Regulation of chalcone synthase
in Trifolium subterraneum

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

October 1994
This is my own original work, except for the analysis presented in Figure 3.8.
Abstract

The aim of this study was to characterize the expression patterns of the chalcone synthase (CHS) multigene family in *Trifolium subterraneum* with a view to understanding the molecular mechanisms by which this plant gene family is regulated. To provide a convenient model by which these molecular mechanisms might be assessed, CHS gene expression was analyzed in the very early stages of the symbiotic interaction between *Rhizobium leguminosarum* biovar *trifolii* and *T. subterraneum* roots. The *Rhizobium* inoculation was contrasted with wounding treatment, which is a treatment known to induce CHS as part of the plant’s induced defence response.

Induction of CHS was observed within 6 h following inoculation with the wild type *R. l.* bv. *trifolii* strain ANU843 and the *R. l.* bv. *trifolii* strain ANU845(pRI4003) which harbours a multicopy plasmid containing the strain ANU843 *nod* genes. No induction was observed following inoculation with the nodulation deficient *R. l.* bv. *trifolii* strain ANU845 (pSym−) or the different host range *R. meliloti* strain 1021 which nodulates *Medicago sativa* but not *T. subterraneum*. Inoculation with *R. l.* bv. *trifolii* strain ANU845(pRt151) which contains the *nodDABC* genes and causes root hair curling without forming infection threads did not induce CHS after 6 h. These results suggested the lipo-oligosaccharide signal molecule (LOS) produced by the *nod* gene products was the factor inducing CHS expression. This was examined by the addition of a crude preparation of *R. l.* bv. *trifolii* strain ANU845(pRI4003) LOSs to the plant growth medium in sufficient amounts to cause root hair curling. LOSs addition had no effect on CHS expression over 36 h. Wounding of the root tissue induced CHS within 2 h. An analysis of which CHS family member was expressed showed CHS5 is the major induced CHS gene following both inoculation with the wild type *R. l.* bv. *trifolii* strain ANU843 and the contrasting wounding treatment. Conserved sequences are identified within the CHS5 promoter and the *Glycine max Gmchs7* promoter, a promoter which has similar expression patterns. The other CHS gene family members CHS1, CHS2, CHS3 and CHS6 are shown to be expressed in root tissues in addition to the previously uncharacterized CHS9.

An analysis of the exudates from dark grown plants 3 days after wild type *R. l.* bv. *trifolii* strain ANU843 inoculation detected a new *nod* gene inducing flavonoid that was tentatively identified as 4′,7-dihydroxyflavone. This new compound was not detectable in the exudates of similar dark grown plants 3 days after wounding.
treatment or in plants 1 day after *R. l. bv. trifolii* strain ANU843 inoculation. An analysis of exudates from plants with some light in the growth conditions 5 days after wild type *R. l. bv. trifolii* strain ANU843 inoculation showed a new *nod* gene inducing compound that was different to that found in the 3 day dark grown plant exudates. It is not clear whether the new *nod* gene inducing activity is linked with the observed *CHS* expression, although evidence is discussed suggesting a link between the *CHS* expression and the exudation of new flavonoids.

The results presented in this thesis correlate a defined stimulus with the induction of a specific *CHS* gene family member and the synthesis of a new exuded flavonoid compound. These findings support the view that the induction of the phenylpropanoid pathway is involved in synthesizing flavonoid *nod* gene inducing compounds and in response to *Rhizobium* infection. The response to *Rhizobium* inoculation is discussed in the context of the plant’s defence response. The analysis of *CHS* gene expression described in this thesis provides the first steps towards the future identification of regulatory DNA binding proteins and the development of transgenic plants containing *CHS* promoters linked to reporter genes.
Acknowledgements

I wish to thank...

- my supervisor Michael Djordjevic for his enthusiasm, assistance, encouragement and continuing commitment to this project,
- Barry Rolfe for his useful suggestions, critical comments and passion for science,
- Jeremy Weinman,
- the members of the Plant Microbe Interactions Group,
- Tony Arioli and Paul Howles for their molecular biology assistance,
- Bill Parker and Charles Hocarth for assistance in piloting the HPLC,
- Neal Gowen for his timeless energy in making plans and theories happen,
- Tony Arioli, Margreet de Boer, Kevin Broderick, John Redmond, John Campbell, Ian McKay, Bill Creaser and John de Majnik for helpful discussions,
- Chris Pittock, John de Majnik, Kevin Broderick, Margreet de Boer and Louise Roddam for proof reading,
- James Popple for his time and assistance with \LaTeX,
- Shaun Humphreys for his assistance with Designer and PhotoWorkshop,
- Lynette Preston for her expertise running the 373A sequencer,
- Meat Research Corporation for providing the funding,
- A. W. Howard Memorial Trust for a travel scholarship,
- my family for their continuing support of yet one more folly,
- Ingrid McKenzie, Tim Chapman and Liz Milbourne for their friendship,
...and finally,
- Cp Pickering for partnership, support, encouragement and insight;
  \emph{waake akupendaye}. 
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<td>4CL</td>
<td>4-coumarate CoA ligase</td>
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<td>ABA</td>
<td>abscisic acid</td>
</tr>
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<td>ACP</td>
<td>acyl carrier protein</td>
</tr>
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<td>ADH</td>
<td>alcohol dehydrogenase</td>
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<tr>
<td>AS</td>
<td>anthocyanin synthase</td>
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<tr>
<td>b/HLH/Z</td>
<td>basic/helix-loop-helix/zipper</td>
</tr>
<tr>
<td>BMM</td>
<td>Bergersen's modified media</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cinnamic acid 4-hydroxylase</td>
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<td>copy DNA (DNA synthesized from RNA)</td>
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<td>CHI</td>
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<td>CHS</td>
<td>chalcone synthase</td>
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<td>CoA</td>
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<td>DHF</td>
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<td>double stranded DNA</td>
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<td>2'-deoxythymidine 5'-triphosphate</td>
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<td>EDTA</td>
<td>Na2-ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>exo-polysaccharide</td>
</tr>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>F3H</td>
<td>flavanone 3-β-hydroxylase</td>
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<td>FAS</td>
<td>fatty acid synthase</td>
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<td>F media</td>
<td>Fahraeus media</td>
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<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<td>HR</td>
<td>hypersensitive response</td>
</tr>
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<td>Description</td>
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<tr>
<td>HRGP</td>
<td>hydroxyproline-rich glycoprotein</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>LOS</td>
<td>lipo-oligosaccharide</td>
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<tr>
<td>LPS</td>
<td>lipo-polysaccharide</td>
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<tr>
<td>MOPS</td>
<td>4-morpholinepropane-sulphonic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>OD</td>
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<td>ONPG</td>
<td>O-nitrophenyl β-D-galactopyranoside</td>
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<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PR</td>
<td>pathogenesis related (of proteins)</td>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>SSPE</td>
<td>salt, sodium phosphate and EDTA solution</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>UF3GT</td>
<td>UDPglucose flavonoid 3-oxy-glucotransferase</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet (of light)</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoyl β-D-galactopyranoside</td>
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Chapter 1

General Introduction

1.1 Introduction

Plants are generally sedentary, remaining in one particular niche throughout their lives. During this time the plant may be subject to “attack” by other organisms or environmental stresses. Both of these stresses can be deleterious to the continued viability of the plant. As a result of these various stresses, plants have developed certain strategies of response which may be adaptive and/or protective. The response may be either preformed (physical or chemical) or induced by the triggering stimuli (physical/mechanical, chemical and environmental). Preformed responses include lignification, protective enzymes (such as chitinases and glucanases), protective compounds (polyphenols, waxes, flavonoids, etc.) and protective structures (thorns, etc.). The induced responses include the synthesis of specific molecules (such as flavonoids, phytoalexins, protease inhibitors, antimicrobial compounds (such as thionins) and enzymes (such as chitinases and glucanases), cell wall reinforcement and repair (by the deposition of callose, lignin, wall bound phenolics, the accumulation of hydroxy-rich glycoproteins (HRGPs) and glycine rich proteins) and the synthesis of a series of pathogenesis related proteins (PR proteins; reviewed Dixon 1986; Lamb et al. 1989; Scheel and Parker 1990).

The plant’s induced response to an invading pathogen depends on the particular infection strategy of the pathogen. The pathogen’s strategy may be purely physical/mechanical (necrotrophic) or a more subtle invasion (biotrophic). Invasion by biotrophs will not proceed if the pathogen does not have the ability to grow on (parasitism) or infect the particular plant (colonization; Gabriel 1986). If the pathogen is able to infect the plant, the response of the plant then follows the particular pathogen’s strategy determined by molecular signals from both the host plant and pathogen (reviewed Dixon and Lamb 1990). The response of the plant to the pathogens that infect may be an induction of the defence response at some time after infection followed by programmed host cell death of infected tissues (interaction incompatible/plant resistant, hypersensitive response (HR)),
or the infecting pathogen may be able to continue to infect and grow on the host plant without triggering the defence response (interaction compatible/plant susceptible, water soaking).

The genes coding for the enzymes of the phenylpropanoid pathway are among the genes activated as part of the plant’s induced defence response to invading pathogens in both physical/mechanical and the more subtle forms of an incompatible infection (reviewed Ebel 1986; Lawton and Lamb 1987; Hahlbrock and Scheel 1989; Scheel and Parker 1990; Stafford 1990). This pathway is a marker of both gene expression and the elaboration of a plant induced response to the invading pathogen (Dixon 1986). This thesis examines the regulation of the gene(s) encoding chalcone synthase (CHS; a key gene of the phenylpropanoid pathway) and the flavonoid compounds resulting from this expression. These parameters are examined in the well characterized interaction between the legume Trifolium subterraneum and its symbiotic partner, the soil bacteria Rhizobium leguminosarum biovar trifolii. The results are discussed in the terms of plant defence and the regulation of the T. subterraneum-R. l. bv. trifolii symbiosis.

The remaining sections in this Chapter contain a review of the phenylpropanoid pathway (§1.2) and its regulation (§1.3), and a detailed review of the available literature concerning CHS genes and CHS enzymes (§1.4). The detail illustrates the complexity of these biological systems and the significance of the task necessary to understand the mechanisms regulating both CHS genes and CHS enzymes. This is followed by an analysis of the role of CHS gene expression and phenylpropanoid pathway derived flavonoids (produced through the action of CHS) in plant functions, including a detailed summary of the interaction between legumes and Rhizobium (§1.5).

1.2 Phenylpropanoid pathway

“Flavonoids” is a general, collective term to describe a class of plant metabolites derived through glycolysis, shikimate (or aerogenate) and finally the phenylpropanoid pathways (Figure 1.1). In the following discussion the term “flavonoid” is confined to a subgrouping of phenylpropanoid compounds produced through the CHS branch pathway (Figure 1.1). The phenylpropanoid pathway involves the conversion of phenylalanine (or tyrosine) to coumaroyl CoA (the general pathway; Figure 1.3; §1.2.1) and then to a variety of related compounds (Figures 1.1, 1.3 and 1.4). Compounds produced through the CHS branch pathway have a basic structure of 2 aromatic rings (A and B rings) joined by a 3 carbon chain (C ring; Figure 1.2). These compounds include flavonoids in addition to aurones, phlobaphenes, chalcones, flavonoids, isoflavonoids, flavanones, flavanols, anthocyanins, tannins, etc. The connecting C ring chain is closed in all of these compounds except the chalcones and dihydrochalcones. The remaining compounds are divided into subclasses on the basis of oxidation and substitution by hydroxylation, methylation, glycosylation, prenylation, sulfation, acylation and
Carbohydrate metabolism

Shikimate pathway

Phenylpropanoid pathway

CHS branch

Figure 1.1: Diagram of the pathways leading to the phenylpropanoid pathway and the diversity of compounds produced through the phenylpropanoid pathway.
Figure 1.2; Generalized structure of flavonoid compounds

Figure 1.3; General phenylpropanoid pathway. Phenylalanine (and tyrosine) are converted to trans-cinnamic acid by PAL and then 4-coumarate by 4CL. This is then converted to coumaroyl CoA by 4CL. The coumaroyl CoA is the substrate for the CHS branch pathway. Modified from Dixon et al. (1983).
polymerization (reviewed Harborne 1988; Stafford 1990). These substitutions also generate the diversity of these compounds found in plants (Harborne 1988).

Flavonoid compounds are widely distributed in plants (Wollenweber and Dietz 1981), with at least 3500 reported compounds (Harborne 1980, 1988). These compounds have a variety of different functions within the plant (reviewed Dixon and Lamb 1990; Stafford 1990). These diverse functions include lignin biosynthesis for structural development and defence (Hahlbrock and Grisbach 1979), (iso)flavonoids and anthocyanins for pigmentation and UV-protection (Hahlbrock and Grisbach 1979), proanthocyanidins (or tannins) to reinforce plant tissues (Moore 1972), phytoalexins with anti-microbial and anti-fungal activities (coumarins, Beier and Oerti 1983; Scheel et al. 1986; (iso)flavonoids, Dixon et al. 1983), signalling molecules in the rhizosphere (reviewed Rolfe 1988), modulators of hormone transport and/or action (Binns et al. 1987; Jacobs and Rubery 1988; Brunn et al. 1992), as possible metabolic regulators of phenylpropanoid enzyme levels (Bolwell et al. 1989) and pollenator attractants (reviewed Malamy and Klessig 1992; Koes et al. 1994). The pathway products have found a variety of other uses unrelated to functions within the plant including food flavour additives (Horowitz 1964), medicines and therapeutic drugs (reviewed Cody et al. 1986, 1988) such as the inhibition of carcinogen activation (Cassady et al. 1988), anti-viral activities (Vrijsen et al. 1988), anti-inflammatory agents (Gabor 1972), anti-ulcer activity (Takai et al. 1972), limiting arthritis (Malhotra et al. 1976), anti-malarials (Nkunya 1992) and inhibitors of HIV infection (Li et al. 1993).

1.2.1 General pathway

The general pathway (or Group I enzymes) is common to all of the phenylpropanoid pathway products, except coumarin biosynthesis (Ebel et al. 1974) and salicylic acid biosynthesis (Ryals et al. 1992; Figures 1.1 and 1.3). Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) catalyses the first committed step in the biosynthesis of the phenylpropanoid compounds from the compound L-phenylalanine. PAL catalyses the deamination of L-phenylalanine to trans-cinnamic acid. PAL can also deaminate L-tyrosine to form 4-coumarate (Hanson and Havir 1981; Dixon et al. 1983). Cinnamic acid 4-hydroxylase (C4-H; EC 1.14.13.11) is the second enzyme of the general phenylpropanoid pathway and catalyses the conversion of trans-cinnamic acid to 4-coumarate (reviewed Dixon et al. 1983). The enzyme 4-coumarate CoA ligase (4-CL; EC 6.2.1.12) then converts the molecule 4-coumarate to its corresponding CoA ester, coumaroyl CoA (reviewed Dixon et al. 1983).

1.2.2 Coumarins

Furanocoumarins function as phytoalexins in some plant systems including Petroselium hortense (reviewed Hahlbrock and Scheel 1989) and act as anti-inducers
of nod genes in the legume-Rhizobium symbiosis (Djordjevic et al. 1987b). The furanocoumarin pathway is not as yet elucidated, with several postulated enzyme steps still to be verified. The 4-coumarate from the general pathway (Figure 1.3) acts as a substrate for subsequent ortho-hydroxylation and cis-trans isomerization followed by various methylase activities (reviewed Hahlbrock and Scheel 1989).

1.2.3 Lignins

Lignins function as physical support for the plant and as a physical barrier to pathogens during normal growth (reviewed Vance et al. 1980; Lewis and Yamamoto 1990). Lignins are polymeric compounds made up from the dehydrogenative polymerisation of phenyl propane monomers. The 4-coumarate from the general pathway (Figure 1.3) is converted to cinnamic acid which is then converted to a cinnamoyl alcohol before being variously polymerized to form lignin (reviewed Vance et al. 1980; Lewis and Yamamoto 1990).

1.2.4 Salicylic acid

Salicylic acid (SA) probably functions as a signalling molecule leading to the onset of systemic acquired resistance (Gaffney et al. 1993; reviewed Ryals et al. 1992) and as a thermogenesis agent in flowering (reviewed Malamy and Klessig 1992; Raskin 1992). SA is produced from trans-cinnamic acid, an intermediates of the general pathway (Figure 1.3). The metabolic pathway for SA (2-hydroxybenzoic acid) remains unclear. Two pathways have been proposed and there is evidence to support both proposals (reviewed Ryals et al. 1992). In both cases the precursor trans-cinnamic acid is derived from phenylalanine through the action of PAL. The trans-cinnamic acid is then converted to either 2-hydroxy cinnamic acid by cinnamate 2-hydroxylase and to SA through the action of β-oxidation and an acetyl CoA intermediate (Klambt 1962), or the trans-cinnamic acid is oxidized to benzoic acid and then hydroxylated to SA by a postulated α-hydroxylase (El-Basyouni et al. 1964; Yalpani et al. 1993). SA produced through both proposed pathways has also been reported in the same plant (Ellis and Amrhein 1971).

1.2.5 CHS branch pathway

The compounds produced through the CHS branch pathway (Figures 1.1 and 1.4) have a variety of functions (reviewed Koes et al. 1994; discussed further below; §1.5). The enzyme chalcone synthase (CHS; EC 2.3.1.74) catalyses the first committed step of this branch (Figure 1.4), condensing 3 molecules of malonyl CoA (derived from carboxylation of acetyl CoA) with a single molecule of 4-coumaroyl CoA to produce naringenin chalcone (Figure 1.4; discussed §1.4.3 below). Chalcone isomerase (CHI; EC 5.5.1.6) catalyses the conversion of naringenin chalcone to narigenin flavone (and the reverse reaction to some degree). This is a
Figure 1.4: Flavonoid biosynthetic pathway (modified from Koes et al. 1994). CHS - chalcone synthase, CHI - chalcone isomerase, DFR - dihydroflavanol 4-reductase, IFS - isoflavonoid synthase, F3H - flavanone hydroxylase, FLS - flavanol synthase, AS - anthocyanin synthase, UF3GT - UDP-glucose:flavonoid 3-O-glycosyltransferase.
Figure 1.5; 6'-deoxy- and 6'-hydroxychalcone activity of CHS and co-acting enzyme CHR with the co-factor NADPH.
stereospecific isomerization of the chalcone to its corresponding flavanone. The reaction occurs spontaneously, but is accelerated by the CHI enzyme. The narigenin flavone is then converted into (iso)flavonoids, phlobaphenes, aurones, anthocyanins or tannins etc. through substitution, isomerization, condensation, reduction, glycosylation and polymerization (reviewed Stafford 1990).

1.3 Regulation of the phenylpropanoid pathway

The regulation of the phenylpropanoid pathway from the expression of the gene to the functioning enzyme is a complex process and occurs at a variety of levels. Each of these levels is itself subject to regulation, and together they enable toxic phenylpropanoid compounds to be delivered in an active form to the appropriate location and at the appropriate time. The primary level of regulation of the phenylpropanoid pathway is transcription (reviewed Lawton and Lamb 1987). DNA binding proteins interact with defined sequences within the structural genes increasing or decreasing the rate(s) of RNA polymerase activity. Following transcription, there is probably regulation of translation and compartmentalization of the active proteins, their substrates and end products.

1.3.1 Transcription regulation

The phenylpropanoid pathway has been shown to respond to a variety of interactions and developmental cues by increasing the rate at which the phenylpropanoid genes are transcribed (Bell et al. 1984; Schmelzer et al. 1984; Ryder et al. 1984; Cramer et al. 1985; Lawton and Lamb 1987). Induction of gene expression following these interactions and cues requires regulation of transcription. This occurs at various levels from access of regulatory proteins to DNA (opening of the chromosome) to the presence of regulatory proteins to transcribe both regulatory genes and structural genes. For example, developmental cues are known to induce the transcription of myb like and myc like genes which code for proteins known to regulate the expression of phenylpropanoid pathway structural genes. The regulation of expression of structural genes by the products of regulatory genes and the regulation of expression of the regulatory genes generates considerable complexity into the mechanisms controlling gene expression. The following discussion illustrates the levels of complexity by examining some of the available information on transcriptional regulation of the phenylpropanoid pathway.

Structural genes

Structural genes are defined as that part of genomic DNA at a locus containing the sequence coding for the protein and any sequences both 5' and 3' linked to that sequence at that locus that is not an open reading frame and that influences
the expression of the former sequence and any sequences within the open reading frame that are excised prior to translation (introns). The introns and 5' and 3' regions can contain sequences (cis elements) that affect the transcription of the coding sequence. These sequences provide an avenue for the regulation of gene expression.

The tissue and developmental specific expression of genes is considered to be achieved through interactions between (multiple) enhancers and (multiple) promoter elements, and possibly with intermediary factors (Johnson and McKnight 1989). cis elements are composed of modules between 7–20 bp in length (Dynan 1989) which act as recognition/binding sequences for regulatory trans factors (reviewed Schibler and Sierra 1987). These modules are usually located adjacent to the transcription start site, but may be found at some distance. Complex patterns of interactions between cis elements and trans factors then determine the specific patterns of transcription by RNA polymerase (reviewed Tijan and Maniatis 1994).

In many plants, multiple copies of phenylpropanoid structural genes exist. For example T. subterraneum has at least 4 PAL and 9 CHS genes (Arioli 1992; Howles 1992; Arioli et al. 1994; Howles et al. 1994). These multiple copies arise by gene duplication and the redundancy in homology and overlapping function enables considerable diversity in regulation and function to be achieved by the plant (reviewed Campbell 1983; Schibler and Sierra 1987). Different structural genes coding for the same function may be differentially regulated by the presence of different sequences within the untranslated regions which are recognized by specific DNA binding proteins. Sequences such as the RNA polymerase binding site, the TATA box, have been well characterized in a variety of different organisms (reviewed Tijan and Maniatis 1994). Sequences within the 5' region of phenylpropanoid genes have been shown to regulate the expression of these genes, although this work has been conducted in artificial cultures (such as tissue and suspension cultures) and may not reflect the true function of these sequences within the whole plant (Dangl 1992). Some of the cis elements found in phenylpropanoid genes are set out in Table 1.1. The presence of similar elements within the promoters of different phenylpropanoid pathway genes may then enable coordinated induction of the pathway genes (discussed below §1.3.3).

