of the nah and sal operons, activating transcription of the sal promoter only in the presence of salicylate (a salt or ester of salicylic acid). It is interesting to note that the NGR234 nodD1 gene does not interact with salicylic acid.

The expression of A. tumefaciens vir genes (required for plant cell transformation and crown gall formation) is dependent upon a two-component regulatory system. The virA gene product interacts with simple phenolic compounds to activate virG, and the resultant VirG protein induces expression of the remaining vir genes (Winans et al. 1986). Inducers of the vir genes include acetosyringone, α-hydroxyacetosyringone, acetovanillone, syringaldehyde, and sinapinic acid (Stachel et al. 1985a), all of which are single-ring, simple phenolic compounds. Acetosyringone production is significantly increased in wounded tissue (Stachel et al. 1985b), and other active compounds are found at sites of lignin production such as root elongation zones and tissue damage repair sites. Rhizobium nod genes are also induced by acetosyringone, acetovanillone, and syringaldehyde in the presence of the NGR234 nodD1 gene but not by sinapinic acid. Thus, some wound-induced compounds, lignin production intermediates, and degradation products will induce NGR234 nodD1-dependent nod genes. Interestingly, vir gene activating compounds have also been found in extracts of oats and of wheat seeds (Usami et al. 1988). These are apparently different from acetosyringone, possibly being a phenolic structure conjugated to a hydrophobic molecule. Usami et al. (1988) also postulate the existence of inhibitors of vir gene induction in monolycotyledonous tissues.

There has been no reported DNA or protein homology between virA and either nodD or nahR, yet all have a common ability to interact with simple phenolic-inducing compounds. It is interesting to speculate on the possible evolutionary relationship between these genes; perhaps virA development diverged early on from an ancestral gene, and nahR and nodD developed subsequently. Alternatively, the genes may have converged from separate ancestral genes to perform a similar role.

Identification of simple phenolic compounds as inducers of nodD-dependent nod genes from Rhizobium has allowed a clearer definition of the chemical nature of the induced nodD interaction that may contribute to the further development of a model for the mechanism of action of the nodD-encoded protein. Further investigation of the nature of this interaction involving the mutation of the NGR234 nodD1 gene to alter the affinity of the nodD-encoded protein for phenolic compounds and the precise characterization of such mutations will enable definition of the critical domains of the protein involved in the inducer-nodD interaction.

**Table 4. Characteristics of Australian wheat varieties examined**

<table>
<thead>
<tr>
<th>Variety</th>
<th>Characteristics</th>
<th>Induction levels ( (\text{max}) )</th>
<th>Vanillin/isovanillin (^a) concentration (^b)</th>
<th>Vanillin concentration (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASW</td>
<td>Australian Standard White</td>
<td>850</td>
<td>+</td>
<td>1.3 × 10^{-4}</td>
</tr>
<tr>
<td>Halberd</td>
<td>Hard, dry-land variety; low boron intake</td>
<td>900</td>
<td>+</td>
<td>4.2 × 10^{-4}</td>
</tr>
<tr>
<td>Suneca</td>
<td>Hard, high-yield variety, will not germinate if wet during harvest</td>
<td>1,100</td>
<td>+</td>
<td>4.0 × 10^{-4}</td>
</tr>
<tr>
<td>Hartog</td>
<td>Hard, high-yield variety</td>
<td>980</td>
<td>+</td>
<td>4.4 × 10^{-4}</td>
</tr>
<tr>
<td>Vulcan</td>
<td>Hard, high-yield variety</td>
<td>1,280</td>
<td>+</td>
<td>2.8 × 10^{-4}</td>
</tr>
<tr>
<td>Rosella</td>
<td>Soft, long-season variety</td>
<td>1,010</td>
<td>+</td>
<td>1.0 × 10^{-4}</td>
</tr>
<tr>
<td>Eradu</td>
<td>Soft, rapid-maturing variety</td>
<td>720</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Niatong</td>
<td>Soft, late-maturing, lower protein variety</td>
<td>990</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)max: maximal levels of induction of fraction A2 equivalents expressed in units of β-galactosidase activity (Miller 1972) for comparison, DIF at 10^{-4} M gives 700 units, background of H2O gives 110 units.

\(^b\)Presence or absence of vanillin/iso vanillin determined by reverse-phase HPLC analysis and UV spectrum scanning as described in the text.

\(^c\)Estimate of the amount of vanillin/iso vanillin in wheat variety extracts, expressed as moles per 100 g of seeds.

**ACKNOWLEDGMENTS**

We wish to thank Bridget Roberts and Marie Oakes for skillful technical assistance, Michael Bailey for helpful discussions, and Jeremy Weinman for critical reading of the manuscript. We would also like to acknowledge the cooperation of John Moss from the CSIRO Bread Research Institute and Colin Wrigley from the CSIRO Wheat Research Unit.

K. K. Le Strange is supported by an Australian National University Postgraduate award. G. L. Bender was the recipient of a National Research Fellowship funded by the Australian Department of Employment, Education and Training.

Vol. 3, No. 4, 1990 219