**Regulatory genes**

Regulatory genes are defined as structural genes at a locus containing the sequence coding for a protein that influences the expression of a structural gene(s) at a different locus. Genes regulating transcription of the phenylpropanoid pathway structural genes have been identified in *Zea mays*, *Petunia hybrida*, *Arabidopsis thaliana* and *Antirrhinum majus* (reviewed Dooner et al. 1991; Koes et al. 1994). These genes have been identified most often as anthocyanin biosynthesis mutants because of the ease of scoring the phenotype (regulatory gene phenotypes; Table 1.2) and they appear to be functionally homologous across species (Goodrich et
§1.3 Regulation of the phenylpropanoid pathway

Table 1.1: Some of the regulatory elements in phenylpropanoid structural genes.

<table>
<thead>
<tr>
<th>element</th>
<th>promotor sequence</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-E2 enhancer</td>
<td>CAGGTG</td>
<td>Sen and Baltimore (1986)</td>
</tr>
<tr>
<td>G-box</td>
<td>CACGTG</td>
<td>Gilmartin et al. (1990)</td>
</tr>
<tr>
<td>ABA responsive H-box</td>
<td>CCTACC(N)-CT</td>
<td>reviewed Cushman et al. (1993)</td>
</tr>
<tr>
<td>P-box</td>
<td>CTAACT</td>
<td>Loake et al. (1991)</td>
</tr>
<tr>
<td>Myb binding P-box</td>
<td>$\frac{1}{2}$AAC$\frac{2}{3}$G</td>
<td>Yu et al. (1993)</td>
</tr>
<tr>
<td>Myc binding</td>
<td>CACGTG</td>
<td>Biedenkapp et al. (1988)</td>
</tr>
<tr>
<td>box I consensus</td>
<td>$#CTA^CACCCTAAC\frac{A}{C}A$</td>
<td>Prendergast and Ziff (1991)</td>
</tr>
<tr>
<td>box II consensus</td>
<td>$CCAC\frac{A}{C}AC\frac{4}{7}AAC\frac{2}{7}CC$</td>
<td>Lois et al. (1989)</td>
</tr>
<tr>
<td>sugar box 2</td>
<td>-</td>
<td>Tsukaya et al. (1991)</td>
</tr>
<tr>
<td>sugar box 3</td>
<td>-</td>
<td>Tsukaya et al. (1991)</td>
</tr>
<tr>
<td>GT-1 box</td>
<td>GGTAAA</td>
<td>Gilmartin et al. (1990)</td>
</tr>
<tr>
<td>SBF-1</td>
<td>GGTAAA</td>
<td>Arias et al. (1993)</td>
</tr>
<tr>
<td>AT-1 box</td>
<td>AATATTTTTATT</td>
<td>Gilmartin et al. (1990)</td>
</tr>
<tr>
<td>CAAT box</td>
<td>CCAAT</td>
<td>McKnight and Tjian (1986)</td>
</tr>
</tbody>
</table>

al. 1992). The action of regulatory genes (such as myb and myc like genes) and the regulation of regulatory genes provides considerable potential (and complexity) for transcriptional regulation. This is illustrated by the following description of some of the mechanisms of the myb and myc like regulatory genes which effect at least some of the transcriptional regulation of the phenylpropanoid pathway genes. The diversity and complexity of regulation is shown by the pigmentation phenotypes of the regulatory genes listed in Table 1.2.

The phenylpropanoid pathway is regulated in Z. mays by the C1 (myb like) and R (myc like) gene families (Dooner et al. 1991), in A. majus by the delila and eluta genes, in P. hybrida by the an1, an2, an4 and an11 genes and in A. thaliana by the ttg gene (reviewed Dooner et al. 1991; Koes et al. 1994). These regulatory proteins in the different species are similar (Koes et al. 1994). However, patterns of regulation across the species are different (Koes et al. 1994). For example, in Z. mays the R and C1 genes regulate CHS, CHI, DFR and UF3GT (Dooner et al. 1991) while in A. majus the regulation by similar genes is of the DFR and UF3GT steps only (Quattrocchio et al. 1993; Martin and Gerats 1993). The gene products from different regulatory genes probably interact to effect regulation (Roth et al. 1991; Goff et al. 1992).

Transgenic plants have provided some insight into the actions of the myb like and myc like regulatory gene products on the regulation of the phenylpropanoid pathway genes. For example, transfer of the monocotyledon Z. mays R and C1 genes into the dicotyledenous A. thaliana and Nicotiana tabacum showed that the
General Introduction

§1.3

The gene augmented anthocyanin biosynthesis in both plant species and trichome production in *A. thaliana* (Lloyd et al. 1992). The gene *Ct* by itself had no visible effects (Lloyd et al. 1992). However, hybrid transgenic *A. thaliana* with both the *R* and *Ct* genes produced anthocyanins in roots, petals and stamens which do no normally produce anthocyanins (Lloyd et al. 1992).

1 - myc like genes

The *Z. mays* *R* gene family has been shown to regulate the expression of the phenylpropanoid pathway genes (reviewed Dooner et al. 1991; van der Meer et al. 1993). The functionally duplicate *R* and *B* loci genes (Styles et al. 1973; Gerats et al. 1984) belong to a class of helix-loop-helix transcription factors (reviewed Ludwig and Wessler 1990) which are characteristics of the *Myc* transcriptional factors (Ludwig et al. 1989; reviewed Marcu et al. 1992). *Myc* proteins contain a conserved basic region/helix-loop-helix motif/leucine zipper (b/HLH/Z), an acidic domain and a nuclear localization domain (reviewed Marcu et al. 1992). The helix-loop-helix motif enables complexes with other proteins to be formed (Davis et al. 1990) and the basic region probably interacts with the DNA (Davis et al. 1990). The acidic region may be involved in transcriptional regulation (van der Meer et al. 1993).

In *Z. mays*, this class of transcription factors is made up of the *R* locus (*P* and *S* genes, and the mutable *R* allele *R*-nj), the linked *Lc* and *Sn* genes and the unlinked *B* genes (*B*-1 and *B*-Peru). The *R* and *B* loci probably arose through gene duplication and the *R* locus subsequently diverged through recombinational events (Robbins et al. 1989; van der Meer et al. 1993). These genes confer developmental and tissue specific expression on the phenylpropanoid structural genes (Gavazzi et al. 1986) and have high sequence homology with variation in the 5' untranslated region (Ludwig et al. 1989; Perrot and Cone 1989; Tonelli et al. 1991). The sequence homologies between different *R* family members suggest that the tissue specific expression results from differences in the proteins or differences in the expression patterns of particular members (Ludwig et al. 1989; Ludwig and Wessler 1990; Goff et al. 1990). Differential expression of the *B* genes has been shown to result from differences in the 5' sequences and the accumulation of anthocyanins has been shown to result from the level of gene expression (Radicella et al. 1992). The *ttg* gene in *A. thaliana* (Lloyd et al. 1992) and the *delila* gene in *A. majus* (Goodrich et al. 1992) are probably homologues of the *Z. mays* *R* gene (Lloyd et al. 1992).

Members of the *R* gene family interact with the Myc like consensus sequence motif 5'-CAGGTG-3' (Roth et al. 1991; Goff et al. 1992) which has direct homology to the K-E2 enhancer motif (Sen and Baltimore 1986). In the avian myelocytomatosis virus the Myc protein has been shown to bind to a similar 6 bp 2-fold symmetric DNA site 5'-C<sub>-3</sub>A<sub>-2</sub>C<sub>-1</sub>G<sub>1</sub>T<sub>+2</sub>G<sub>+3</sub>-3' (Papoulas et al. 1992) with the His<sub>836</sub> contacting (or close to) the thymine 5-methyl group at 2-fold symmetry related positions -2 and +2 (Dong et al. 1994). The crystallographic
§1.3 Regulation of the phenylpropanoid pathway

structure has not been determined, although this structure and function has been determined for the homologous DNA binding protein Max (b/HLH/Z; Ferre-D’Amare et al. 1993,1994). Mutagenesis of the binding site 5’-CAGGTG-3’ in a reporter construct containing the Bz1 gene promoter in combination with a constitutive B-Peru and biolistically introduced into Z. mays embryonic callus reduced activity 29.3 fold confirming the involvement of this site in the regulation of anthocyanin biosynthesis (Goff et al. 1990). The Myc binding consensus 5’-CACGTG-3’ has been shown to occur in many of the phenylpropanoid pathway genes (reviewed Dangl 1992) but the differences between this sequence and the R gene binding site remains to be determined.

2 - myb like genes

The Z. mays C1 gene family has also been shown to regulate expression of the phenylpropanoid pathway genes (reviewed Dooner et al. 1991; van der Meer et al. 1993). The C1 (or Pl, a homolog of C1; Cone et al. 1993ab) gene is similar to the Myb proto-oncogenic transcription factors (Paz-Ares et al. 1986; reviewed Luscher and Eismann 1990; Shen-Ong 1990). The myb related genes have been reported in Z. mays (Grotewold et al. 1991), Hordeum vulgare (Marocco et al. 1989), A. majus (Jackson et al. 1991) P. hybrida (Avila et al. 1993; van der Meer et al. 1993) and A. thaliana (Oppenheimer et al. 1991; Shinozaki et al. 1992). The myb gene products are nuclear proteins which act in response to external signals (Herrlich and Ponta 1989) and have been shown to be expressed in a variety of tissues (Marocco et al. 1989; Wissenbach et al. 1993). Expression of different gene copies determining tissue and stimulus (for example light) specific expression most probably results from differential regulation of the gene(s) rather than different activities of the functioning proteins (Cone et al. 1993b). Tight regulation may be achieved through specific transcription and degradation of the mRNA (Laird-Offringa 1992). In animals the highly conserved amino-terminal region of the Myb protein (Peters et al. 1987; Klempnauer and Sippel 1987) binds to the conserved nucleotide sequence 5’-T/CAC/G-3’ (Biedenkapp et al. 1988). However in plants the Myb homolog encoded by the P gene binds to 5’-CC/GACC-3’ (Grotewold et al. 1994). The tryptophans in the terminal region are speculated to be involved in stacking and/or charge transfer interactions (Anton and Frampton 1988). This protein also contains a potential α-helical leucine zipper motif (Biedenkapp et al. 1988) which is presumed to mediate protein-protein interactions (Landschulz et al. 1988). Further, there is no significant homology with consensus sequences for helix-turn-helix or zinc-finger DNA binding proteins (Biedenkapp et al. 1988).

The Z. mays C1 gene regulates the expression of the anthocyanin biosynthesis genes C2 (CHS; Dooner 1983), A1 (DFR; Reddy et al. 1987) and Bz1 (UF3GT; Roth et al. 1991). The C1 gene has been cloned (Cone et al. 1986; Paz-Ares et al. 1986) and shown to encode a protein with two major domains, a basic N-terminus and an acidic carboxyl terminus (Paz-Ares et al. 1986). The basic
domain has 40% homology to the myb-proto-oncogenes (Paz-Ares et al. 1986). It is presumed the Cl protein is a transcriptional activator with the acidic domain effecting transcriptional regulation. This is supported by the finding that the Cl-1 allele, which lacks the acidic region as a result of a frame shift insertion, has a dominant inhibitory effect on anthocyanin biosynthesis (Paz-Ares et al. 1990), and that imperfect excision revertants of transposon induced mutants of Cl regain some pigmentation (Wienand et al. 1990). Furthermore, bombardment of the coding region of a Cl gene linked to a constitutive promotor into Cl recessive callus tissue restored anthocyanin biosynthesis (although a B gene was also required; Goff et al. 1990; Grotewold et al. 1994), and in vitro synthesized Cl protein has been shown to bind to the anthocyanin biosynthesis gene A1 (Wienand et al. 1990). However, the Cl homolog P gene product has been shown to bind and activate transcription of the A1 gene (Grotewold et al. 1994). Interestingly, the P gene product required an R or B gene product and did not activate transcription of the Bz1 gene like the Cl gene. This finding suggested that Myb homologs could differentially regulate gene expression and co-act with other common factors (Grotewold et al. 1994).

Other regulatory genes

Some of the genes which control anthocyanin biosynthesis genes also regulate other plant processes. For example, in Z. mays the Vpl gene (viviparous) which has reduced sensitivity to ABA and is involved in seed dormancy (Hattori et al. 1992) regulates Cl gene expression leading to anthocyanin accumulation in the aleurone and embryo tissues (McCarty et al. 1989). The vpl mutants have multiple enzyme deficiencies in several different pathways (Dooner 1985) indicating that the Vpl gene product has a variety of functions in addition to regulating Cl in the aleurone (McCarthy et al. 1989).

The Clf gene which is involved in crinkled leaves also has a regulatory effect on anthocyanin biosynthesis, as clf plants have reduced levels of CHS and UF3GT enzyme activities (Dooner 1983).

1.3.2 Compartmentalization

The complexity of the interaction between the shikimate pathway, the TCA cycle the general phenylpropanoid pathway and the CHS branch pathway, and the large range of pathway intermediates and products necessitates an organised metabolic process that does not permit the mixing of the intermediate substrate pools or interference of the end products with cellular activities (Hrazdina 1992). Regulation of the various component parts of the pathways through transcription accounts for some of the control (discussed above §1.3.1). However, regulation may also be achieved through compartmentation of the substrates, enzymes, reactions and the end products. This compartmentation can occur at the level
§1.3 Regulation of the phenylpropanoid pathway

Table 1.2: Phenylpropanoid regulation genes. Listing modified from Styles et al. (1973) and van der Meer et al. (1993).

<table>
<thead>
<tr>
<th>gene</th>
<th>location of pigmentation</th>
<th>reference for cloned gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>scutellum, aleurone of kernal</td>
<td>Cone et al. (1986)</td>
</tr>
<tr>
<td>C1-1</td>
<td>dominant allele of C1</td>
<td>Paz-Ares et al. (1986)</td>
</tr>
<tr>
<td>Pl</td>
<td>organs other than seed</td>
<td>Paz-Ares et al. (1990)</td>
</tr>
<tr>
<td>R-P</td>
<td>plant (anthers, coleoptile, sheath)</td>
<td>Cone et al. (1993a,b)</td>
</tr>
<tr>
<td>R-S</td>
<td>seed (aleurone)</td>
<td>Perrot and Cone (1989)</td>
</tr>
<tr>
<td>R-nj</td>
<td>aleurone, anthers, roots, coleoptile</td>
<td>Dellaporta et al. (1988)</td>
</tr>
<tr>
<td>Lc</td>
<td>leaf midrib, ligule, auricle, glume, lemma, palea, pericarp</td>
<td>Ludwig et al. (1989)</td>
</tr>
<tr>
<td>Sn</td>
<td>scutellar node, mesocotyl, leaf base, midrib, glume, pericarp</td>
<td>Consonni et al. (1992)</td>
</tr>
<tr>
<td>B-1</td>
<td>coleoptile, blade, sheath, seedling, auricle, husk, culm, tassel branch</td>
<td>Chandler et al. (1989)</td>
</tr>
<tr>
<td>B-Peru</td>
<td>aleurone, scutellum, coleoptile, culm blade sheath, seedling auricle, husk tassel branch</td>
<td>Chandler et al. (1989)</td>
</tr>
<tr>
<td>Vp1</td>
<td>aleurone, embryo</td>
<td>McCarthy et al. (1989)</td>
</tr>
<tr>
<td>Clf</td>
<td>aleurone</td>
<td>-</td>
</tr>
</tbody>
</table>

The general phenylpropanoid pathway enzymes appear to be located in the “cytoplasm” (Wagner and Hrazdina 1984) rather than the “microsomal fraction”, despite some conflicting evidence (McClure 1979). Using a pea mutant that contains large air spaces between the epidermal and mesophyll layers (allowing the epidermal layers to be cleanly peeled away from the mesophyll layers including the vascular bundles; Marx 1978; Hoch et al. 1980) it was found that PAL and 4CL were in both tissue fractions while CHS was located in the epidermis and sub-epidermis (Hrazdina et al. 1982; Kojima et al. 1979; Cosio and McClure 1984). Similar CHS localization has been shown in P. crispum seedlings (Jahnen and Hahlbrock 1988a,b; Schmelzer et al. 1988), Spinacia oleracea (Beerhues et al. 1988) and Glycine max (Cosio et al. 1985). However, in addition to the epidermis and sub-epidermis CHS has been reported in the mesophyll in Secale cereale (Jahne et al. 1993), Z. mays (Jahne et al. 1993) and Avena sativa (Knogge and Weissenbock 1986). CHS has also been reported in anthers (Sutfeld et al. 1978; Kehrel and Wiermann 1985), stigmas (Pollak et al. 1993), guard cells (Beerhues et al. 1988), chloroplasts (Beerhues et al. 1988) and vacuoles (Hrazdina et al. 1978; Hopp et al. 1985).

The best current evidence suggests that the CHS branch pathway end prod-

There is some evidence to suggest phenylpropanoid pathway substrates are consecutively assembled (or channelled) by enzyme complexes (reviewed Hrazdina 1992). This may account for some of the regulation of the pathway metabolites and ensure the end products are suitably compartmentalized. Similar enzyme complexes have been postulated for glycolysis (Moorehead and Plaxton 1988) and the Calvin cycle (Gontero et al. 1988) and the synthesis of the cyanogenic glycoside dhurrin (Conn 1981). Microcompartmentation of CHS as part of a multienzyme complex was first presented by Stafford (1981). This was modified to incorporate the membrane-bound evidence (Hrazdina and Wagner 1984; Ibrahim et al. 1987). Stafford (1990) has proposed a model that has gained some support (Hrazdina 1992; Ibrahim 1992) and compares favourably with the earlier model proposed by Hrazdina and Wagner (1985), although there are some differences (for example, the membrane side on which PAL is found). These models place CHS on the cytoplasmic surface of the ER (Wagner and Hrazdina 1984; Hrazdina et al. 1987; Ibrahim 1992) in either a linear array of enzymes in the membrane or aggregate(s) of pathway enzymes attached to the membrane. However, these models do not account for the variety and complexity of the end products, nor the external agents inducing the pathway(s) or endogenous factors controlling constitutive enzymes (Stafford 1990). They are clearly working hypotheses.

The means by which the flavonoids are transported from their site of synthesis to their site of accumulation remains unresolved (Hrazdina 1992). By analogy to other systems it has been proposed that the transport process occurs through specific vesicle structures (Hrazdina 1992; Parham and Kaustinen 1977; Hrazdina et al. 1987). These vesicles secrete their contents by exocytosis to external surfaces (Charest et al. 1986), the vacuole (Stafford et al. 1987) or form specialised cells (Zobel 1986). There is also some evidence to suggest flavonoids are transported from their tissue of synthesis to distant tissues (Beerhues et al. 1989; van Tunen et al. 1990; Vogt et al. 1994).

1.3.3 Coordinate regulation

There is evidence that the induction of the phenylpropanoid pathway is coordinated with the shikimate pathway. The pools of phenylalanine and tyrosine are
insufficient to account for the accumulation rates of phenylpropanoid compounds (measured in certain tissues at 22 µmol carbon g⁻¹ fresh weight h⁻¹, Cressy 1968; Cressy and Zucker 1974; Margna 1977) unless coordination of the phenylpropanoid and shikimate pathways are presumed (reviewed Hanson and Havir 1981; Jensen and Fischer 1987; Stafford 1990). This coordination also occurs at the level of gene expression. Increases in DAHP mRNA, the first enzyme of the shikimate pathway, and PAL mRNA was shown following mechanical injury (physical wounding) of Solanum tuberosum (Dyer et al. 1989). This suggests that precursors for the phenylpropanoid pathway are derived from sugars or phosphoenolpyruvate pools rather than phenylalanine or tyrosine (Hrazdina 1992) and that the genes of the pathway enzymes are coordinately regulated (Dyer et al. 1989).

There is considerable evidence to suggest that there is coordinate regulation of the different enzymes within the phenylpropanoid pathway (reviewed Dixon et al. 1983; Hahlbrock and Scheel 1989; Stafford 1990). UV irradiation of of P. hortense and G. max cultures resulted in increases in the levels of PAL and 4CL enzyme levels (Ebel et al. 1974; Hahlbrock et al. 1976). Similar coordination has also been demonstrated at the level of gene expression (van Tunen et al. 1988; Kubasek et al. 1992). Further, phenylpropanoid pathway intermediates have been shown to affect gene expression. Loake et al. (1992) found p-coumarate stimulated the P. vulgaris CHS promotor in M. sativa protoplasts and concluded there was internal pathway regulation through feed forward stimulation of gene expression by pathway intermediates.

### 1.3.4 Co-suppression

There appears to be an additional level of coordinate regulation determined by the genetic component of the plant in response to introduced gene copies in transgenic plants (co-suppression; reviewed Jorgensen 1990). Van der Krol et al. (1990a) evaluated the effect of increased gene expression on flower pigmentation by transforming P. hybrida plants with additional CHS and DFR gene copies. In up to 25% of transformants additional gene copies reduced floral pigmentation regardless of the promotor and the gene construct (whether chimeric or intact genomic). Napoli et al. (1990) introduced a chimeric CHS gene into P. hybrida and found the introduced gene blocked anthocyanin biosynthesis and that the level of CHS mRNA was reduced in a coordinated way (in trans) so that the introduced gene was not responsible for the reduction in CHS levels. Interestingly, the introduction of antisense CHS gene(s) inhibits flower pigmentation (van der Krol et al. 1988ab, 1989ab; Stevenson 1991), although this does not appear to affect the levels of expression of other phenylpropanoid pathway genes such as CHI and DFR (van der Krol et al. 1990a; Stevenson 1991).
1.4 Chalcone synthase

1.4.1 Historical survey

Birch and Donovan (1953) hypothesized the presence of an enzyme with the characteristics of CHS. Kreuzaler and Hahlbrock (1972) reported for the first time the formation of 5, 7, 4'-trihydroxyflavonone (naringenin chalcone) from p-coumaroyl CoA and malonyl CoA in an illuminated parsley cell suspension culture. The formation of this compound was postulated to occur through the formation of the A ring from the malonate and the B ring from the phenyl ring of p-coumarate in a 2 enzyme reaction. These enzymes were designated “chalcone synthase” which condensed the malonate molecules, and “chalcone-flavone isomerase” which isomerizes the chalcone to a flavonone (Figure 1.1). An intermediate chalcone was observed but it was not clear whether this was an artifact of the enzyme purification steps. Subsequently purification and partial characterization of the “flavanone synthase” enzyme was achieved (Kreuzaler and Hahlbrock 1975a; Hrazdina et al. 1976; Kreuzaler et al. 1978, 1979).

Kreuzaler et al. (1983) isolated cloned cDNAs from dark-grown UV irradiated cell suspension cultures of *P. crispum* by differential hybridization, hybrid-selection and hybrid-arrested translation. CHS clones were identified from fungal elicitor induced *Phaseolus vulgaris* cells using the *P. crispum* clone (Ryder et al. 1984). Transposon tagging was used to identify the CHS gene (at the C2 locus) in *Z. mays* (Wienand et al. 1986). The CHS gene(s) have been identified in a range of other plants and in most cases these clones have been identified by homology to the *P. vulgaris* and *P. crispum* CHS genes (Table 1.3). CHS genes have also been identified in *Pinus sylvestris* from cross-hybridization to a *Arachis hypogaea* stilbene synthase (Fliegmann et al. 1992).

1.4.2 CHS evolution

CHS is proposed to have arisen with the class of *Bryophyte* (Musci) approximately 500 million years ago, preceding ferns, gymnosperms and angiosperms (Stafford 1990; Koes et al. 1994). There exists a strong similarity between the enzymes CHS and β-ketoacyl-ACP synthase type II fatty acid synthase (FAS) which has led to the suggestion that CHS may have arisen by gene duplication from this FAS (Kreuzaler et al. 1979; Stafford 1990). CHS is not considered to be similar to the fatty acid synthase type I because it lacks a pantethenyl residue (Kreuzaler et al. 1979; Stafford 1990).

Using cDNA probes specific for the CHS gene, sequences have been isolated from many plants and then compared in order to determine their evolutionary relationships. Niesbach-Klosgen et al (1987) compared various diverse CHS sequences and found greater than 66% homology at the nucleotide level and 80% homology at the amino acid level between transcripts. A comparison between the plant types on the basis of nucleotide comparison and splitting the genes
Table 1.3: Cloned CHS genes.

<table>
<thead>
<tr>
<th>plant</th>
<th>locus</th>
<th>reference</th>
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<tbody>
<tr>
<td>Antirrhinum majus</td>
<td>nivea</td>
<td>Spirille and Forkmann (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sommer and Saedler (1986)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td></td>
<td>Feinbaum and Ausubel (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wingender et al. (1989)</td>
</tr>
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<td>Hordeum vulgare</td>
<td></td>
<td>Niesbach-Klosgen et al. (1987)</td>
</tr>
<tr>
<td>Magnolia liliiflora</td>
<td></td>
<td>Niesbach-Klosgen et al. (1987)</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td></td>
<td>Junghans et al. (1993)</td>
</tr>
<tr>
<td>Matthiola incana</td>
<td>F</td>
<td>McKhann and Hirsch (1994)</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td></td>
<td>Ryder et al. (1984)</td>
</tr>
<tr>
<td>Petroselium crispum</td>
<td></td>
<td>Hermann et al. (1988)</td>
</tr>
<tr>
<td>Petroselium hortense</td>
<td></td>
<td>Kreuzaler et al. (1983)</td>
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<td></td>
<td></td>
<td>Reimold et al. (1983)</td>
</tr>
<tr>
<td>Petunia hybrida</td>
<td></td>
<td>Reif et al. (1985)</td>
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<td>Koes et al. (1987,1989a)</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td></td>
<td>Fliegmann et al. (1992)</td>
</tr>
<tr>
<td>Pisum sativum</td>
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<td>An et al. (1993)</td>
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<td></td>
<td></td>
<td>Harker et al. (1990)</td>
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<tr>
<td>Ranunculus acer</td>
<td></td>
<td>Niesbach-Klosgen et al. (1987)</td>
</tr>
<tr>
<td>Sinapis alba</td>
<td></td>
<td>Batschauer et al. (1991)</td>
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<td></td>
<td></td>
<td>Ehmann and Schafer (1988)</td>
</tr>
<tr>
<td>Trifolium subterraneum</td>
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<td>Arioli (1992)</td>
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<tr>
<td></td>
<td></td>
<td>Howles (1992)</td>
</tr>
<tr>
<td>Vitis vinifera</td>
<td></td>
<td>Sparvoli et al. (1994)</td>
</tr>
<tr>
<td>Zea maize</td>
<td>C2</td>
<td>Wienand et al. (1986)</td>
</tr>
</tbody>
</table>
into exons and introns suggests the *P. crispum CHS* may have evolved differently from other known *CHS* genes. However, the conserved position of the intron in both monocotyledon and dicotyledon transcripts suggests this intron was already present when the division between monocotyledons and dicotyledons occurred.

Koes et al. (1986) analysed the origins of *CHS* genes by examining the cultivars from which the hybrid line *P. hybrida* (V30) was derived. Using Southern blot analysis and specific probes they were able to show the *CHS* genes of the hybrid lines were not typically derived from a particular cultivar and that the copy number was variable.

### 1.4.3 CHS enzyme activity

A chalcone intermediate in the phenylpropanoid pathway was shown to exist following radiolabelling experiments in the hypocotyls of *G. max* infected with *Phytophthora megasperma* using radioactively labelled 2',4,4'-trihydroxychalcone (Keen et al. 1972). CHS enzyme activity was later found in UV irradiated *P. hortense* cell suspension cultures (Kreuzaler and Hahlbrock 1975a). The *CHS* gene is the most highly expressed phenylpropanoid pathway gene and can reach levels of 2% of the total rate of protein synthesis (Schröder et al. 1979).

The CHS enzyme (EC 2.3.1.74) is a dimer of approximate 82 kDa requiring no cofactors (reviewed Stafford 1990). The enzyme catalyses a condensation reaction sequentially adding 3 molecules of malonyl CoA to a single molecule of 4-coumaroyl CoA to form an open chain tri-β-ketoacyl thioester which cyclizes to form the aromatic A ring (Hahlbrock 1981; Heller and Hahlbrock 1980; Kreuzaler and Hahlbrock 1972, 1975a). This reaction catalysed by CHS has been shown to also produce “release” or “derailment” products. Partially purified CHS preparations from *P. hortense* in the presence of high concentrations of 2-mercaptoethanol and dithioerythritol produce 5,7,4'-trihydroxyflavonone (naringenin, 3 malonyl molecules), 4-OH-6-[4-OH-styryl]2-pyrene (bis-noryangonin, 2 malonyl molecules) and 4-OH-5,6-dihydro-6-(4-hydroxyphenyl)2-pyrene (p-hydroxy-benzalacetone, 1 malonyl molecule; Kreuzaler and Hahlbrock 1975b; Hrazdinà et al. 1976). These compounds are accounted for by the sequential addition of the malonyl CoA units. In some plants 4-coumaroyl CoA is used exclusively as a substrate (for example; *M. incana*, Spribille and Forkmann 1981; *A. majus*, Spribille and Forkmann 1981; *P. vulgaris*, Whitehead and Dixon 1983) while caffeoyl CoA and feruloyl CoA can act as substrates in other plants (reviewed Heller and Forkmann 1988). The CHS enzyme is made up of 2 identical subunits each of approximately 42 kDa and is believed to have at least 2 active SH groups on the enzyme (Kreuzaler and Hahlbrock 1975a; Kreuzaler et al. 1979). Site directed mutagenesis studies have identified a strictly conserved cysteine which is essential for enzyme activity and may represent the active site (Lanz et al. 1991). The malonyl CoA decarboxylation takes place before the condensation reaction and provides the acetyl carbanion which then acts as the nucleophile in the subse-
sequent condensation reaction (Kreuzaler et al. 1978). Stoessl and Stothers (1979) using \([1,2,^{13}C_2]\)acetate incorporation and NMR measurements showed that the C-5 carbonyl group in the enzyme bound, open chain polyketide precursor is reduced before the ring formation. The addition of the malonyl CoA acetate units during the condensation to form the A ring of the 5-hydroxy compounds is a random orientation which occurs biosynthetically (Light and Hahlbrock 1980; Dewick et al. 1982). However, the orientation of the malonyl CoA acetate units is not always random. In the 6-hydroxychalcones the acetate units are orientated in a non-random manner (Stoessl and Stothers 1979; Dewick et al. 1982).

The CHS enzyme reaction has been shown to produce chalcones with a hydroxyl group at position 5 of the A-ring (phloroglucinol-type A-ring hydroxylation; 6'-hydroxychalcone). These hydroxyl groups originate from the CoA-thiol ester linkage of the malonyl CoA molecules (Dixon et al. 1983). However, a major class of molecules produced by CHS action do not possess this hydroxy group at position 5 (the 6'-deoxychalcone; Ayabe et al. 1988). Welle and Griesbach (1988) purified a 34 kDa single polypeptide protein reductase from *G. max* that acted with CHS in the presence of NADPH to produce this other class of molecules (Figure 1.5). The reductase catalyzed the transfer of the pro-R hydrogend of \(\left[4-{^3}H\right]\)NADPH to the substrate. Welle and Griesbach (1989) found a parallel induction of the reductase with CHS following elicitor treatment of suspension cultures and produced polyclonal rabbit antibodies that did not cross react with CHS. Then Welle et al. (1991) used these polyclonal antibodies to screen a cDNA library of elicitor induced tissue and identified distinct reductase genes. Expression analysis of reductase and *CHS* gene constructs in *Eschericia coli* confirmed that the reductase co-acts with CHS in the presence of NADPH to produce the 6'-deoxychalcones. Interestingly independent regulation of the *CHS* and *CHS* plus reductase pathways is suggested by different time courses of appearance of the resulting isoflavonoids following cotyledon wounding in *P. vulgaris* (Whitehead et al. 1982). Hybridization of the reductase clone showed that similar genes exist in *P. vulgaris* and *A. hypogaea*, although there was no apparent hybridization to *Dacus carota*, *P. crispum* or *Pisum sativum* DNA even though *P. sativum* is known to produce 4,2',4-trihydroxychalcone (pisatin), a deoxychalcone (Welle et al. 1991).

There appears to be some post-translational control of CHS enzyme activity. Schroder et al. (1979) examined light induced CHS activity in cell cultures and compared the decrease in CHS enzyme activity with the radioactivity of immunoprecipitated enzyme subunits. The CHS activity was found to decline. The decline in enzyme activity was much greater than the precipitated enzyme, suggesting the loss in catalytic activity could not be accounted for by enzyme degradation. Schroder and Schafer (1980) used a sensitive radioimmunoassay to examine the effects of light on CHS activity and CHS protein, and found an accumulation of the inactive CHS enzyme during the later stages of enzyme induction.
1.4.4 Genetic analysis of CHS

Following a variety of interaction and developmental cues products of the phenylpropanoid pathway (flavones, flavonols, isoflavones and anthocyanins etc.) accumulate and the activity of the pathway enzymes, including CHS increase (Dixon et al. 1983; Chappell and Hahlbrock 1984; Cramer et al. 1985; Lawton and Lamb 1987). The general regulation of the phenylpropanoid pathway is discussed above (§1.3). The induction of CHS activity has been shown to be the result of increased CHS mRNA activity (Hille et al. 1982; Lawton et al. 1983b; Ebel et al. 1984; Grab et al. 1985) and the rate of CHS mRNA synthesis (Bell et al. 1984; Schmelzer et al. 1984; Ryder et al. 1984; Cramer et al. 1985; Lawton and Lamb 1987). The transcriptional nature of CHS therefore makes this gene (and its family) an ideal candidate for genetic analysis in an attempt to understand the mechanisms of plant gene regulation.

Structural genes

Structural genes code for the CHS protein (enzyme) responsible for the catalytic biochemical step. The regions upstream (5’) of the start codon, downstream (3’) of the stop codon and within the intron contain potential regulatory sequences which can affect the transcription of the CHS gene(s) (discussed above, §1.3.1). Structural gene(s) coding for CHS have been isolated from a variety of plants (Table 1.3). CHS occurs as a single copy gene in some plants (for example A. thaliana, Feinbaum and Ausubel 1988) and as multigene gene in other plants (for example P. hybrida, Koes et al. 1989a; T. subterraneum, Arioli 1992, Howles 1992). Most legumes have been shown to have multiple CHS copies. Multiple copies of a gene have been proposed to enhance the evolution of differential regulation of the different copies by increasing the potential for variability in the regulatory (5’, 3’ and intron) sequences (Campbell 1983).

The 5’ sequences contain the majority of known regulatory motifs that control qualitative and quantitative gene expression. The 5’ regions of several CHS genes have been analysed by sequence and deletion analysis of 5’ untranslated regions incorporated into reporter gene constructions to determine sequences and regions that affect the transcription of the gene (Table 1.1). Analysis of the A. majus promotor has revealed TATA and CAAT boxes (Sommer and Saedler 1986), a 5’-TACCAT-3’ box (Sommer et al. 1988), a 5’-CACGTG-3’ box (G box; Staiger et al. 1989), orientation independent UV inducible elements (Lipphardt et al. 1988) and positive regulatory elements (Fritze et al. 1991). In other plants negative regulatory “silencer” elements (Dron et al. 1988; Arias et al. 1993), UV light elements (Schulze-Lefert et al. 1989ab; Weissshaar et al. 1991) and tissue specific elements (van der Meer et al. 1990) have also been found. Similar analysis of other plants has revealed conservation of some of these elements. For example, the sequence 5’-CACGTG-3’ (G box) has been found in the promotor of CHS genes from T. subterraneum (Arioli 1992; Howles 1992), Z. mays (Niesbach-Klosgen et
Regulatory genes

Regulatory genes encode proteins that influence the transcription of structural genes (discussed above §1.3.1). A range of regulatory genes affecting CHS gene expression have been identified and in some instances found to comprise families of related genes (Table 1.4). These genes do not all affect the expression of CHS and the effects on CHS differ from one plant species to another. For example, in Z. mays the R(S) gene regulates CHS and other phenylpropanoid genes in the aleurone layers (Martin et al. 1991) while in A. majus the delila gene is an absolute requirement for some of the phenylpropanoid genes but has little effect on CHS in flowers (Martin et al. 1991).

In P. sativum CHS expression is absent in mutants at the a and a2 loci resulting in colourless petals. Using S1 nuclease protection analysis Harker et al. (1990) showed that the loci a and a2 did not affect CHS expression in roots, but a2 blocked expression in the petals and a reduced expression in the petals. Further, induction of CHS by the abiotic elicitor CuCl$_2$ was independent of the a and a2 loci. These regulatory genes have not been further characterized. Other regulatory genes have been identified. In P. hortense cDNAs have been characterized which code for proteins that bind to the P. crispum box II (Weisshaar et al. 1991) during light exposure of dark grown cells, in A. majus the CG-1 protein which binds to the G box (5’-CACGTG-3’) has been purified (Staiger et al. 1989,1991) and transcript competition experiments have shown a requirement for the G box and H box proteins in P. vulgaris CHS15 expression (Arias et al. 1993). Two H box proteins in P. vulgaris KAP1 and KAP2 which bind to the P. vulgaris CHS15 have been purified and shown to move from the cytoplasm to the nucleus following elicitation (Yu et al. 1993).

1.4.5 Allelic interactions

The analysis of CHS gene expression has revealed some unusual genetic mechanisms that are still poorly understood. Paramutation is one such interaction. Paramutation is an interaction between alleles that leads to a direct, heritable change at the locus with high frequency and invariably within the time span of a generation (Brink 1973). Paramutation has been documented at the R locus (Brink 1973) and B locus (Coe 1966) in Z. mays, and at the nivea locus (CHS gene) in A. majus (Harrison and Carpenter 1973; reviewed Dooner et al. 1991). In A. majus crosses between the female niv allele (white flowered, niv-44) and the male niv$^{res}$ allele (veriegated flowered, niv-53) results in a majority of the F$_1$ with a granular flush over the corolla. Selfing gives an F$_2$ with the same granular flush and no veriegated progeny are observed in Mendelian segregation for several generations (Harrison and Carpenter 1973). Molecular analysis revealed
Table 1.4: Summary of regulatory genes known to affect CHS.

<table>
<thead>
<tr>
<th>plant species</th>
<th>element</th>
<th>reference</th>
</tr>
</thead>
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<tr>
<td>Antirrhinum majus</td>
<td>delila</td>
<td>Almeida et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Martin et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>CG-1</td>
<td>Staiger et al. (1991)</td>
</tr>
<tr>
<td>Zea mays</td>
<td>R(S)</td>
<td>Ludwig et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dooner (1983)</td>
</tr>
<tr>
<td></td>
<td>R(Sn)</td>
<td>Tonelli et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Cl</td>
<td>Dooner (1983)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>Grotewold et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Vp1</td>
<td>Dooner (1983)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>ttg</td>
<td>Koornneef (1990)</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>SBF-1</td>
<td>Arias et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>KAP1</td>
<td>Yu et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>KAP2</td>
<td>Yu et al. (1993)</td>
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a transposon (Tam-1) in the promotor of ninrec (nin-53; Bonas et al. 1984) and a transposon (Tam-2) in the first intron-exon boundary of the gene nin (nin-44; Upadhyaya et al. 1985). Molecular analysis of F1 generation of a nin/ninrec cross found no major rearrangement of the genome (Krebbers et al. 1987) and excision of the Tam-2 did not alter the paramutation phenotype (Krebbers et al. 1987). Models have been proposed to explain the experimental observations which suggest the presence of suppressors in the genetic background (Krebbers et al. 1987) or that Tam-2 is a defective element that is affected by a viable Tam-1 (Hehl et al. 1987; Hudson et al. 1987).

### 1.4.6 Stilbene synthase

CHS and stilbene synthase (EC 2.3.1.-) are involved in the biosynthesis of flavonoids and stilbenes respectively. These 2 enzymes share many characteristics and most probably diverged from a common ancestor. Both enzymes can use the same substrates and catalyze similar condensing type enzyme reactions which sequentially add acetate units via malonyl CoA to coumaroyl CoA, cinnamoyl CoA or dihydrocinnamoyl CoA. The 2 enzymes differ in their action only by the folding of enzyme-bound intermediates before the new aromatic ring is closed to produce the end products (that is, the stilbene reaction involves a reduction during the A-ring formation, Schroder et al. 1988; Schroder and Schroder 1990). Two forms of stilbene synthase have been examined. Stilbene synthase I (resveratrol synthase) produces resveratrol and derivatives and stilbene synthase II produces pinosylvin and derivatives. Stilbene synthases have been cloned from *Pinus sylvestris* (Fliegmann et al. 1992), *Vitis vinifera* (Melchior and Kindl 1991; Sparvoli et al. 1994).

Stilbene synthase from *A. hypogaea* shows approximately 70% nucleotide homology to CHS consensus sequences and the intron in *A. hypogaea* cuts the same codon in both enzyme sequences (Schroder et al. 1988). Similarly, stilbene synthase from *Pinus sylvestris* is approximately 70% homologous to the *P. sylvestris* CHS, but only 65% homologous to the *A. hypogaea* stilbene synthase (Fliegmann et al. 1992). The protein is also similar to CHS with 90 kDa for the total and 45 kDa for each subunit (Schoppner and Kindl 1984). However, purified stilbene synthase from *A. hypogaea* was shown not to have CHS activity and stilbene synthase antibodies were found not to cross-react with CHS (Schoppner and Kindl 1984).

The compounds resulting from the stilbene synthase branch function to prevent wood decay (Hart and Shrimpton 1979) and in disease resistance as phytoalexins (Derecks and Creasy 1989; Ingham 1976). These compounds can be induced following UV irradiation (Vornam et al. 1988; Schoppner and Kindl 1984; Langcake and Pryce 1976, 1977) and pathogen interaction (Langcake and McCarthy 1979). *N. tabacum* plants, which do no contain stilbene synthase but have the substrates present for the CHS branch pathway, have been transformed with stilbene synthase gene(s) from *V. vinifera* and shown to express the stilbene synthase gene and synthesize resveratrol (Hain et al. 1990). In such transformed *N. tabacum* plants stilbene synthase is induced by UV irradiation (Hain et al. 1990) and fungal elicitor (Hain et al. 1990), and transformants show increased disease resistance to the pathogen *Botrytis cinerea* compared to control plants (Hain et al. 1993).

1.5 Role of phenylpropanoid pathway in plant functions

The phenylpropanoid pathway end products have been shown to have functions in a variety of plant processes. These functions are illustrated by the following discussion. The description of the involvement of flavonoids in the legume-*Rhizobium* symbiosis has been expanded to include additional details about the this interaction as this topic forms the core of the experiments described in later Chapters.

1.5.1 Plant hormone induction

The plant hormones gibberellins are involved in plant development (reviewed Jones 1973). These compounds have been shown to be involved in seed germination, shoot elongation, flower development and fruit formation. Gibberellins also affect anthocyanin pigmentation (Wiess and Halevy 1989). The gibberellin GA$_3$ is involved in stimulating the phenylpropanoid pathway in flowers (Zielsin
et al. 1974; Wiess et al. 1990) and inhibition of the pathway in *D. carota* cell suspension cultures (Hinderer et al. 1984).

In *P. hybrida* the gibberellin GA₃ is synthesized in the stamens and transported to the corollas where they induce development of the corolla and anthocyanin pigmentation (Wiess and Halevy 1989). The effect of the GA₃ on the phenylpropanoid pathway was found to be independent of its effects on growth (Wiess and Halevy 1989). In both *in vivo* and *in vitro*, CHS protein and CHS transcript were shown to increase following GA₃ application (Wiess and Halevy 1989; Wiess et al. 1992), and GA₃ was shown to be necessary to maintain CHS transcription (Wiess et al. 1992). Cyclohexamide treatment (an inhibitor of protein synthesis) suggested GA₃ induced CHS indirectly (Wiess et al. 1992).

The viviparous gene *Vpl* is the only member of this class of genes that affect sensitivity to the hormone ABA resulting in germination defects (reviewed Neill et al. 1985). The *Vpl* gene also affects the phenylpropanoid pathway in the aleurone and embryo tissues (see above discussion §1.3.1). The *Vpl* gene product probably regulates the phenylpropanoid pathway by regulating *Cl* and has a role in enabling specific seed tissues to respond normally to developmental signals, such as ABA (van der Meer et al. 1993).

### 1.5.2 Pollen function and tube growth

Flavonoids in pollen cells and stigmas affected pollen germination and pollen tube growth (Sedgley 1975) and they are essential for successful fertilization (Coe et al. 1981; reviewed Mascarenhas 1993). The absence of CHS activity in *P. hybrida* and *Z. mays* mutants results in deficient pollen function and development (Coe et al. 1981; van der Meer et al. 1992a; Taylor and Jorgensen 1992). The flavonoid(s) necessary for successful fertilization may come from either the pollen or the stigmas, as placing flavonoid deficient pollen onto normal stigmas as well as the reverse, results in normal seed production (Mo et al. 1992; Taylor and Jorgensen 1992). The addition of the flavonoids kaempferol to *P. hybrida* and *Z. mays* CHS mutants (Mo et al. 1992) and quercitin, kaempferol, and myricetin to immature *N. tabacum* and *P. hybrida* (Ylstra et al. 1992) has been shown to restore germination and seed set. This condition is known as conditional male fertility (Taylor and Jorgensen 1992). The restoration of pollen function in pollen from male sterile plants could be accounted for by permeation of the amphiphilic flavonoid (kaempferol) across the plasma membrane and into the kaempferol deficient pollen confirming the stigma as the source of the active flavonoid (Vogt et al. 1994). These compounds are proposed to be derived from existing aglycones in anthers and stigmas through glycosidase activity (Pollak et al. 1993). These compounds are not synthesized in the anthers or stigmas, but rather in the tapetal cells (van Tunen et al. 1990) and then transported to the stigmas and pollen cells (Beerhues et al. 1989). Interestingly, flavonoids involved in pollination and induced following wounding in the floral tissues of *P. hybrida*
were shown to be the same, with wound induced kaempferol enhancing plant fecundity (Vogt et al. 1994).

CHS protein has been detected in developing stigmas (Pollak et al. 1993) and transcript and protein levels increase following pollination (Vogt et al. 1994). The increased levels of CHS transcript and subsequent detection of increased levels of kaempferol over hours does not correlate with the kaempferol being involved in pollen germination, which occurs within minutes (Vogt et al. 1994). To account for this difference Vogt et al. (1994) propose that the later kaempferol associated with the CHS induction acts as a phytoalexin (allelopathic function) to prevent bacterial and fungal growth in/on the stigma following fertilization when the stigma begins to break down. The pollen tube “wounding” the stigma triggers the wound inducible CHS as well as other classical wound-induced proteins (Atkinson et al. 1993).

Koes et al. (1994) also suggest that active expression of phenylpropanoid biosynthetic genes in the ovary and the production of a flavonoid gradient between the ovary and stigma surface might provide a direction for the pollen tube to grow towards the ovary.

1.5.3 Glutathione induction

Glutathione (g-L-glutamyl-L-cysteinyl-glycine) is a low molecular weight thiol involved in the plant metabolic processes (reviewed Meister and Anderson 1983). It has been shown to induce plant defence genes, including CHS (Wingate et al. 1988). Glutathione has various proposed functions in higher plants (Rennenberg 1982) and appears to protect the plant against oxidative damage following physical stresses caused by irradiation (Meister and Anderson 1983), heat (Nieto-Sotelo and Ho 1986) and heavy metals (Grill et al. 1985) as well as a role in disease resistance and microbial infection (Wingate et al. 1988; Edwards et al. 1991; Robbins et al. 1991). Lawton et al. (1990) showed glutathione changed the chromatin structure within the CHS promoter.

1.5.4 Light induction

Plants have an array of mechanisms for protection against damaging light which include increasing leaf thickness, reflection and absorbance of the harmful light with light absorbing compounds (reviewed Beggs et al. 1986). Of the light incident on plants, UV has been shown to damage DNA, RNA and proteins (Stapleton 1992). Flavonoids are proposed to function as UV protectants by absorbing the harmful UV light (Hahlbrock and Grisbach 1979; reviewed Koes et al. 1994). Flavonoids have been shown to accumulate in the epidermal layers (Schmelzer et al. 1988) and the genes responsible for their synthesis have been shown to be induced following UV exposure (Chappel and Hahlbrock 1984; Schmelzer et al. 1988; Feinbaum et al. 1991; Kubasek et al. 1992). Using a range of A. thaliana
mutants that did not produce flavonoids (including a CHS deficient mutant), Li et al. (1993) were able to show that control plants exposed to UV-B induced PAL and CHS and produced flavonoid compounds and that these compounds had a direct effect on the ability of the control plant to overcome the harmful effects of the UV-B radiation compared to the mutants (Tevini et al. 1991; Middleton and Teramura 1993). These results demonstrate a role for flavonoids as UV protectants.

1.5.5 The plant defence response and wounding

Flavonoids are involved as part of the plant’s preformed and induced defence responses (reviewed Ebel 1986; Lawton and Lamb 1987; Hahlbrock and Scheel 1989; Dixon and Harrison 1990; Scheel and Parker 1990; Stafford 1990). The preformed defences include lignification, protective compounds and protective structures. The induced defence response is best illustrated by example. The fungal pathogen *Nectria haematococca* either has (Pad+) or does not have (Pad-) the ability to metabolize the plant host produced isoflavonoid pisatin. The Pad− strains are unable to infect the host *P. sativum* while Pad+ strains were pathogenic and tolerant to pisatin (Kistler and Van Etten 1984). Transformation of a Pad− fungus with a gene able to reverse the Pad− phenotype restored the ability to metabolize pisatin and restored pathogenicity (Ciuffetti et al. 1988). These experiments showed that the ability to metabolize the isoflavonoid were responsible for the plant susceptibility and therefore that the isoflavonoid was the plant’s defence to this particular fungus. The induction of the phenylpropanoid compounds (Whithhead et al. 1982), enzyme activities (Whitehead et al. 1982) and genes (including *CHS*; Collinge and Slusarenko 1987) have been shown to be characteristic in a range of other plant pathogen interactions and upon physical/mechanical wounding (reviewed Dixon 1986; Lawton and Lamb 1987; Collinge and Slusarenko 1987; Hahlbrock and Scheel 1989; Lamb et al. 1989; Bowles 1990) and in response to an array of elicitors (reviewed Lamb et al. 1989; Bowles 1990).

1.5.6 The mycorrhiza interaction

Fungi form symbiotic associations with plants. Like the legume-*Rhizobium* interaction (§1.5.7), the development of the symbiosis between the plant and fungus involves a series of complex interactions. The fungus attaches to the root surface, differentiates to form appressoria, the hyphae enter the root and differentiate to form specialized structures that enable nutrient exchange (reviewed Brudrett et al. 1985; Smith and Gianinazzi-Pearson 1988). Plant tissues infected by the fungi forming a mycorrhiza respond by the production of chitinases (Spanu et al. 1989), peroxidases (Spanu and Bonfante-Pasolo 1988), induction of phenylpropanoid pathway enzyme activities (Volpin et al. 1994) and genes (Harrison and Dixon 1993), and the accumulation (Morandi et al. 1984) and exudation
(Harrison and Dixon 1993) of flavonoid compounds. Flavonoid molecules may also function as signalling molecules in the early stages of the interaction (Phillips 1992) in addition to promoting colonization (Siqueira et al. 1991) and enhancing germination and hyphal growth (Gianinazzi-Pearson et al. 1989). Mycorrhizal plants also show increased resistance to root pathogens and drought stress which may be attributed to flavonoids (Jeffries 1987; Smith 1988).

1.5.7 The **Rhizobium-legume symbiosis**

*Rhizobium*, *Bradyrhizobium* and *Azorhizobium* can form nitrogen fixing nodule structures on the roots of the plant family Leguminosae and members of the plant genus *Parasponia* (*Ulmaceae*). The process by which these nodule structures are formed involves a complex series of recognition interactions between the bacteria and plant host, entry of the bacteria into the host plant, the differentiation of the plant tissues into a nodule structure (or modified lateral root) and the exchange of nutrients between the plant and bacteria in the resulting nitrogen fixing symbiosis (Halverston and Stacey 1986). Compounds produced through CHS enzyme action in the phenylpropanoid pathway have been correlated with the early stages of this interaction, functioning in the early recognition exchanges between the plant root and bacteria (reviewed Rolfe 1988; Long 1989; Djordjevic and Weinman 1991; Hirsch 1992; Fischer and Long 1992; Verma 1992; Frassen et al. 1992), in the infection process (reviewed Djordjevic et al. 1987a) and have been proposed to function in the differentiation and development of functioning nitrogen fixing nodules (reviewed Hirsch 1992).

**Events in the symbiotic interaction**

In the field, the interaction between the plant and infecting rhizobia is subject to a considerable array of competing and inhibiting factors (Robson and Bottomley 1991). The concentration of aluminium (Wood et al. 1984), phosphate deficiencies (Mullen et al. 1988), calcium deficiencies (O’Hara et al. 1988), nitrate (reviewed Streeter 1988), salinity (Singleton and Bohlool 1984), extreme pH (Munns 1986) and extremes of temperature (Gibson 1977) are all known to affect the interaction. These factors affect both the plant and invading microbes. For example, low pH reduced the exudation of flavonoids from *T. subterraneum* roots following inoculation (Richardson et al. 1988) and low phosphate reduces the amount of LOS produced by bacteria (McKay and Djordjevic 1993). As a result of these extraneous factors affecting and limiting the successful interaction between the plant and bacteria, laboratory experimentation uses defined media, sterile plants and monoculture inoculum. Under these conditions the characteristic events in the interaction have been examined and defined.

There are a series of recognizable developmental stages leading to a successful nitrogen fixing interaction between the plant host and infecting rhizobia (reviewed Halverston and Stacey 1986; Djordjevic et al. 1987a; Gloudemans and Bisseling...
1989; Brewin 1991; Franssen et al. 1992; Hirsch 1992). These stages include root hair attachment, root hair curling, infection thread formation, root hair invasion, stimulation of cortical cell division, the release of the invading bacteria into the divided cortical cells and then nodule maturation and nitrogen fixation. There are a few exceptions to the generalized scheme presented in the following discussion (Hirsch 1992) and there is considerable diversity in the details of the interaction (Ridge et al. 1992).

Prior to the saprophytic soil rhizobia attaching to the roots the bacteria are attracted towards the roots by the release of carbohydrates, amino acids and flavonoids from the plant roots and seed coats (reviewed Phillips 1992). Flavonoids from the seed coats and roots (Phillips 1992) act as chemoattractants at very low concentrations (Aguilar et al. 1988; Armitage et al. 1988; Caetano-Anolles et al. 1988; Kape et al. 1991), although some flavonoids have no chemoattractant activity (Caetano-Anolles et al. 1988). It is not clear at this stage whether soil microbes generally respond to seed coat or root released flavonoids, or both (Phillips 1992). Some of the flavonoids released into the rhizosphere are thought to be taken up by the rhizobia and induce the expression of the nod genes (reviewed Long 1989). These genes code for proteins involved in the synthesis of the LOS molecules which are involved in the early interaction events and also have a role in determining host specificity (discussed below). The attracted bacteria attach to root hairs. Lectins (Lugtenberg et al. 1991; Kijne 1992), adhesins (Smit et al. 1987; Lugtenberg et al. 1991), extracellular microfibrils (Halverson and Stacey 1986), pili (Vespe and Bauer 1986) and exopolysaccharides (reviewed Gray and Rolfe 1990) have been implicated to have roles in rhizobial recognition and attachment, although the details remain unclear.

Within hours of attachment to the root hairs, the bacteria cause deformation of some root hairs and severe curling (shepherd’s crooks) in a number of these root hairs. The bacteria become trapped within the pocket of the shepherd’s crook. The bacteria in this pocket then interact with the root hair cell wall (for example, through pectinase activity, Mateos et al. 1992) and penetration of the root hair begins by invagination and deposition of plant cell wall material (Ridge 1992). The root hairs most susceptible to infection resulting in a nodule are those just behind the root tip at the time of infection (Bhuvaneswari et al. 1981). Only a small number of these successful interactions lead to a nitrogen fixing nodule (Nutman 1959). This shepherd’s crook is thought to be important for a successful interaction (Kijne 1992) and most probably results from localized stimulation of the root hair tip (Kinje 1992). Correlated with the infection is an increase in CHS expression (Estabrook and Sengupta-Gopalan 1991; Recourt et al. 1992; Junghans et al. 1993) and the production of a range of new flavonoids in the root exudates which have nod gene inducing activity (van Brussel et al. 1992; Recourt et al. 1991; Dakora et al. 1993ab; Schmidt et al. 1991,1994). A schematic representation of some of the molecular interactions between the plant
and infecting rhizobia are shown in Figure 1.6.

Following entry into the infection thread the rhizobia divide, moving down the growing infection thread and into the root cortex cells which are stimulated to divide and form a nodule primordia (Calvert et al. 1984). The infection thread grows towards the nodule primordia (reviewed Brewin 1991). In the root cortex zone, the bacteria are encapsulated in plant derived membrane and released into the cortex cells. These encapsulated bacteria then differentiate into bacteroids (Hennecke 1990). The interaction between the bacteria and plant involves a complex metabolic exchange (reviewed Werner 1992).

Bacterial symbiotic genes

Several different groupings of bacterial genes are involved in the symbiotic interaction. These include the *nod* and *nol, exo, nps, nif, ntr, fix* and other poorly defined genes (reviewed Long 1989; Werner 1992). The *nod* (and *nol*) genes are transcribed in response to seed coat and plant root released phenylpropanoid compounds (reviewed Long 1989). The *nod* (and *nol*) genes (Table §1.5) may be divided into three classes; the common *nod* genes, the *nodD* regulatory gene(s) and host-specificity *nod* genes. In the fast growing *Rhizobium* these genes are located on a large indigenous symbiotic plasmid (Sym plasmid) while in the slow growing *Bradyrhizobium* these genes are found on the chromosome (reviewed Kondorosi 1989). Some of these *nod* genes code for proteins (Table 1.5) involved in the biosynthesis of LOS molecules (Lerouge et al. 1990; Spaink et al. 1991; reviewed Spaink 1992). These molecules have been shown to cause root hair curling and cortical cell divisions in the plant (Truchet et al. 1991) but their function remains to be determined (Spaink 1992). The common *nod* genes (*nodABC*J) are found in all *Rhizobium, Bradyrhizobium* and the *Azorhizobium*. These genes are structurally conserved and may generally be interchanged between genera without loss of function (Kondorosi et al. 1984; Djordjevic et al. 1985; reviewed Kondorosi 1989). Bacterial strains deleted for these common *nod* genes are unable to curl root hairs, induce cortical cell division or form infection threads resulting in a complete abolition of nodulation (Long 1989). The *nodABC* genes code for proteins involved in the synthesis of the core *nod* factor molecule (Spaink 1992). These core factor enables the bacteria to cause cortical cell division (Dudley et al. 1987) and root hair deformation (Kondorosi et al. 1984; Rossen et al. 1984; Djordjevic et al. 1985; Plazinski et al. 1994) The *nodIJ* genes code for proteins involved in the transport of the *nod* factor (McKay and Djordjevic 1993).

The *nodD* gene is present as either a single copy gene or in multiple copies with variable regulation and is the only *nod* gene constitutively expressed in the free living bacteria (reviewed Long 1989). The *nodD* gene(s) in the presence of some flavonoid compounds from the plant host then acts as a transcriptional regulator of all the other *nod* gene operons (reviewed Gyorgypal et al. 1991). The flavonoids can act as inducers (Redmond et al. 1986; Peters et al. 1986; Djordjevic et al.
1987b; Zaat et al. 1987; Peters and Long 1988; and synergistically, Hartwig et al. 1989) and anti-inducers (Firmin et al. 1986; Djordjevic et al. 1987b; Peters and Long 1988) of nod gene expression, and a range of other phenolics also induce nod gene expression (Kape et al. 1991). Different plants produce different arrays of nod gene inducing flavonoids and anthocyanins (Redmond et al. 1986; Peters et al. 1986; Zaat et al. 1987; Peters and Long 1988) and the nodD is presumed to interact directly with these plant compounds (Horvath et al. 1987; Spaink et al. 1987; Djordjevic and Weinman 1991; Long and Staskewicz 1993). NodD proteins from different Rhizobium respond to different flavonoid compounds determining to some extent host specificity (Spaink et al. 1987; Recourt et al. 1991; Werner 1992).

The remaining nod (and noo) genes determine the specificity (host range) and rate of nodulation between the plant and bacteria so that only a bacteria with the correct functioning compliment of nod genes (in addition to nodDABCIJ) will form nodules on the particular host plant without delay. The biological function of some of these genes have been determined and others remain unknown (Table 1.5; reviewed Spaink 1992; Fisher and Long 1992; Werner 1992).

1.6 Scope of this thesis

T. subterraneum is an agriculturally important legume in Australia as a pasture legume providing a nutritious feed for pasture livestock and as an important source of fixed nitrogen for pasture improvement and rotation cereal crops (Gladstone and Collins 1983). The poor soils in Australia necessitate the use of fertilizers at substantial financial and environmental expense, and pests and pathogens account for a considerable amount of lost production. An understanding of the mechanisms by which flavonoids are involved in the interaction between the plant and bacteria (symbiotic and pathogenic) may enable considerable advances to be made in manipulating these interactions for enhancing agricultural production. An analysis of the involvement of phenylpropanoid pathway gene expression and its involvement in plant-microbe interactions is therefore a worthy challenge.

The analysis of plant gene regulation is also a necessary step in manipulating genes for the benefit of agriculture. The mechanisms of plant gene regulation are poorly understood as a result in part of the lack of suitable technologies to elucidate the component parts and also because of the complexity of the mechanisms involved. The phenylpropanoid pathway has advanced the understanding of plant gene regulation because mutations in both structural and regulatory genes result in easily scored colour phenotypes. Classical genetic analyses has enabled the characterization of these phenotypes and molecular analyses has enabled the characterization of the genes. The molecular analyses have shown considerable complexity in the mechanisms of regulation and highlighted the need for well characterized model systems in which to tease apart the contributing factors. This analyses has also been limited by poorly understood developmental
Figure 1.6: Rhizobium infection of legume plant roots. A schematic representation of (A) the early infection events during the Rhizobium infection of a legume root, and (B) the signal exchange between the legume and the infecting Rhizobium during the early preinfection stages. Diagrams from Djordjevic and Weinman (1991).
### Table 1.5: Functions and predicted functions of *nod* genes; modified from Fisher and Long (1992), Spaink (1992) and Werner (1992).

<table>
<thead>
<tr>
<th><em>nod</em> gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>Required for Nod production</td>
</tr>
<tr>
<td>C</td>
<td>Homology to chitin and cellulose synthases - <em>N</em>-acetylglucosaminyl transferase</td>
</tr>
<tr>
<td>D</td>
<td>Transcriptional regulator of <em>nod</em> genes</td>
</tr>
<tr>
<td>EFG</td>
<td>Proposed to synthesize and modify acyl chain - β-ketoacylsynthase, acyl carrier protein and dehydrogenase or β-ketoreductase respectively</td>
</tr>
<tr>
<td>HPQ</td>
<td>Involved in sulphonation of Nod factor - sulphotransferase, ATP sulphurylase subunit and ATP sulphurylase subunit respectively</td>
</tr>
<tr>
<td>IJ</td>
<td>Involved in transport of Nod factor</td>
</tr>
<tr>
<td>K</td>
<td>No known function</td>
</tr>
<tr>
<td>L</td>
<td>Adds acetyl group to Nod factor - acetyltransferase</td>
</tr>
<tr>
<td>M</td>
<td>Involved in synthesis of sugar units</td>
</tr>
<tr>
<td>N</td>
<td>Host specificity in <em>Vicia hirsuta</em> nodulation</td>
</tr>
<tr>
<td>O</td>
<td>Ca$^{2+}$ channels</td>
</tr>
<tr>
<td>R</td>
<td>Host range <em>R. l.</em> bv. <em>trifolii</em></td>
</tr>
<tr>
<td>S</td>
<td>No known function</td>
</tr>
<tr>
<td>T</td>
<td>Host specificity in <em>T. subterraneum</em> nodulation</td>
</tr>
<tr>
<td>U</td>
<td>No known function</td>
</tr>
<tr>
<td>VW</td>
<td>Regulates unknown target gene(s)</td>
</tr>
<tr>
<td>X</td>
<td>Extends host range of <em>P. sativum</em> cv. “Afghanistan”</td>
</tr>
<tr>
<td>Y</td>
<td>No known function</td>
</tr>
<tr>
<td>Z</td>
<td>Adds 2-O-methylfucose residue</td>
</tr>
<tr>
<td>1A</td>
<td>Host range <em>B. japonicum</em></td>
</tr>
</tbody>
</table>
cues which are responsible for triggering the expression of both the structural and regulatory genes.

The need for well characterized (model) systems for the analyses of plant gene expression enables correlations to be made between specific stimuli, the resulting gene expression and the consequences of that gene expression (enzyme activity). A comparison between stimuli and the resulting gene expression then has the potential to highlight differences in the mechanisms of regulation and therefore delineating the mechanisms of regulation. The research described in this thesis takes advantage of the detailed understanding of the symbiotic interaction between the legume *T. subterraneum* and the soil bacteria *Rhizobium*. The products of the phenylpropanoid pathway have been shown to be involved in this interaction and phenylpropanoid pathway genes have been shown to be induced following *Rhizobium* inoculation. Linking the morphological events following *Rhizobium* inoculation with the expression of gene(s) and then an analyses of the resulting flavonoid(s) provides considerable potential for elucidating some aspects of regulation. A complete understanding of gene regulation is unlikely given the complexity and the restraints of time.

Therefore, the research described in this thesis determines the involvement of the *CHS* multigene family in the very early stages of the *T. subterraneum-R. l. bv. trifolii* symbiosis with a view to characterizing some of the events leading to gene expression and identifying potential regulatory mechanisms. This treatment is then contrasted with physical wounding of the plants. This treatment was chosen because it provides a trigger that is immediate, visible and known to induce *CHS* gene expression and has been postulated to share some similarities with *Rhizobium* infection.

The research presented in Chapter 3 characterizes the interaction between the plant and bacteria in time and then places the *CHS* gene expression into that morphological context. A survey of expressed *CHS* genes then identifies which *CHS* gene family members are triggered by the stimuli and identifies potential regulatory regions within the promotor. The same analyses is made of plants subject to physical wounding.

The research presented in Chapter 4 examines the flavonoid compounds produced after both *Rhizobium* inoculation and wounding treatments. Using analytical techniques and a sensitive bio-assay the flavonoids are assessed and the production of a new flavonoid is determined.

Together, the results presented in Chapters 3 and 4 link morphologically defined stimuli with the expression of a particular gene and the synthesis of a new flavonoid compound. These results are then examined in the context of the involvement of *CHS* gene expression and flavonoids in the legume-*Rhizobium* interaction and the possible regulatory mechanisms resulting in gene expression. This research provides a causal link between stimulus, gene expression and the consequences of that expression.
Chapter 2

Materials and Methods

2.1 Plant material and culture

2.1.1 Plant material

*T. subterraneum* cultivar “Karridale” was used for all the plant experimentation. Certified seed stocks were obtained from Cleanseeds Pty Ltd, 45 Molonglo Road, Bungendore, NSW, Australia, 2621.

2.1.2 Plant culture

Sterilization and germination

Seeds were stored in sealed airtight containers at 4°C until use. Seeds were surface sterilized by gentle shaking for 5 min in 3.25% NaClO solution (1.5% available Cl) followed by vigorous washing in 4 changes of sterile dH₂O. The seeds were then allowed to soak in sterile dH₂O for 20 min. The seeds were germinated on BMM plates (Table 2.6) for 24 h at 4°C in the dark to synchronize germination and then at 22°C for 24 h in the dark. For all experiments germinated seedlings showing no signs of contamination were selected for experimentation.

Routine plate culture

Routine plate culture was used to grow plants for mRNA isolation and to confirm that *Rhizobium* inoculations resulting in nodule formation. For routine plate culture between 4–10 (9 cm Petri dish) and 10–40 (15 cm Petri dish) seedlings were placed onto F media plates (Table 2.1), the plants treated (*Rhizobium* inoculation or wounding) and lids placed over each plate. For seedlings used to isolate mRNA (Chapter 3) the plates were wrapped in aluminium foil and placed directly into the growth cabinet at 22°C in the dark for the duration of the experiment. To confirm that *Rhizobium* inoculation resulted in nodule formation in the experiments described in Chapter 3, after *Rhizobium* inoculation extra plates from each
2.2 Materials and Methods

### Table 2.1: Fahraeus media (F media; Rolfe et al. 1980).

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Stock concentration</th>
<th>Final concentration</th>
<th>Amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂.H₂O</td>
<td>10.0 g/l</td>
<td>0.7 mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>12.0 g/l</td>
<td>0.5 mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>10.0 g/l</td>
<td>0.7 mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>Na₂HPO₄.12H₂O</td>
<td>15.0 g/l</td>
<td>0.4 mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>C₆H₅O₇Fe.5H₂O</td>
<td>0.5 g/l</td>
<td>0.02 mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>Gibson’s trace elements</td>
<td>Table 2.2</td>
<td></td>
<td>1 ml</td>
</tr>
</tbody>
</table>

pH 6.5
For solid media added 15.0 g/l agar.

Treatment were closed with a 1 cm x 1 cm sterile aluminium foil spacer at the top edge, sealed with Nesco film (Bando Chemical Industries, Kobe, Japan) and then punctured with 3 slits around the spacer at the top of the plate. A brown paper “skirt” was attached to the plate to protect the roots from direct light. The plates were then placed upright into growth cabinets with standard 16 h day/8 h night regime at 22° C day/18° C night. Plants were examined for nodule formation 9–12 days after inoculation.

**Cellulose acetate membrane culture**

Cellulose acetate membrane culture was used to grow plants for flavonoid extraction and exudate collection. Cellulose acetate membranes (SM111, Sartorious, Goettingen, Germany) were washed in dH₂O and sterilized prior to use. The membranes were placed onto the surface of 15 cm Petri dishes of F media (Table 2.1). Ten germinated plants were placed onto the membrane, the plants treated (Rhizobium inoculation or wounding) and lids placed over each plate. Where growth conditions did not involve light, the plants were wrapped in aluminium foil and placed into a growth cabinet at 22° C in the dark. Where the growth conditions incorporated light, a 1 cm x 1 cm sterile aluminium foil spacer was placed at the top edge, the plate sealed with Nesco film, 3 large slits punctured around the spacer and a paper skirt attached to the plate to protect the roots from direct light. The plates were then placed upright into growth cabinets with a standard 16 h day/8 h night at 22° C day/18° C night.

2.2 Bacterial material and culture

#### 2.2.1 Bacterial culture media

LB media (Table 2.3) and 2YT media (Table 2.5) were used to grow *E. coli* strains at 37° C. BMM (Table 2.6) and BIII (Table 2.7) were used to grow *Rhizobium* inoculation or wounding.)
Table 2.2: Gibson’s trace elements (Gibson 1968).

<table>
<thead>
<tr>
<th>chemicals</th>
<th>stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₄</td>
<td>2.8 g/1</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>2.0 g/1</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>220.0 g/1</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>80 mg/1</td>
</tr>
<tr>
<td>H₂MnO₄·H₂O</td>
<td>90 mg/1</td>
</tr>
</tbody>
</table>

zobium strains at 28° C. All media were made up in dH₂O using the chemicals and reagents from the sources listed in Table 2.11, or reagent grade chemicals from Sigma (Castle Hill, NSW). All media was autoclaved (20 min at 120° C). Antibiotics were filter sterilized and added following autoclaving to a final concentration of 50 µg/ml for spectinomycin, 2 µg/ml for tetracycline, 100 µg/ml for carbenicillin and 100 µg/ml for ampicillin.

2.2.2 Eschericia coli strains

E. coli were used to maintain, amplify and select manipulated DNA molecules. E. coli strain NM522 (Gough and Murray 1983; F’ episome, recA⁺) was used to select insertions using blue/white selection and to produce ssDNA for sequencing. E. coli strain DH5 (recA⁻) was used to amplify and maintain plasmids on stock plates.

2.2.3 Eschericia coli culture

E. coli strains containing plasmids were maintained as long term stocks in stab cultures at room temperature. Stab cultures were made up by placing a scoop of bacteria into tubes containing 1.5 ml of nutrient agar LB media (Table 2.3). As a backup, strains were also maintained as long term stocks by placing a scoop of bacteria into 25% glycerol, allowed to stand overnight and then placed at −70° C. Plasmid constructs were maintained as DNA stocks and stored separately in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at −70° C. Plasmids were introduced into E. coli strains by electroporation (§2.4.7)

E. coli strains in regular use were stored as streaked colonies on LB plates (Table 2.3; with the appropriate antibiotic selection) or M9 plates (see 2.4; with the appropriate antibiotic selection). Streaked colonies were grown overnight at 37° C and then stored at 4° C. These plates were re-streaked regularly. For liquid culture E. coli were grown up in either LB media (Table 2.3) or 2YT media (Table 2.5) at 37° C with vigorous shaking.
Table 2.3: Luria broth media (LB media).

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

For solid media added 15.0 g/l w/v agar.
For stab cultures added 15.0 g/l nutrient agar.

Table 2.4: Minimal media (M9 media).

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9 minimal salts</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O (1M)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Thiamine (1mg/ml)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Difco Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: 2YT media.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>
2.2.4 *Rhizobium* culture

*Rhizobium* strains were maintained as long term stocks in stab cultures at room temperature in the same way as *E. coli* (§2.2.3) except that the slopes were made up with BMM (Table 2.6) containing 1 g/200 ml w/v CaCO₃. Strains were also maintained as glycerol stocks in the same way as *E. coli* (§2.2.3). Where the bacteria contained plasmid constructs, the DNA was isolated and stored separately in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at −70° C.

*Rhizobium* in regular use were maintained on BMM plates (Table 2.6) with the appropriate antibiotic selection at 4°C. These plates were streaked out regularly and the bacteria grown for approximately 20 h at 28°C before being stored at 4°C until use. *Rhizobium* strains for plant inoculation and *nod* gene inducing assays were grown up in BMM (Table 2.6) or BIII (Table 2.7) at 28°C with vigorous shaking. The cultures were inoculated from freshly grown bacteria on BMM (Table 2.6) plates with the appropriate antibiotic selection.

The number of *Rhizobium* cells in an inoculation dose were determined by dilution plating. Samples of the inoculant were diluted in sterile dH₂O and plated out onto BMM plates (Table 2.6), incubated at 28°C and the number of colonies counted.
Table 2.7: BIII media (Hollingsworth et al. 1984).

<table>
<thead>
<tr>
<th>chemicals</th>
<th>stock</th>
<th>final concentration</th>
<th>amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per 100 ml (w/v)</td>
<td>per 100 ml (w/v)</td>
<td></td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>23 g</td>
<td>0.23 g/l</td>
<td>1 ml</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>10 g</td>
<td>0.10 g/l</td>
<td>1 ml</td>
</tr>
<tr>
<td>Na-glutamate</td>
<td>11 g</td>
<td>1.10 g/l</td>
<td>10 ml</td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
<td></td>
<td>10 g</td>
</tr>
<tr>
<td>Trace elements</td>
<td>Table 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamins</td>
<td>Table 2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For solid media added 15.0 g/l w/v agar.

Table 2.8: 1000× BIII trace elements stock.

<table>
<thead>
<tr>
<th>chemicals</th>
<th>amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>6.6 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>145 mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>125 mg</td>
</tr>
<tr>
<td>CoSO$_4$.7H$_2$O</td>
<td>70 mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>MnCl$_2$.4H$_2$O</td>
<td>4.3 mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>108 mg</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$</td>
<td>125 mg</td>
</tr>
<tr>
<td>Nitrilo triacetate</td>
<td>7.0 g</td>
</tr>
</tbody>
</table>

Filter sterilized.

Table 2.9: 1000× BIII vitamin stock.

<table>
<thead>
<tr>
<th>vitamin</th>
<th>amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>20 mg</td>
</tr>
<tr>
<td>$p$-amino benzoic acid</td>
<td>20 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>20 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>20 mg</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>20 mg</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>20 mg</td>
</tr>
<tr>
<td>Ca pantothenate</td>
<td>20 mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>120 mg</td>
</tr>
</tbody>
</table>

Made up in 0.05 M Na$_2$HPO$_4$ (pH 7.0).
Filter sterilized.
Table 2.10: Gamborg and Eveleigh (1968) trace elements.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount added per 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO₄·4H₂</td>
<td>10.0 g</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>3.0 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.25 g</td>
</tr>
</tbody>
</table>

2.3 Nucleic Acid Isolation

2.3.1 Stock solutions and materials

Chemicals, reagents and enzymes were obtained from the sources set out in Table 2.11. Where the source is not identified reagent grade materials were used. The dH₂O used in all instances was distilled, deionized and sterilized by autoclaving (20 min at 120° C).

Phenol

Phenol was purchased as redistilled crystals (International Biotechnologies, New Haven, CT). Before use, the phenol was saturated and the pH adjusted in the following manner. The phenol was melted at 65°C in a water bath. An equal volume of 1 M Tris-HCl (pH 7.0) was added, the solutions mixed and the phases allowed to settle. The pH of the aqueous phase was then checked using Whatman Full Range pH 1-14 paper indicator. The aqueous layer was then removed and fresh 1 M Tris-HCl (pH 7.0) added. Again the phases were mixed and the pH of the aqueous phase checked. This procedure was repeated until the aqueous phase was pH 7.0. The aqueous phase was then removed and replaced with TE (10 mM Tris-HCl pH 7.0, 1 mM EDTA), shaken, allowed to settle and removed. Solid 8-hydroxyquinolone was added to a final concentration of 0.1% w/v. The buffered phenol was then aliquotted and stored at −20°C in the dark. Before use the phenol was melted in a 65°C waterbath.

Buffers

Buffers for all manipulations were made up in dH₂O. The pH was then measured with a PHM82 Standard pH Meter (Radiometer, Copenhagen) and the appropriate pH adjustments made with NaOH and HCl stock solutions, also made up in dH₂O. Where buffers were provided by the manufacturer, they were used according to the manufacturer’s instructions.
### Table 2.11: Chemicals, reagent and enzymes used and their sources.

<table>
<thead>
<tr>
<th>chemicals and reagents</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>agar</td>
<td>Difco Laboratories, Detroit, MI</td>
</tr>
<tr>
<td>agarose</td>
<td>Progen Industries, Darra, Queensland</td>
</tr>
<tr>
<td>agarose (low melting)</td>
<td>GIBCO BRL, Gaithersburg, MD</td>
</tr>
<tr>
<td>$\alpha^{32}$P-dATP</td>
<td>Amersham International, Buckinghamshire</td>
</tr>
<tr>
<td>ampicillin</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Boehringer Mannheim, Castle Hill, NSW</td>
</tr>
<tr>
<td>ethidium bromide</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>IPTG</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>M9 mineral salts</td>
<td>GIBCO BRL, Gaithersburg, MD</td>
</tr>
<tr>
<td>nutrient agar</td>
<td>GIBCO BRL, Gaithersburg, MD</td>
</tr>
<tr>
<td>polyethylene glycol</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>polaroid film</td>
<td>Polaroid Ltd, Hertfordshire</td>
</tr>
<tr>
<td>rATP</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Boehringer Mannheim, Castle Hill, NSW</td>
</tr>
<tr>
<td>tryptone</td>
<td>Difco Laboratories, Detroit, MI</td>
</tr>
<tr>
<td>Xgal</td>
<td>Sigma, Castle Hill, NSW</td>
</tr>
<tr>
<td>yeast extract</td>
<td>Difco Laboratories, Detroit, MI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>enzymes</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klenow fragment</td>
<td>Pharmacia, North Ryde, NSW</td>
</tr>
<tr>
<td>BamHI</td>
<td>Boehringer Mannheim, Castle Hill, NSW</td>
</tr>
<tr>
<td>BamHI</td>
<td>Pharmacia, North Ryde, NSW</td>
</tr>
<tr>
<td>BamHI</td>
<td>Progen Industries, Darra, Qld</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Boehringer Mannheim, Castle Hill, NSW</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Pharmacia, North Ryde, NSW</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Progen Industries, Darra, Qld</td>
</tr>
<tr>
<td>reverse transcriptase</td>
<td>GIBCO BRL, Gaithersburg, MI</td>
</tr>
<tr>
<td>DNase</td>
<td>Stratagene, La Jolla, CA</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>Boehringer Mannheim, Castle Hill, NSW</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>Bresatec, Thebarton, SA</td>
</tr>
</tbody>
</table>
Silanizing glass and plastic wares

For use in RNA extraction and storage (and the isolation and analysis of flavonoids, §2.5.3) all glasswares were silanized. Either the glass was soaked in 5% v/v dimethyldichlorosilane in chloroform for 5 min, or placed in a evacuation chamber with a beaker containing 5% v/v dimethyldichlorosilane in chloroform, evacuated and released twice. The glass was then air dried for 3–4 h, rinsed in H<sub>2</sub>O and then baked at 180°C for 2–3 h. Some plastics were also siliconized using the same procedure except that the baking steps were carried out at 65°C.

2.3.2 DNA isolation

Plasmid DNA – CsCl method

*E. coli* cells were grown up in 500 ml LB media (Table 2.3) overnight with the appropriate antibiotic selection. Where amplification of the plasmid was necessary, spectinomycin (to 100 mg/ml) was added to the cells at OD<sub>600</sub> 0.4–0.6.

Cells were harvested by centrifugation in a Sorvall RC5D using a GSA rotor at 5000 rpm for 10 min. The supernatant was poured off and the pellet resuspended in 9.5 ml cold STE (10 mM Tris-HCl pH 7.0, 1 mM EDTA and 25% w/v sucrose). To this was added 1.5 ml freshly prepared lysozyme (20 mg/ml in 0.25 M Tris-HCl pH 8.0) and 5 ml 0.5 M EDTA pH 8.0. The resuspension was swirled intermittently for at least 20 min. Then 15 ml of Brij/DOC (1% Brij 58 and 0.4% w/v Na-deoxycholate in 10mM Tris-HCl pH8.0, 1mM EDTA) was rapidly added and mixed thoroughly. This was then placed at 4°C for at least 20 min and often overnight. The debris was then pelleted by centrifugation in a Sorval RC5D using an SS34 rotor at 15 000 rpm for 60 min at 4°C. Following this centrifugation the supernatant was decanted into a chilled measuring cylinder and NaCl to 3% w/v and 50% PEG 6000 to ¼ volume were added. This was then placed at 4°C for at least 2 h and often overnight. The DNA was then pelleted by centrifugation in a Sorvall RC5D using an SS34 rotor at 5 000 rpm for 10 min. The supernatant was removed and the precipitate dissolved in 5 ml TESalt (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 100 mM NaCl). To this was added 8.0 g CsCl and 0.6 ml ethidium bromide (10 mg/ml). This was allowed to stand at 4°C for at least 30 min and often overnight before being centrifuged in a Sorvall RC5D using a SS34 rotor at 10 000 rpm for 30 min at 4°C. The solution was decanted and the density adjusted to 1.59–1.61 mg/ml with TESalt and then loaded into a 50Ti polyallomer tube. This was then ultracentrifuged in a Sorvall OTD75B using a Ti50 rotor at 40 000 rpm for 40 h at 18°C. Following centrifugation the lower band was removed with a 19 guage needle and extracted 4 times against n-butanol (saturated with a solution of 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and then 2 times against di-ethyl ether (saturated with a solution of 10 mM Tris-HCl pH 8.0, 1 mM EDTA). The remaining phase was then dialzed against 3 changes of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).
Plasmid DNA — Mini method

The following method was based on that described by Birnboim and Doly (1979). *E. coli* cells were grown up in either 5 ml LB media (Table 2.3) or 2 ml 2YT media (Table 2.5) at 37° C. Then 1.5 ml of cells were pelleted in an Eppendorf centrifuge and the supernatant discarded. The pellet was resuspended in 100 µl TEG (25 mM Tris-HCl pH 7.5, 10 mM EDTA and 50 mM glucose). To this was added 200 µl of a fresh solution of 1% SDS and 0.2 M NaOH (made up from separate solutions of 10% SDS and 2 M NaOH). The tube was then shaken and placed on ice. When complete lysis had occurred, 150 µl ice cold acetate solution (3 M K-acetate and 5 M glacial acetic acid) was added, the tube shaken and returned to the ice for at least 10 min. The tube was then centrifuged for 5 min and the supernatant collected. Depending on the subsequent use of the plasmids, they were either immediately precipitated or subjected to further purification. The plasmids were precipitated (§2.4.2) and resuspended in up to 100 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Where plasmids free of contaminating proteins were required the supernatant was extracted twice against 500 µl phenol and 500 µl chloroform. The aqueous phase was then precipitated (§2.4.2) and resuspended in up to 100 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). For dsDNA sequencing (§2.4.6), plasmids were further purified using a Qiagen column (DIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions.

ssDNA method

ssDNA was isolated from *E. coli* strains that had been grown up on M9 mininal media (Table 2.4) containing the appropriate plasmid selection antibiotic. A single colony of *E. coli* was placed into 2 ml of 2YT media (Table 2.5) containing the appropriate selection antibiotic. Helper phage (1 µl VCSM13 at 1 x 10^{13} pfu/ml; Stratagene Cloning Systems, San Diego) was added and the culture incubated at 37° C with vigorous shaking. After 1.5 h kanamycin was added to a final concentration of 70 µg/ml. The culture was then incubated for a further 16 h at 37° C with vigorous shaking. Then 1.5 ml of the culture was centrifuged at 4° C for 5 min. The supernatant was placed into a fresh tube and centrifuged for a further 5 min at 4° C. The supernatant was then placed into a fresh tube containing 300 µl of 25% polyethyleneglycol 8000 and 2.5 M NaCl, mixed by inversion and then allowed to stand on ice for at least 45 min. The tube was centrifuged for 5 min at 4° C and as much of the supernatant as possible was carefully removed. The pellet was resuspended in 100 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and then extracted against 100 µl TE saturated phenol (§2.3.1, 100 µl chloroform/isoamylalcohol (24:1) and 1 µl 10% SDS with vigorous vortexing for 1 min, allowed to stand at room temperature for 5 min, vortexed vigorously for 1 min and then centrifuged for 10 min. The supernatant (80 µl) was removed to a fresh tube containing 8 µl 3 M Na-acetate (pH 5.2) and 200 µl of ethanol. The
tube was placed at $-20^\circ$C for at least 20 min and centrifuged for 30 min. The resulting pellet was washed twice with 70% ethanol, dried briefly under vacuum and resuspended in 30 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The samples were then checked for yield by running 3 µl on a 1% agarose gel and staining with ethidium bromide (§2.4.3).

2.3.3 Plant RNA isolation

Plant material for RNA isolation was harvested and placed directly into liquid N$_2$. This material was then stored at $-80^\circ$C until extraction. At the time of extraction, material was placed into a pre-cooled mortar containing an equal amount of fine ground glass (Schott, West Germany). The material was then ground to a fine homogeneous powder in liquid N$_2$. The powder was then placed into a stirring beaker containing a 3:2 (total volume 10 ml per 1 g plant material) mixture of extraction buffer (100 mM LiCl, 1% SDS, 100 mM Tris-HCl pH 9.0, 10 mM EDTA) and phenol (§2.3.1). This mixture was then placed into a 50 ml tube on a rotary shaker for 10 min whereupon a volume of chloroform equal to the phenol was added. This was shaken on the rotary shaker for a further 30 min. The sample was then centrifuged in a Clements 200 centrifuge at 4000 rpm for 60 min, the aqueous upper phase removed and placed into a fresh tube. The same volume of equilibrated phenol and chloroform were then added to the bottle. This mixture was placed on a rotary shaker for 10 min and then centrifuged as described above for 30 min. Again the aqueous upper phase was placed into a new tube and an equal volume of chloroform was added. The mixture was placed onto a rotary shaker for 10 min and then centrifuged for 40 min as described above. The aqueous upper phase was divided into fresh Eppendorf tubes and $\frac{1}{3}$ volume of 8 M LiCl added. This was then allowed to precipitate for at least 24 h. The precipitate was then collected, washed twice with 80% ethanol, vacuum dried for 15 min and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Total RNA was quantified by spectrophotometry and the quality checked on 1% agarose denaturing gel (§2.4.3).

2.4 Nucleic acid manipulation

2.4.1 Restriction enzyme digestion

Restriction enzyme digestions were performed with commercially available enzymes according to the manufacturer’s instructions.

Single enzyme digestions were carried out by placing between 0.1 and 4 µg DNA in solution into a fresh Eppendorf tube with $\frac{1}{10}$ total reaction volume of 10× manufacturer’s supplied reaction buffer, between 2 and 10 Units of enzyme (diluted in the manufacturer’s supplied dilution buffer) and made up to the final volume with dH$_2$O. The total reaction volume was between 20 and 50 µl.
Double enzyme digestions were carried out in a similar manner. If a buffer compatible to both enzymes was not available the enzyme digestions were carried out sequentially, the DNA being salt precipitated (see §2.4.2) and resuspended in the appropriate buffer. Where a buffer compatible with both enzymes was available the same reaction as described above was set up reducing the amount of dH$_2$O added to compensate for the additional enzyme volume.

All digestions were carried out at 37° C for at least 20 min or according to the guide that 1 unit of enzyme cuts 1 µg DNA in 1 h at 37° C. Following digestion, the enzyme(s) was inactivated either by heating (at 65° C or 85° C for 20 min according to the manufacturer’s instructions) or by the addition of 0.5 M EDTA pH 8.0 to a final concentration of 12.5 mM EDTA.

2.4.2 Salt precipitation

DNA in solution was precipitated either by addition of $\frac{1}{10}$ volume 3 M Na-acetate pH 5.2 and 3 volumes of absolute ethanol or $\frac{1}{2}$ volume 7.5 M NH$_4$-acetate and 2 volumes of isopropanol. Following this addition, the sample was then placed at -20° C for at least 30 minutes and then centrifuged in an Eppendorf centrifuge at 12 000 rpm at 4° C or centrifuged in an Eppendorf centrifuge at 12 000 rpm at room temperature respectively. The pellet was then washed 2 times with 70% ethanol (in dH$_2$O), dried briefly under vacuum and then resuspended in either TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), T$_{10}$E$_{0.1}$ (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or dH$_2$O.

2.4.3 Electrophoresis

DNA

DNA molecules were separated on horizontal slab agarose gels (0.8-1% w/v) in a 50× dilution of TAE buffer (50× stock, 2 M Tris-acetate, 1 M EDTA). Low melting point agarose was used for purification of DNA (§2.4.7). Standard agarose was used for all other purposes. The progress of the gels was monitored by incorporating a loading dye (10% glycerol w/v, 100 mM EDTA, 0.03% bromophenol blue w/v pH 8.0). Gels were standardly run at 40 mA/100 V (12 Vcm$^{-1}$). Following electrophoresis the DNA was visualized on a UV transilluminator and photographed. Ethidium bromide (10 mg/ml) was either added to the agarose (1 µl in 400 ml agarose solution) or the gels were stained following electrophoresis in a bath (5 µl in 500 ml dH$_2$O).

RNA

Care was taken to ensure all equipment and solutions were RNase free. The RNA samples were diluted to 12 µl with dH$_2$O and incubated for 5 min at 65° C in 12 µl formamide, 3 µl 10×MOPS buffer (0.2 M MOPS, 0.05 M Na-acetate
If a buffer were carried along the digested enzymes was not an account. Using the digestion, for 20 min in M EDTA,

pH 7.0, 0.01 M EDTA) and 7 µl formaldehyde. The samples were then placed on ice and 6 µl loading dye added (50% v/v glycerol, 0.2 mg/ml bromophenol blue pH 8.0). Samples were loaded onto a denaturing gel (1% w/v agarose, 10% v/v 10 x MOPS and 16% v/v formaldehyde) and run using a ten fold dilution of 10 x MOPS buffer. Gels were standardly run at 40 mA/100 V (12 Vcm⁻¹). Following electrophoresis the RNA was visualized (after staining; 5 µl ethidium bromide (10 mg/ml) in 500 ml dH₂O bath or blotted directly; §2.4.8).

2.4.4 cDNA synthesis

cDNA was synthesized from 8 µg total RNA (§2.3.3) by SuperScript Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) using a poly(T)₁₇ primer incorporating a BamH1 site and a T3 primer sequence (5’- ATTAACCCTCACTAAAGGATCC(T)₇₃-3’) in a total volume of 25 µl according to the manufacturer’s instructions. Following cDNA synthesis 175 µl dH₂O was added and the sample stored at -20° C. For the purposes of dye labelled PCR the 9 µg total RNA was first DNase (Stratagene, La Jolla, CA) treated according to the manufacturer’s instructions, and then used directly in cDNA synthesis as described above.

2.4.5 Polymerase chain reaction

The polymerase chain reaction (PCR) was routinely carried out using a total reaction volume of 50 µl overlaid with mineral oil or wax and cycled at 95° C for 1 min, an annealing temperature in the range 45° C-60° C for 2 min and the an extension for 3 min at 72° C in a thermal cycler. The reaction components were according to the manufacturer’s instructions, but in most instances using 2 Units Taq DNA polymerase with the supplied reaction buffer, 0.1 mM dNTPs, 100 pmol each primer and 1.5-2.0 mM MgCl₂. Primers were obtained from the Biomolecular Resource Facility, John Curtin School of Medical Research, ANU.

2.4.6 Sequencing

The “PRISM” Ready Reaction Dye Primer Cycle Sequencing Kit was purchased from Applied Biosystems (Forster City, CA) with the -21M13 forward dye primers (5’-TGTAAAACGACGGCCAGT-3’). The kit was used according to the manufacturer’s instructions with ssDNA or dsDNA (§2.3.2). Analysis was done at the Biomolecular Resource Facility (John Curtin School Medical Sciences, ANU) using the Applied Biosystems 373A DNA Sequencing System. Sequence data was analysed using the SEQ suite of sequencing programs available at the Research School of Biological Sciences, ANU. This includes the programs described by Devereux et al. (1984).
2.4.7 Cloning of DNA inserts

Preparation of vector and insert

For subsequent ligation both the vector and insert were digested with enzymes (§2.4.1) to give cohesive ends. Following digestion the enzymes were heat killed according to the manufacturer’s instructions. Inserts generated by PCR (§2.4.5) were subject to extraction in 1 volume of phenol and chloroform followed by salt precipitation of the aqueous phase prior to restriction enzyme modification. All inserts were subject to gel purification on a 1% low melting agarose gel (§2.4.3) and then purified using either the “Prep-a-gene” DNA Purification Matrix kit (Bio-Rad, Hercules, CA) or the “Magic PCR Prep” DNA Purification System kit (Promega, Madison, WI) according the the manufacturer’s instructions. If the vector was cut so as to liberate a portion of the multiple cloning site, then the vector was cleaned with either the “Prep-a-gene” DNA Purification Matrix kit or the “Magic PCR Prep” DNA Purification System kit according to the manufacturer’s instructions. Otherwise the vector was salt precipitated (§2.4.2), washed twice in 70% ethanol and resuspended in dH₂O.

Ligation

Before ligation reactions were set up, the components to be ligated were carefully purified and quantified. All ligations were done with T4 Ligase using the “One-Phor-All Plus” buffer according to the manufacturer’s instructions. For cohesive end ligations, the DNA samples (vector and insert) were added to 1× ligation buffer, 1 mM rATP and T4 DNA ligase (5 Units) and incubated at 16° C for at least 12 h. Following ligation the enzyme was heat inactivated at 65° C for 10 min. Ligation was checked by loading an aliquot onto a 1% agarose gel (§2.4.3) and where satisfactory the remaining aliquot was salt precipitated (§2.4.2) and resuspended in 20 µl dH₂O. A 10 µl aliquot was then used for electroporation.

Electroporation of E. coli

Plate cultures of E. coli strain NM522 cells (§2.2.3) were grown up freshly on LB plates (Table 2.3) from stocks maintained on M9 minimal media plates (Table 2.4) at 4° C. Single colonies were placed into 5 ml of LB media (Table 2.3) and shaken vigorously for 6–7 h. Then 1.5 ml of this culture was placed into an Eppendorf tube and centrifuged at 10 000 rpm for 30 s. The supernatant was poured off and the pellet resuspended in 1 ml ice cold dH₂O. After brief vortexing to resuspend the pellet the tube was centrifuged as above. These steps were repeated twice. Then the pellet was resuspended in 80–100 µl ice cold dH₂O and maintained on ice. The sample was then used for electroporation within 15 min. The DNA to be electroporated was added to the competent cells and electroporated using the Gene Pulser (Bio-Rad, Hercules, CA) with a 2 mm gap cuvette at 2500 V and 25 µF capacitance (about 12.5 kVcm⁻¹). Following electroporation the cells were
placed into room temperature LB media (Table 2.3) and allowed to recover with gentle shaking for 40 min at 37°C. Aliquots were then plated out onto LB plates (Table 2.3) with the appropriate antibiotic selection.

**Selection of transformants**

Where blue/white (β-galactosidase/lacZ) plasmid selection was possible, transformed *E. coli* strain NM522 were plated out onto LB plates (Table 2.3) containing 0.05 mg/ml w/v X-gal and 0.12 mg/ml w/v IPTG and the appropriate antibiotic selection. A range of dilutions were plated out and the plates placed at 37°C overnight. The following morning white colonies were selected, grown in LB media (Table 2.3) and the plasmids isolated (§2.3.2) and restriction digested (§2.4) to confirm the transformation and verify the plasmid. Plating out the transformants and verification was repeated as appropriate. Where blue/white selection was not possible, transformed *E. coli* were plated out as described above onto LB plates (Table 2.3) with the appropriate antibiotic selection and then colony blotted (§2.4.8) and probed (§2.4.9) to confirm the transformation and verify the plasmid.

**2.4.8 Blotting**

**RNA blotting**

RNA was separated on denaturing gels as described above (§2.4.3). Following electrophoresis the gel was placed face down onto 3 layers of blotting paper prewetted in 0.05 M NaOH in a blot apparatus. The apparatus was comprised of a central platform with a reservoir at each end. The reservoirs were filled with 0.05M NaOH. Next, a cut to size nylon Hybnd N+ membrane (Amersham International, Buckinghamshire, UK) was placed onto the gel with 3 layers of blotting paper cut to size and wetted in 0.05 M NaOH. Absorbant paper towels were then layered above the set up and a weight (about 400 g) placed over the paper towels. The capillary transfer was allowed to proceed for 4-6 h, whereupon the transfer system was dismantled and the nylon membrane placed into a bottle and washed twice in room temperature 2× SSPE (diluted from 20×SSPE stock; 3.6 M NaCl, 0.2 M Na phosphate pH 7.0, 0.02 M EDTA) taking the temperature up to 42°C each time in a rotary oven (Hybaid Limited, Middlesex, UK). The blot was then ready for hybridization (§2.4.9).

**Colony blotting**

Cut circles of the nylon membranes were placed onto the agar surface for approximately 1 min and the orientation marked. The membranes were then removed to absorbent paper soaked in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 15 min. The membranes were transferred to absorbant paper soaked in neutralizing solution (0.5 M Tris-HCl pH 7.2 and 1 mM EDTA) for 7 min and then
placed on a fresh absorbant paper soaked in neutralizing solution for a further 7 min. The filters were washed briefly in 2×SSPE (diluted from 20× stock; 3.6 M NaCl, 0.2 M Na phosphate pH 7.0, 0.02 M EDTA) and allowed to air dry. The DNA was then covalently bound to the nylon membrane by exposure to a UV source (250 mJ, UV Stratalinker 1800, Stratagene, La Jolla, CA). Following linking, the membranes were washed in room temperature 2×SSPE to remove the remaining cell debris. The blot was then ready for hybridization (§2.4.9).

**Plasmid blotting**

Restriction enzyme digested plasmids (§2.4) were separated on slab agarose gels (§2.4.3). Following electrophoresis the gel was placed into a denaturing solution (1.5 M NaCl, 0.5 M NaOH) until the loading dye turned a pale yellow whereupon the gel was transferred into a neutralizing solution (0.5 M Tris-HCl pH 7.2, 1 mM EDTA) with at least one change of the neutralizing solution. The gel was then placed face down onto blotting paper pre-wetted in 0.4 M NaOH in a blot apparatus as described above, except that the reservoirs were filled with 0.4 M NaOH. The transfer was allowed to proceed for 4–6 h. Following transfer the apparatus was dismantled and the blot was placed into a bottle and washed twice in room temperature 2×SSPE (diluted from 20×SSPE stock; 3.6 M NaCl, 0.2 M Na phosphate pH 7.0, 0.02 M EDTA) taking the temperature up to 65°C each time in a rotary oven (Hybaid Limited, Middlesex, UK). The blot was then ready for hybridization (§2.4.9).

**RNA slot blotting**

RNA (5–10 µg) was placed into a tube with an equal volume of solution FFMNaA (500 µl formamide, 162 µl formaldehyde, 100 µl 10×MOPS (0.2 M MOPS, 0.05 M Na acetate pH 7.0, 0.01 M EDTA)). This was heated to 65°C for 5 min and crashed on ice. To this was added an equal volume of 20×SSPE (3.6 M NaCl, 0.2 M Na phosphate pH 7.0, 0.02 M EDTA) and $\frac{1}{10}$ volume dye (50% v/v glycerol, 0.2 mg/ml bromophenol blue pH 8.0). This solution was then applied to a slot blot apparatus (Hybri-slot, GIBCO BRL, Gaithersburg, MD) with Hybond N+ (Amersham International, Buckinghamshire, UK) pre-wetted in 2×SSPE (diluted from 20×SSPE stock). Following blotting the RNA was fixed by placing the membrane face up onto blotting paper soaked in 0.05 M NaOH for 5 min. The blot was briefly rinsed in 2×SSPE and was then ready for hybridization (§2.4.9).

**2.4.9 Hybridization**

**Probe making**

Target DNA for probe making was derived from PCR amplification (§2.4.5) of the desired insert or gel purification in low melting agarose of a digested plasmid
fragment (§2.4.7 and §2.4.3). Probes were made just prior to use. Between 2–
25 ng target DNA was placed into a tube containing ice cold 1 μl 10× Klenow
buffer (0.5 M Tris-HCl pH 7.5, 0.1 M MgCl₂, 10 mM DTT, 0.5 mg/ml BSA) and
10 μl H₂O. The tube was then heated to 95°C–98°C for 5 min, momentarily
centrifuged, and quenched on watery ice for 5 min. To this was added ice cold
1 μl 20 mM dCTP, 1 μl 20 mM dGTP, 1 μl 20 mM dTTP, 3 μl 10× Klenow
buffer, 5 μl [α-³²P dATP], 2 μl dH₂O, 5 μl random hexemers (N₆ 3.3 μg) and
2 Units Klenow fragment DNA polymerase (Pharmacia Biochemicals, Milwaukee,
WI). The sample was incubated for 4–6 h at 37°C. Following incubation 1 μl
0.25 M spermine tetrachloride was added and the sample placed on ice for at least
15 min. The sample was then centrifuged for 10 min, the supernatant removed
and the pellet resuspended in 50 μl 10 mM EDTA, 0.5% SDS (w/v). To this was
added 50 μl 10 mg/ml sheared (to approximately 300 bp) salmon sperm DNA.

For DNA hybridization, the probe was heated to 98°C for 5 min and then
added directly to the hybridization solution. For RNA hybridization the probe
was added to an equal volume of 1:1 buffered phenol (§2.3.1) and chloroform,
mixed vigorously and centrifuged. The aqueous layer was placed into a fresh tube.
The probe was heated to 98°C for 5 min and added directly to the hybridization
solution.

### DNA hybridization

Blots were pre-hybridized for at least 1 h in 3 ml 20×SSPE(3.6 M NaCl, 0.2 M
Na phosphate pH 7.0, 0.02 M EDTA), 0.5 ml Denhardt’s solution (2% w/v BSA,
2% w/v Ficoll-400, 2% w/v PVP 36000), 0.5 ml 10% w/v SDS and 6 ml dH₂O
in a bottle in a rotary oven (Hybaid Limited, Middlesex, UK). The temperature
of hybridization was calculated according to the principles set out in Sambrook
et al. (1989). If the calculated temperature was above 65°C, it was ignored and
hybridization was carried out at 65°C. After pre-hybridization the probe was
added directly to the bottle and hybridized according to the time defined by the
formula;

\[
time \text{ (in h)} = 2.5(Cot_{1/2})
\]

\[
Cot_{1/2} = 2 \left( \frac{1}{x} \right) \left( \frac{y}{5} \right) \left( \frac{z}{10} \right)
\]

where, \( x \) = weight in μg of probe added, \( y \) = complexity of probe in kb and
\( z \) = volume of reaction in ml.

Hybridization for 12 h was usually sufficient. Following hybridization the
hybridization solution was removed and room temperature 2×SSPE added and
taken up to the hybridization temperature. This solution was removed and re­
placed with 2×SSPE, 0.1% w/v SDS for 20 min at the hybridization temperature
and then repeated. If a high stringency was required a further wash at the hy­
bridization temperature was carried out with 0.1×SSPE, 0.1% w/v SDS for 20
min. The blot was then placed into a tray containing 2×SSPE at the hybridization temperature and allowed to return to room temperature with gentle shaking. The blot was then placed onto 2×SSPE wetted blotting paper, sealed in cling wrap and placed into a PhosphorImager cassette.

**RNA hybridization**

Blots were pre-hybridized for at least 1 h at 42°C in 2.5 ml 20×SSPE (3.6 M NaCl, 0.2 M Na phosphate pH 7.0, 0.02 M EDTA), 5.0 ml formamide, 0.5 ml Denhardt’s (2% w/v BSA, 2% w/v Ficoll-400, 2% w/v PVP 36 000), 0.1 ml 10% w/v SDS and 1.9 ml dH₂O in a bottle in a rotary oven (Hybaid Limited, Middlesex, UK). After pre-hybridization the denatured probe was added directly to the bottle and the hybridization carried out at 42°C. Hybridization was usually carried out for at least 12 h. Following hybridization the blot was washed twice in room temperature 2×SSPE (diluted from 20×SSPE stock) taking up to 42°C each time. This was followed by 2 washes in 1×SSPE, 0.1% w/v SDS at 42°C for 20 min each. The blot was then placed into a tray with 42°C 2×SSPE and allowed to return to room temperature with gentle shaking. The blot was then placed onto 2×SSPE wetted blotting paper, sealed in cling wrap and placed into a PhosphorImager Cassette.

**Analysis of hybridization**

Hybridization was analyzed using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**2.5 Isolation of flavonoid compounds**

All reagents used were of HPLC grade and all glass and plastic wares were silanized and baked before use (§2.3.1). Plants were germinated and cultured on cellulose acetate membranes as described above (§2.1).

**2.5.1 Tissue extraction**

At the time of sampling the roots for a time point, the roots were pooled and placed into liquid N₂ and stored at −80°C until extraction. Frozen root tissue was extracted by placing the tissue in a pre-cooled mortar and pestle with ground glass. The root tissue was homogenized to a fine powder and then placed into a tube containing 30 ml methanol. The tube was placed onto a rotary shaker for 4 h and then centrifuged in a Clements 200 centrifuge at 7000 rpm for 20 min. The methanol was placed into a fresh tube and reduced to under vacuum. The residue was extracted with 2 phase water/petroleum ether (10 ml/10 ml) and the petroleum ether phase discarded. The remaining phase was extracted against an
equal volume of water saturated n-butanol and the n-butanol fraction collected. The n-butanol was reduced under vacuum and the residue dissolved in 200 μl methanol.

### 2.5.2 Exudate isolation

Following culture, membranes were cut into smaller pieces, placed into 70 ml methanol and shaken vigorously for 12 h. The methanolic extract was centrifuged at 5000 rpm in a Clements 200 centrifuge for 30 min. The supernatant placed into a fresh tube, the pellet was re-extracted in 5 ml methanol, centrifuged and the supernatants pooled. The pooled extracts were reduced under vacuum to 2 ml. For further analysis of nod gene inducing compounds, the pooled methanolic extract was diluted to 40% in dH₂O and then fractionated on a Sep Extra-Sep column (C18 100 mg/1.0 ml Activon Scientific Products, Thornleigh, NSW) with 40%, 45% and 50% methanol with dH₂O (v/v). The eluate was then concentrated under vacuum to 200 μl.

### 2.5.3 Analysis of isolated flavonoid compounds

#### HPLC

Samples were run on an HPLC system (Waters RCM-100 module, Waters, Milford, MA) using a C-18 reverse phase column (10 cm × 8 mm diameter cartridge packed with 5 μm NVC18, Waters, Milford, MA) with a 30–80% methanol (with water, both acidified with 1% acetic acid) gradient at 4 ml/min over 25–30 min. Absorbance was measured at 280 and 350 nm on a Waters 490 Programmable Multiwavelength detector (Waters, Milford, MA). Fractions were collected every 1 min where appropriate. Data was collected every 0.5 s and analysed using the Maxima 820 software (Dynamic Solutions, Ventura, CA).

#### Bacterial assay

The nod gene inducing activity of the flavonoid compounds was assayed using *R. l. bv. trifolii* strain ANU845(pRt032::M114) containing the nodA-lacZ reporter construct (McIver et al. 1989). This gene fusion enabled the nod gene inducing activity of the various fractions to be accessed by quantifying β-galactosidase activity. β-galactosidase activity was assayed spectrophotometrically using a method modified from Miller (1972) as described by Djordjevic et al. (1987b). *R. l. bv. trifolii* strain ANU845(pRt032::M114) cultures were grown up for 20 h in 2 ml BMM media (Table 2.6) at 28° C. A portion of this culture was then placed into 2 ml fresh BMM media and grown up to OD₆₀₀ 0.2–0.25 at 28° C. The induction with appropriate controls was set up by placing 200 μl of cells with 1200 μl H₂O and 200 μl of inducing sample (for control used 10⁻⁶ M dihydroxyflavone, a known inducer; Djordjevic et al. 1987b) into an tube and incubating this mixture
at 28°C in the dark for 2 h. The β-galactosidase activity was then assayed in triplicate by placing 400 µl (un)induced cells into a fresh tube and adding 400 µl Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM mercaptoethanol, pH 7.5), 12 µl chloroform and 25 µl 0.1% SDS (w/v). The remaining cells were placed on ice for later OD₆₀₀ reading. To each of the assay tubes 160 µl ONPG (4 mg/ml in 0.1 M phosphate buffer pH 7.0) was added and the tubes placed in a 30°C waterbath for 20-40 min. The time was recorded accurately. At the completion of incubation (when colour developed in the induced controls) 400 µl 1 M Na₂CO₃ was added to stop the reaction. The cells were then briefly centrifuged to remove cell debris and the OD₄₂₀ measured. The OD₆₀₀ of the cells on ice was measured.

The β-galactosidase activity was calculated as follows;

\[ x = 1000 \left( \frac{a}{btv} \right) \]

where, \( t = \) time of ONPG addition to stop of reaction in min, \( v = \) volume (ml) of culture used in ONPG reaction (that is 0.4), \( x = \) units of β-galactosidase activity (Miller Units), \( a = \text{OD}_{420} \) and \( b = \text{OD}_{600} \).
Chapter 3

Rhizobium inoculation and physical wounding

3.1 Summary

The gene(s) encoding CHS in the legume *T. subterraneum* was induced within 6 h after inoculation by the wild type *R. l. bv. trifolii* strain ANU843 and *R. l. bv. trifolii* strain ANU845(pRI4003), a recombinant strain harbouring a multicopy plasmid containing the strain ANU843 nod genes. No induction was found in uninoculated controls or plants inoculated with either the nodulation deficient *R. l. bv. trifolii* strain ANU845 (pSym−) or *R. meliloti* strain 1021, which is capable of nodulating *M. sativa* but not *T. subterraneum*. Inoculation of plant roots with *R. l. bv. trifolii* strain ANU845(pRt151) which contains only the nodDABC genes led to a pronounced root hair curling response but failed to induce CHS at 6 h. Morphological examination of the interaction between the *T. subterraneum* and *Rhizobium* in this system showed that root hair distortion (a marker of the early events in the interaction) was clearly apparent within 10 h after inoculation. This indicated that CHS induction could occur before any detectable sign of rhizobial penetration of root hairs. The addition of a crude preparation of *R. l. bv. trifolii* strain ANU845(pRI4003) lipo-oligosaccharide signals (Nod metabolites) to the plant growth medium had no effect on the expression of CHS over 36 h, although root hair distortion was apparent over this time. These treatments were then contrasted with physical wounding. Wounding the plants led to a rapid induction of CHS which occurred within 2 h. Sequence analysis of cloned CHS cDNA from pools sampled after *Rhizobium* inoculation or wounding treatments showed the gene designated CHS5 was the major induced CHS species in both treatments. Conserved sequences were found in promoters of CHS5 and *G. max Gmchs7* - a gene which has similar expression patterns in *G. max*. These findings support the view that the induction of the phenylpropanoid pathway is involved in the very early events of the *Rhizobium* infection of legumes.
3.2 Introduction

The enzyme CHS occurs at a key branch point in the phenylpropanoid pathway in plants. The CHS genes respond through transcription to a variety of developmental and environmental stimuli (Dixon et al. 1983; Hahlbrock and Scheel 1989; Dixon and Lamb 1990). The pathway has been subject to considerable analysis in a wide variety of plants (Stafford 1990). The compounds produced through the CHS branch of the pathway include phenylpropanoid signalling molecules (flavonoids and isoflavonoids) which function in the early events of the symbiotic association between legumes and the Rhizobium as inducers of bacterial nod gene expression (Djordjevic and Weinman 1991; §1.5.7). These compounds have also been shown to have a variety of other functions (Stafford 1990), although their function in roots remains unclear.

During the Rhizobium infection process bacterial penetration and proliferation is combined with the induction of a developmental response leading to the formation of a new meristem and nodule structure. The flavonoid products of CHS expression could have a role in either or both of these processes (Hirsch 1992). The following experiments therefore focus on the role of CHS in the very early pre-infection events.

To date CHS expression has been examined in a limited number of rhizobial interactions. Wingender et al. (1989) found no induction of CHS in inoculated G. max root tissues during infection by B. japonicum over 10 h. However, higher levels of CHS expression was found in nodulated root tissue 16 and 28 days after inoculation. Estabrook and Sengupta-Gopalan (1991) examined induction of CHS gene(s) following infection of G. max by B. japonicum over 4 days and were able to show different copies of the CHS gene family were specifically induced by 24 h after inoculation. Use of a plant supernodulation mutant (nts382) provided evidence that the induction of CHS copies was the result of postinfection events. Inoculation with a heterologous strain (R. meliloti) on G. max was found to increase the expression of the CHS gene family whereas the B. japonicum infection led to a specific subset of CHS transcripts. Stokkermans et al. (1992) also examined CHS expression in G. max after inoculation with B. japonicum and found only a slight increase in CHS expression 6 h after inoculation compared to 3 h inoculated and control samples. In V. sativa subspecies nigra, CHS induction was shown to peak 12 h after inoculation with R. l. bv. viceae (Recourt et al. 1992). This induction was correlated with an increased secretion of new phenylpropanoid compounds into the rhizosphere which could be detected 7 days after inoculation (Recourt et al. 1991).

The nod genes code for enzymatic gene products involved in the synthesis of lipo-oligosaccharides (LOS; Lerouge et al. 1990) which are essential for successful nodule formation. Isolated LOS has been shown to induce root hair deformation, cortical cell division and induce the expression of the early plant nodulin genes (Truchet et al. 1991; Bisseling et al. 1992; Horvath et al. 1993). Recourt et
Materials and Methods

3.3 Materials and Methods

3.3.1 Bacterial culture and plant inoculation

The *R. l. bv. trifolii* strains ANU843, ANU845(pRI4003), ANU845(pRt151) and *R. meliloti* strain 1021 (Table 3.1) were grown up to OD$_{600}$ 0.6 in BI11 medium (Hollingsworth et al. 1984; §2.2.4) at 28°C and centrifuged (Sorvall RC5D) at 5000 rpm for 5 min. The supernatants from each strain was removed and the pellet resuspended in liquid F medium (Rolfe et al. 1980; §2.2.4). These bacteria (approximately $2 \times 10^8$ cells/ml in 20 ml; §2.2.4) were allowed to recover for 1 h and then flooded onto 40 germinated seedlings (see below) on F medium plates (Rolfe et al. 1980; §2.2.4) for 1 h at 22°C in the dark. The bacterial suspension was then poured off and the plates placed at 22°C in the dark for the duration of the experiment.

3.3.2 Plant germination and growth

Seeds of *Trifolium subterraneum* were sterilized and germinated as described in §2.1. Following *Rhizobium* inoculation or wounding lids were placed over the plates, which were wrapped in foil and placed into an incubator at 22°C in the dark. Incident light was excluded by carrying out all manipulations in a darkened room to avoid the interference of light induced CHS expression in the subsequent analysis.

al. (1992) showed a *Rhizobium* strain deleted for the *nod* genes (pSym cured and unable to nodulate the host plant) was unable to induce CHS expression while under the same conditions the wild type could induce expression. One hypothesis for the induction of CHS expression was that the bacterial LOS act as elicitor-type molecules (Spaink 1992; Recourt et al. 1992), but at this time there was no direct evidence showing LOS induced CHS.

Earlier work from the laboratory isolated and characterized genomic clones of 8 members of the CHS multigene family in subterranean clover cultivar "Karridale". This characterization established that CHS occurs in this cultivar in a family of at least 9 members in a minimum of 3 clusters (Arioli 1992; Howles 1992). The results presented in this Chapter analyze the expression of the CHS genes following the interaction between the plant host and both the symbiotic infective bacteria *R. l. bv. trifolii* strain ANU843 and the LOS produced by the products of the *nod* genes in this strain. *Rhizobium* strains that produce different LOS were also inoculated onto *T. subterraneum* and the plant responses examined. The expression of CHS in the symbiotic interaction is then compared and contrasted with the expression of CHS following physical wounding.
Table 3.1: *Rhizobium* strains used in this Chapter.

<table>
<thead>
<tr>
<th>strain</th>
<th>description</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. l. bv. trifolii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>wild type</td>
<td>Rolfe et al. (1980)</td>
</tr>
<tr>
<td></td>
<td>pSym+ Nod+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>on <em>T. subterraneum</em></td>
<td></td>
</tr>
<tr>
<td>ANU845</td>
<td>pSym- Nod-</td>
<td>Djordjevic et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>on <em>T. subterraneum</em></td>
<td></td>
</tr>
<tr>
<td>ANU845(pRI4003)</td>
<td>Nod+</td>
<td>Innes et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>on <em>T. subterraneum</em></td>
<td></td>
</tr>
<tr>
<td>ANU845(pRt151)</td>
<td>Hac+ Ccd- Nod-</td>
<td>Plasinski et al. (1994)</td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>pSym+ Nod+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>on <em>M. sativa</em></td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.3 Time course of root hair curling

Germinated plants were inoculated with *R. l. bv. trifolii* strain ANU843 as described above or with F medium alone (control) and then examined and photographed using bright field microscopy at various times after inoculation. Roots were soaked for 5 min in a 0.004% solution of methylene blue prior to examination to aid visualization of the root hairs.

### 3.3.4 RNA analysis

For the RNA analysis 40 germinated seedlings were treated either by inoculation, with LOS or physically wounded with a 5 mm long scalpel blade incision in the root tissues. At the time of sampling the roots were excised and placed in liquid nitrogen and then stored at −70°C until the extraction of the RNA. Total RNA was isolated from roots by hot phenol extraction and LiCl precipitation (§2.3.3). The total RNA was quantified spectrophotometrically and checked for degradation on a 1% formaldehyde agarose gel (§2.4.3). For northern analysis, total RNA was separated in a 1% formaldehyde agarose gel (§2.4.3), alkaline blotted onto Hybond N+ (Amersham International, Buckinghamshire, UK; §2.4.8) and then probed with a randomly primed [α-32P]dATP labelled *PstI-XbaI* fragment from the second exon of *CHS2* (Arioli et al. 1994; §2.4.9). For slot blots, 6 μg total RNA was blotted onto Hybond N+ according to the manufacturer’s instructions and then probed with a randomly primed [α-32P]dATP labelled *BamHI* fragment containing the wheat ribosomal RNA gene (Appels and Dvorak 1982; §2.4.8 and §2.4.9). Hybridization was analyzed using a PhosphorImager and the ImageQuant software (§2.4.9).
§3.3 Materials and Methods

3.3.5 Isolation of bacterial signal molecules (LOS)

A strain with the multicopy plasmid carrying the nod gene region (R. l. bv. trifolii strain ANU848(pRI4003)) and a non-nodulating control strain without this plasmid (R. l. bv. trifolii strain ANU845) were grown up in BIII medium (Hollingsworth et al. 1984; §2.2.4) and Nod metabolites extracted from the entire culture in water saturated n-butanol as described by McKay and Djordjevic (1993). One hundred µl of extract was placed into plates, allowed to air dry before molten (55° C) F media (Rolfe et al. 1980; §2.1) was added and allowed to set. Ten selected germinated plants were placed onto each of 4 plates per treatment/time point and the plants cultured as described above. One hundred µl dried extract was sufficient to cause root hair curling on the plates within 24 h.

3.3.6 cDNA synthesis and cloning

cDNA was synthesized from 8 µg total RNA (§2.4.4). Aliquots (10 µl) of this dilution were then used directly in a 30 cycle PCR (§2.4.5; 1 min 95° C, 2 min 52° C and 3 min 72° C with a final extension of 15 min at 72° C) with 100 pmol of each of CHS-BAG-2 (5'-TCGAATTCCATTGTTGGTTCTGATCC-3') and Long-T3 (5'-GCTCGGAATTAACCCTCACTAAAGG-3') primers in a reaction tube containing 1.5 mM MgCl2, 0.1 mM dNTPs, 0.1 mM tetramethylammonium chloride and 2 units Taq DNA polymerase and the supplied reaction buffer (Bresatec, Thebarton, SA). The CHS-BAG-2 sequence is homologous to all the known T. subterraneum cultivar “Karridale” CHS genes (Arioli 1992; Howles 1992). The products were cloned according to standard protocols (§2.4) into pBluescript SK/KS(+) vectors (Stratagene, La Jolla, CA).

3.3.7 Dye labelled PCR

cDNA was synthesized from 9 µg DNase treated total RNA (§2.4.4). Aliquots (20 µl) were then used directly in 30 cycles PCR as described above (§3.3.6) except that the annealing temperature was 46° C for 2 min and different primers were used. 100 pmol of hex amadite labelled (Applied Biosystems, Forster City, CA) CHS-BAG-2 and 100 pmol unlabelled primer specific to CHS5 (DIOR5; 5'-ACGATATATTATTTAACATTG-3') were used. Then 1 µl was loaded onto an Applied Biosystems 373A sequencer (Applied Biosystems, Forster City, CA) equipped with the GENESCAN 672 software (Applied Biosystems, Forster City, CA) according to the manufacturer’s instructions.

3.3.8 Sequencing and analysis

Clones were sequenced using the dye primer PRISM cycle sequencing kit (Applied Biosystems, Forster City, CA; §2.4.6) and analysed on an Applied Biosystems
3.4 Results

3.4.1 Root hair curling

To place the expression of the *CHS* gene(s) into a developmental context a time course of root hair deformation was conducted following inoculation with *R. l. bv. trifolii* strain ANU843. Root hair deformation in the susceptible root zone (Bhuvaneswari et al. 1981) is one of the earliest observable morphological changes in the plant in response to the invading bacteria. Root hair deformation induced by *R. l. bv. trifolii* strain ANU843 was readily apparent 10 h after inoculation compared to uninoculated controls (Figure 3.1). The root hair deformation was apparent in all subsequent sample times after 10 h. Experiments were therefore undertaken over the time frame of 36 h following inoculation.

3.4.2 *CHS* induction following inoculation

Total *CHS* expression in dark grown seedlings was examined over 36 h following *Rhizobium* infection (Figure 3.2). Total RNA was probed with a second exon fragment from *CHS2* that hybridized to all known *T. subterraneum* *CHS* genes (Arioli 1994). *CHS* was induced within 6 h of inoculation by the wild type *R. l. bv. trifolii* strain ANU843. In contrast, inoculation with either the non-nodulating *R. l. bv. trifolii* strain ANU845 (pSym-) or *R. meliloti* strain 1021 did not induce *CHS* gene expression above background levels. The *R. meliloti* strain 1021 nodulates *M. sativa* but not *T. subterraneum* and produces sulphated LOS molecules (Lerouge et al. 1990; Truchet et al. 1991) which differ from the LOS produced by *R.l. bv. trifolii* strain ANU843 which do not contain the sulphate (McKay and Djordjevic 1993). Inoculation with a strain ANU845(pRI4003) induced *CHS* to the same extent as the wild type strain ANU843 within 6 h and declining to 12 h (Figure 3.3). Inoculation of plants with a strain containing a multicopy plasmid coding only the nodABC region (Plasinski et al. 1994) failed to induce *CHS* within 6 h (Figure 3.3), although there appears to be some induction by 12 h. The differences in the relative levels of *CHS* induction observed between Figures 3.2 and 3.3 may be accounted for by differences between experiments.

3.4.3 *CHS* induction following wounding

*CHS* induction following physical wounding was examined to contrast the response apparent following *Rhizobium* inoculation and to determine the specificity
Figure 3.1: Root hair curling. Roots of dark grown plants (A) 2 h, (B) 4 h, (C) 6 h, (D) 8 h, (E) 10 h, (F) 12 h, (G) 18 h and (H) 24 h after inoculation with (1 and 2) F medium and (3 and 4) R. l. bv. *trifolii* strain ANU843 in the region (1 and 3) nearly fully emerged root hairs or (2 and 4) just emerging root hairs behind the root tip.
Figure 3.2; Induction of total CHS expression following *Rhizobium* inoculation. Blots of total RNA samples from uninoculated control, *R. l. bv. trifolii* strain ANU843, *R. l. bv. trifolii* strain ANU845 and *R. meliloti* strain 1021 inoculations. Hybridization was analyzed with ImageQuant software of (A) northern blots (10 µg) probed with a CHS2 second exon fragment, (B) presented graphically and (C) compared to the same samples (6 µg) hybridized to a constitutive marker, rRNA.
Figure 3.3; Induction of total CHS expression following Rhizobium inoculation. Blots of total RNA samples from uninoculated control, *R. l. bv. trifolii* strain ANU843, *R. l. bv. trifolii* strain ANU845(pRI4003) and *R. l. bv. trifolii* strain ANU845(pRtl51) inoculations. Hybridization was analyzed with ImageQuant software of (A) northern blots (10 µg) probed with a CHS2 second exon fragment, (B) presented graphically and (C) compared to the same samples (6 µg) hybridized to a constitutive marker, rRNA.
Figure 3.4: Induction of total CHS expression following wounding treatment. Blots of total RNA samples from control and wounded treatments. Hybridization was analyzed with ImageQuant software of (A) northern blots (10 µg) probed with a CHS2 second exon fragment, (B) presented graphically and (C) compared to the same samples (6 µg) hybridized to a constitutive marker, rRNA.
of CHS gene induction. Physical wounding was chosen as a contrasting treatment to Rhizobium infection to test the hypothesis that the plant responses following wounding shares some similarities with the plant responses during the Rhizobium infection process (Brewin 1991). Further, analysis of another inducing treatment acts as a measure of difference for the induction of symbiosis specific CHS gene(s). Single 5 mm incisions were made in the roots of newly germinated plants and RNA was extracted at various times following treatment. The conditions were identical to those used to examine the induction of CHS following Rhizobium infection. CHS induction was rapidly induced within 2 h (Figure 3.4). The CHS mRNA levels declined to background levels by 6 h in contrast with the plants exposed to the Rhizobium inoculation (Figures 3.2 and 3.3).

3.4.4 LOS induction of CHS

Isolated LOS applied to roots has been shown to cause root hair curling, and in some systems cortical cell division and expression of some of the early nodulin genes (Truchet et al. 1991) and the release of flavonoid compounds into the exudate (Schmidt et al. 1994). A crude preparation containing R. l. bv. trifolii LOS molecules was added to the plate culture medium in sufficient quantities to cause root hair curling after 24 h in plants grown under the same conditions used to examine the induction of CHS after Rhizobium inoculation. This LOS preparation did not induce CHS over the 36 h assay period (Figure 3.5).

3.4.5 Identification of expressed CHS copies

Expressed CHS genes were cloned following cDNA synthesis and polymerase chain reaction (PCR) amplification and sequenced to survey their identity when compared to existing genomic sequences (Arioli 1992; Howles 1992). The PCR fragment lengths varied between 600 and 700 bp depending upon which CHS gene was being amplified. The cDNA was made from total root RNA isolated from the time points up to 36 h after R. l. bv. trifolii strain ANU843 inoculation and up to 12 h after wounding. A total of 32 clones from the various treatment time points were examined (Table 3.2). Sequences identical to the previously designated CHS2 and CHS3 were found in uninoculated 0 h controls in addition to a new CHS sequence (designated CHS9). CHS1, CHS5 and CHS6 were found in 6 h R. l. bv. trifolii strain ANU843 inoculated samples and CHS5 and CHS6 were found in similar 24 h and 36 h R. l. bv. trifolii strain ANU843 inoculated samples. CHS5 and CHS6 were also found in samples from the 2 h of wounding treatment. It is possible that the C-terminal sequence designated CHS9 may be that of the previously designated CHS4 or CHS8 gene, as these genes have not had their C-terminal sequences characterized. A sequence comparisons of each of the expressed CHS species confirmed the sequence and identity of expressed CHS genes and showed the position of the addition of the polyA tail (Figure 3.6).
Table 3.2: Survey of CHS genes expressed.

CHS mRNA from various time points after *R. l. bv. trifolii* strain ANU843 inoculation and wounding treatments was copied into cDNA, amplified by PCR and then cloned and sequenced. The numbers of clones sequenced corresponding to a particular CHS gene are listed showing the time point (h after inoculation or wounding) and treatment pool (wild type *R. l. bv. trifolii* strain ANU843 inoculation or wounding) from which the clone was isolated.

<table>
<thead>
<tr>
<th>CHS gene</th>
<th>0 hr</th>
<th>6 hr</th>
<th>24 hr</th>
<th>36 hr</th>
<th>2 hr</th>
<th>12 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHS1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS5</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>CHS6</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>CHS7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHS9</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

These findings indicate that CHS5 is the predominant CHS species in the mRNA pools in our system under both the inoculation and wounding treatments used, although CHS1 and CHS6 are also present.

From the sequence analysis (Figure 3.6) an antisense PCR primer was designed that was specific to CHS5 in order to confirm the presence of CHS5 as an induced CHS gene. PCR using this primer in combination with the primer that was homologous to all known *T. subterranean* CHS genes confirmed that CHS5 was present 6 h after *R. l. bv. trifolii* strain ANU843 inoculation and that it was absent from the uninoculated control 6 h sample (Figure 3.7).

### 3.4.6 Sequence comparisons

A comparison of the promoter regions could identify a (novel) consensus region which could be responsible for copy specific induction of CHS. The promoter regions of CHS5 and CHS6 (Howles 1992) were compared to the promoter of the *G. max Gmchs7*, which is the CHS gene known to be expressed in the *B. japonicum-G. max* interaction (Estabrook and Sengupta-Gopalan 1991; Akada et al. 1993b). This comparison revealed 4 regions of considerable homology between the gene promoters designated regions I - IV (Figure 3.8A and B). Regions I - III can be accounted for by conserved regions established in earlier analysis (Arioli 1992; Howles 1992), which are also present in the promoters of CHS1, CHS2, CHS3 and partially in that of CHS4 (data not shown). Region IV occurs only in CHS5 and CHS6 of the characterized *T. subterranean* CHS genes and has
Figure 3.5; Induction of total CHS expression following LOS treatment. Blots of total RNA samples from roots treated with LOSs from *R. l. bv. trifolii* strain ANU845 and *R. l. bv. trifolii* strain ANU845(pRI4003). Hybridization was analyzed with ImageQuant software of (A) northern blots (10 µg) probed with a CHS2 second exon fragment, (B) presented graphically and (C) compared to the same samples (6 µg) hybridized to a constitutive marker, rRNA.
Figure 36. CHS cDNA sequence data. CHS mRNA was copied into cDNA from various treatment time points. Amplified by PCR and then cloned. The sequence was then compared to previously cloned genomic CHS data to determine the identity of the cloned sequence. The cDNA sequence data are presented from the 5' end of the poly(A) tail for cDNA strand. The alignment program used was an iterative multiple alignment written by D. K. Smith (RSBS Computer Unit, ANU).
Figure 3.7; Dye labelled PCR of CHS5. GENESCAN of PCR products run on ABI Model 373A sequencer of (A) 6 h uninoculated control and (B) 6 h *R. l. bv. trifolii* strain ANU843. The peak at approximately 663 bp correlates with the expected size of a *CHS5* PCR product using the CHS-BAG-2/DIOR5 primer set.
Figure 3.8: Comparison of *T. subterraneum* CHS5 and CHS6 promoters and the *G. max Gmchs7* promoter. DotPlot alignments (GCG package, Devereaux et al. 1984) of the *Gmchs7* promoter (horizontal axis) against (vertical axis) (A) CHS5 and (B) CHS6 are presented for the 600 nucleotides upstream of the start codon (601-603). Regions of homology are designated regions I - IV. Alignment of the sequences corresponding to region IV (C) shows conserved sequences (boxed) and direct repeats (underlined).
two conserved features which are also evident in the *G. max* *Gmchs7* (Figure 3.8C). The first is the sequence 5'-AA(N)ATAT(N)_2CCAAT(N)ATT-3', which is the same in all three promotors and the second is an 8 base pair direct repeat, 5'-ATAAAATA-3', which is present only in the promotors of *CHS5* and *Gmchs7*.

### 3.5 Discussion

The experiments described in this Chapter set out to define *CHS* gene expression in the context of well defined morphological events in the well characterized *T. subterraneum*- *R. l. bv. trifolii* interaction. Early signs of root hair curling (Figure 3.1) indicates that the early events in the interaction are clearly apparent within 10 h following inoculation. From previous data it is possible that the initiation of events leading to cortical cell division may have occurred and infection thread formation begun within this time (Djordjevic and Weinman 1991). The experiments described in this thesis were therefore designed to examine the induction of the *CHS* gene(s) by the bacteria and isolated LOS within this time frame. To avoid the known effects of light on *CHS* induction plants were incubated in the dark (Koes et al. 1989b). To counter the possible masking of *CHS* induction by the emergence of lateral roots newly germinated seedlings were used (Yang et al. 1992). In this system lateral roots would are not initiated until well after the last time point (36 h). The expression of *CHS* following inoculation was then contrasted with a well characterized *CHS* induction response (physical wounding) in order to highlight and contrast the responses to *Rhizobium* infection and LOS application.

*CHS* expression was induced within 6 h after inoculation with the wild type *R. l. bv. trifolii* strain ANU843, while the pSym− *R. l. bv. trifolii* strain ANU845 and *R. meliloti* strain 1021 (different host range) did not induce detectable *CHS* expression (Figure 3.2). The reports of Estabrook and Sengupta-Gopalan (1991) in *G. max* and Recourt et al. (1992) in *V. sativa* subspecies *nigra* are consistent with the induction of *CHS* by the wild type strain ANU843, although the results presented in this Chapter show the rates of induction are much faster. This rapid induction is significant because the kinetics are similar to those found during incompatible infections with avirulent bacterium, for example *P. syringae* on bean (Jakobek et al. 1993). The absence of induction by the pSym− strain ANU845 is consistent with the results reported by Recourt et al. (1992). However, we did not find induction of *CHS* by *Rhizobium* with a different host range (*R. meliloti* strain 1021), whereas Estabrook and Sengupta-Gopalan (1991) found *R. meliloti* was able to induce expression of the *CHS* gene family on *G. max*. We have clearly shown that *CHS* induction occurs before any recognizable *Rhizobium* penetration of root hairs and therefore *CHS* induction is a preinfection event in *T. subterraneum* and not solely a post-infection phenomenon as concluded by Estabrook and Sengupta-Gopalan (1991).

One possible explanation for the observed *CHS* induction by the wild type
strain ANU843, but not by either the pSym− strain ANU845 or the *R. meliloti* strain 1021 was the LOS molecules excreted by the wild type strain (Spaink 1992; Recourt et al. 1992). To test this possibility, isolated LOS was applied in sufficient amounts to cause root hair curling within the assay period. The isolated LOS did not induce CHS expression (Figure 3.5). Therefore, LOS by itself does not account for CHS induction following inoculation with the wild type strain.

The use of additional *R. l. bv. trifolii* strains that contain different complements of the *nod* genes has the advantage of providing the LOS at the correct site on the plant root with any other factors that may be necessary for successful infection. Inoculation with the *R. l. bv. trifolii* strain ANU845(pRI4003) which harbours a multicopy plasmid containing the strain ANU843 *nod* (and *nif*) genes resulted in the same CHS induction kinetics as the wild type *R. l. bv. trifolii* strain ANU843. However, inoculation with the *R. l. bv. trifolii* strain ANU845(pRt151) which contains only the *nodDABC* genes and distorts and curls root hairs with no infection thread formation (Plazinski et al. 1994) did not induce CHS with the same kinetics as the wild type. These findings suggest root hair distortion does not trigger CHS induction and supports the contention that some form of “perturbation” of the root hair is required to induce CHS, such as that which occurs during root hair wall penetration and infection thread formation.

The contrast between infection and wounding was chosen to test the hypothesis that the plant’s response to *Rhizobium* infection and wounding share some similarities (Brewin 1991). Our results show some similarities. There is a similar rapid induction of CHS gene expression following *Rhizobium* infection and wounding (Figures 3.2, 3.3 and 3.4), and a predominance of the same CHS gene copy in both induced cDNA pools (Table 3.2). The absence of CHS induction following LOS addition (Figure 3.4) and the correlation of similar CHS induction following wounding and successful *Rhizobium* inoculation suggests that some physical “perturbation” of the plant cell wall/membrane may be required for the induction of CHS. Such a “perturbation signal” occurs with physical wounding and at the site of infection by a wild type *Rhizobium* degrading the cell wall soon after attachment (for example by pectinases; Mateos et al. 1992). The consequence of this “perturbation” is the activation of cell wall repair mechanisms and the formation of the infection thread (Robertson and Lyttleton, 1982) with a mobilization of vesicular bodies to the site of “perturbation” in a manner similar to host-pathogen interactions (Ridge and Rolfe 1985). The differences in outcome between the wound response and infection response leaves open the possibility that the *Rhizobium* is in some way modifying the plant’s defence response to enable at least some successful nodule forming infections (Rolfe et al. 1989).

Jakobek and Lindgren (1993) showed that a generalized defence response (including the induction of CHS expression) could be separated from a hypersensitive response and concluded that there were several levels to the defence response of plants to invading pathogens. It is conceivable that wild type *Rhizobium* infec-
Discussion

induces one of the levels of the plant defence response and then limits the later levels. Djordjevic et al. (1988) using a non-nodulating Tn5 induced adenine auxotroph of NGR234, showed that this strain could curl root hairs and initiate an infection thread but the root hairs and adjacent epidermal cells rapidly collapsed within 24 h after inoculation. This localized response closely resembled a hypersensitive response. This observation suggests that a wild type Rhizobium induces the plant’s generalized defence response and then actively limits the later and distinct hypersensitive response to enable further penetration and nodule formation (Djordjevic et al. 1987a). The CHS induction may also have a function in the abortion of infection threads leading to the plant limitation of infection reported by Vasse et al. (1993).

A comparison of the CHS5 and CHS6 promoters with the promoter of the CHS induced by B. japonicum in G. max (Gmchs7, Estabrook and Sengupta-Gopalan 1991; Akada et al. 1993b) revealed sequences conserved between these promoters (Figure 3.8). Regions I-III are common to all of the characterized clover CHS gene promoters (Arioli 1992; Howles 1992) and contain sequences with possible regulatory roles. These include a TATA box in region I, a G-box (5'-CACG1G-3') sequence (Giuliano et al. 1988) in region II and in region III a sequence (5'-CCACCAAACTC-3') homologous to sequences believed to have a role in UV and elicitor induction (Lois et al. 1989). The region IV element is found in CHS5 and CHS6 of the characterized T. subterraneum CHS genes and in the G. max Gmchs7. This suggests that region IV (possibly in combination with the other elements) could be responsible for the Rhizobium and wounding induction of CHS. Further analysis of these promoters is necessary, particularly to assess the possible regulatory functions of the regions II-IV in the plant.

Analysis of the induction of CHS in the very early stages of infection and its consequences will be necessary to elucidate the kinetics of particular CHS genes expressed in the Rhizobium-plant interaction. The results presented here suggest that the early CHS expression following Rhizobium inoculation has similarities with the plant’s response to wounding.
Chapter 4

Changes in the root exudates following Rhizobium inoculation

4.1 Summary

Earlier analysis of CHS gene expression following R. l. bv. trifolii inoculation of T. subterraneum showed induction of CHS5 within 6 h in dark grown plants. Experiments were therefore conducted to examine the time of onset of a detectable change in flavonoid exudates to determine whether the early CHS expression correlated with the exudation of new flavonoid nod gene inducing compounds involved in the early infection or pre-infection events. Plants were grown for 5 days with light or 1 and 3 days in the dark after either Rhizobium inoculation or wounding treatments. Dark growth was chosen to be consistent with the treatments described in Chapter 3. Flavonoid exudates were collected on cellulose acetate membranes and examined by HPLC and nod gene inducing activity (measured using a β-galactosidase nodA-lacZ reporter construct). Exudates were examined from plants 5 days after inoculation with Rhizobium. There was a significant difference in the nod gene inducing activity of total un-fractionated exudates between Rhizobium inoculation and uninoculated control samples. HPLC fractionation combined with a monitoring of nod gene inducing activity of each fraction revealed a nod gene inducing compound in the uninoculated controls and inoculated samples and an additional new compound in the inoculated samples. There was no significant difference in the nod gene inducing activity of total un-fractionated exudates between 3 day dark grown roots after Rhizobium inoculation and control treatments. However, HPLC fractionation combined with a monitoring of nod gene inducing activity for each fraction revealed a single new nod gene inducing compound which was tentatively identified as 4',7-dihydroxyflavone (DHF). Exudates were also collected from wounded plants as a contrast to Rhizobium infection. HPLC profiles of these exudates were considerably different from Rhizobium inoculation and uninoculated control HPLC profiles. There was no detectable changes in nod gene inducing activity of
HPLC fractions of root exudates of 1 day dark grown roots after Rhizobium inoculation. The new nod gene inducing compound in the 5 day inoculated sample was different to that observed in the 3 day sample. The presence of light in the growth conditions changes the HPLC profiles as well as the nod gene inducing compound(s). The pools of UV light absorbing compounds within the roots were examined and some differences between Rhizobium inoculation and the uninoculated controls and wounded roots were detected. The late detection (3 days) of changes in the exudates following Rhizobium inoculation does not correlate with the observed expression of CHS 6 h after inoculation.

4.2 Introduction

Flavonoids have been shown to be important signal molecules in the legume-Rhizobium symbiosis (Redmond et al. 1986; Djordjevic et al. 1987b; Hirsch 1992; §1.5.7). T. repens has been shown to exude flavonoids into the rhizosphere which either induce or antagonize nod gene expression in R. l. bv. trifolii (Djordjevic et al. 1987b). These compounds were exuded from distinct regions of the root, with the inducing compounds exuding from just behind the root tips (Djordjevic et al. 1987b). Following inoculation with infective Rhizobium there are increased amounts of nod gene inducing compounds in root washes from T. repens (Weinman et al. 1988), V. sativa (van Brussel et al. 1990; Recourt et al. 1991) and M. sativa (Dakora et al. 1993b). In V. sativa (Recourt et al. 1991) and M. sativa (Dakora et al. 1993b) this increased nod gene inducing activity was shown to be the result of new flavonoid compounds exuded into the rhizosphere. However, in G. max flavonoids that were already present before inoculation, increased in amount following inoculation (Schmidt et al. 1991,1994). These changes in the exudates following Rhizobium inoculation have only been shown to occur 10 h (Schmidt et al. 1991), 18 h (Schmidt et al. 1994), 2 days (Dakora et al. 1993b) and 7 days (Recourt et al. 1991) after inoculation. It is not clear whether the changing patterns of flavonoid exudates are associated with (a) the signalling in the preinfection stages of the interaction between the host plant and the infecting Rhizobium or (b) involved in the later stages of the infection, or (c) part of an autoregulatory response occurring after infection, or (d) as a byproduct or an artefact of the infection process.

Results in Chapter 3 showed the genes coding for CHS are induced in T. subterraneum within 6 h of inoculation by R. l. bv. trifolii strain ANU843 (§3.4.2). This CHS expression correlated with the very early events of the interaction between the R. l. bv. trifolii strain ANU843 and host plant. The relationship between the early induction of CHS and the appearance of new flavonoids is unclear. The reported late detection of the flavonoid molecules (Recourt et al. 1991; Dakora et al. 1993ab; Schmidt et al. 1994) compared to the very early CHS gene expression raises some question as to whether the early CHS gene expression is related to the biosynthesis of the exuded flavonoid compounds involved in the
early signalling events or has some other function. Other possible functions for flavonoids include a defence response to the infecting \textit{Rhizobium} (HR; Vasse et al. 1993) or the regulation of root mitotic activity necessary for nodule development (Hirsch 1992) through the possible role of flavonoids as auxin transport inhibitors.

This Chapter sets out to examine the time of onset of a detectable change in the flavonoid exudates following \textit{Rhizobium} inoculation to determine whether the early expression of the \textit{CHS} genes correlates with the reported flavonoid exudate changes following inoculation. This was achieved by isolating exudates following \textit{Rhizobium} inoculation and assessing the \textit{nod} gene inducing activity of the exudates. As a control, exudates were isolated from plants 5 days after inoculation in standard light/dark growth conditions using cellulose acetate membranes as described by Recourt et al. (1991). To more closely examine the relationship between \textit{CHS} gene expression and the exudate changes observed following inoculation, exudates were collected from plants 1 and 3 days after inoculation in dark growth conditions similar to those previously used to examine \textit{CHS} gene expression (§3.4.2). Exudates were also collected from physically wounded plants which were shown in Chapter 3 to induce the same \textit{CHS} gene in order to contrast the plant response to \textit{Rhizobium} inoculation. The flavonoid pools within the plants were extracted and examined to determine if these pools changed following inoculation and wounding treatment compared to controls.

4.3 Materials and Methods

4.3.1 Bacterial culture and plant inoculation

The bacterial strains used in these experiments are described in Table 4.1. The \textit{Rhizobium} culture for inoculation was grown up to OD$_{600}$ 0.6 in BIII media (Hollingsworth et al. 1984; §2.2.4) at 28°C and then centrifuged (Sorvall RC5D) at 5000 rpm for 5 min, the supernatant removed and the pellet resuspended in liquid F media (Rolfe et al. 1980; §2.2.4). The resuspended bacteria (approximately $2 \times 10^8$ cells/ml in 20 ml) were allowed to recover for 1 h and then flooded onto the plants for 1 h at 22°C in the dark. The bacterial suspension was then poured off and the plates placed into the appropriate growth cabinets (§2.1.2). For the \textit{nod} gene inducing activity measurements, the bacteria were grown up as described in §2.5.3.

4.3.2 Plant culture

\textit{T. subterraneum} were sterilised, germinated and placed onto cellulose acetate membranes as described in §2.1. Following \textit{Rhizobium} inoculation and wounding, lids were placed over the plates and they were prepared for culture as described in §2.1, and placed into either growth cabinets/incubators with the appropriate...
Changes in the root exudates following Rhizobium inoculation §4.3

<table>
<thead>
<tr>
<th>strain</th>
<th>description</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. l. bv. trifolii</em></td>
<td>wild type (<em>T. subterraneum</em>)</td>
<td>Rolfe et al. (1980)</td>
</tr>
<tr>
<td>ANU843</td>
<td>pSym+ Nod+</td>
<td></td>
</tr>
<tr>
<td><em>R. l. bv. trifolii</em></td>
<td>nodA-lacZ fusion</td>
<td>MclIver et al. (1989)</td>
</tr>
<tr>
<td>ANU845(pRt032::M114)</td>
<td>reporter construct</td>
<td></td>
</tr>
</tbody>
</table>

light conditions (§2.1.2). The dark growth conditions were the same as those used for the experiments described in Chapter 3. All manipulations were carried out in a darkened room to avoid the interference of light induced CHS expression in the subsequent analysis.

4.3.3 Isolation of exudates

Following plant culture the cellulose acetate membranes were cut into smaller pieces, placed into methanol for 12 h with vigorous shaking and extracted as described in §2.5.2. For further analysis of the nod gene inducing compound(s) the total exudates were fractionated as described in §2.5.2 by HPLC.

4.3.4 Extraction of root tissues

The root tissues were extracted as described in §2.5.1. Following extraction the residue was resuspended in methanol ready for HPLC analysis.

4.3.5 HPLC analysis

Samples were run on an HPLC system as described in §2.5.3. For analysis of the root tissue extracts and membrane bound exudates 5% of the methanolic sample was loaded directly onto the HPLC. For further analysis of the nod gene inducing compound the 50% methanol fraction (§2.5.2) was diluted to 40% methanol with water and 10% of the sample was loaded directly onto the HPLC using the same system except that the gradient was 40–60% methanol over 15 min and the absorbance was measured at 280 nm and 328 nm. The DHF standard (Sigma-Aldrich, Castle Hill, NSW) was made up in 40% methanol and loaded directly onto the HPLC.

4.3.6 Measurement of nod gene inducing activity

The nod gene inducing activity of total exudates and HPLC fractions was assessed using the *R. l. bv. trifolii* strain ANU845(pRt032::M114) nodA-lacZ reporter construct as described in §2.5.3. For the total exudate assay 5% of the methanolic sample (§2.5.2) was dried under vacuum and resuspended in 20 µl methanol and 180 µl water prior to assaying. For the HPLC fractions assay, 1 min fractions
Table 4.2: Summary of treatments, growth conditions and analyses. The + represents the experimental treatment, growth condition or analysis reported in this Chapter.

<table>
<thead>
<tr>
<th>treatments</th>
<th>day 1 (dark)</th>
<th>day 3 (dark)</th>
<th>day 5 (light/dark)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizobium wounding</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>material collected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extracts</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>exudates</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HPLC analysis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>extracts</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>exudates</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>bioassay - nod</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gene induction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total exudates</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HPLC fractions</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

were collected, dried down under vacuum and resuspended in 20 µl methanol and 180 µl water prior to assaying for nod gene inducing activity (§2.5.3).

### 4.4 Results

#### 4.4.1 Summary of treatment and conditions

The treatments, growth conditions and analyses used in the experiments described in this Chapter are summarized in Table 4.2.

#### 4.4.2 Extracts from roots - HPLC profiles 5 days after inoculation

The internal pool of flavonoids within the root tissue was examined by extracting from whole root tissue (minus exudate) of plants grown in a standard day/night cycle for 5 days (Figure 4.1). The UV spectrum of HPLC profiles of extracts from uninoculated control plants and Rhizobium inoculated plants were similar but showed differences in the amounts (peak heights) of individual absorbing compounds. No new peaks were detected nor were any lost (Figure 4.1). This suggests that the total internal flavonoid pools remained the same following inoculation.
Table 4.3: nod gene inducing activity of total exudates.

Root exudates were isolated and the β-galactosidase activity assayed (Miller Units) using a nodA-lacZ reporter construct and compared to control (background) and induced (10⁻⁶ M DHF) cells. Results were analyzed by one way ANOVA (F = 297.20, P = 0.0001 and DF = 17), means and standard errors (SE) calculated and significant differences indicated by different alphabet letters using a Fisher PLSD test.

<table>
<thead>
<tr>
<th>sample</th>
<th>activity (Miller Units)</th>
<th>significance</th>
</tr>
</thead>
<tbody>
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<td>a</td>
</tr>
<tr>
<td>control (induced)</td>
<td>786</td>
<td>b</td>
</tr>
<tr>
<td>3 day uninoculated</td>
<td>89</td>
<td>ac</td>
</tr>
<tr>
<td>3 day inoculated exudates</td>
<td>96</td>
<td>c</td>
</tr>
<tr>
<td>5 day uninoculated exudates</td>
<td>119</td>
<td>c</td>
</tr>
<tr>
<td>5 day inoculated exudates</td>
<td>192</td>
<td>d</td>
</tr>
</tbody>
</table>

4.4.3 Exudates from roots - 5 day HPLC profiles and nod gene inducing activity

The exudates from inoculated and uninoculated plants grown under standard day/night cycles for 5 days after absorption onto cellulose acetate membranes. The total unfraccionated exudates were first examined for their nod gene inducing activity using the nodA-lacZ reporter gene construct (strain ANU845 (pRt032::M114)) (Table 4.1). After 5 days, exudates from strain ANU843 inoculated plants showed a significant increase in the total nod gene inducing activity compared to the uninoculated plant exudates (Table 4.3). Rhizobium inoculation also led to changes in the HPLC profiles of root exudates (Figure 4.2). The changes found were predominantly typified by differences in the amounts of various peaks rather than the appearance of new peaks as described by other workers (Recourt et al. 1991), although one new peak was apparent at 7 min (Figure 4.2, panel B). Compared to the uninoculated control (Figure 4.2, panel A), strain ANU843 inoculation (Figure 4.2, panel B) led to the decline of peaks at 3 and 17 min and the increase in peaks at 5 and 16 min. To closely examine which fractions were responsible for the increased nod gene inducing activity, fractions were collected from uninoculated and strain ANU 843 inoculated plants and assessed for nod gene inducing activity (Figure 4.2, panels A and B). Both the uninoculated control and strain ANU843 inoculated exudates showed a peak of nod gene inducing activity after approximately 5 min. The new peak after 7 min in the strain ANU843 inoculated exudates showed a new nod gene inducing activity that was not present in the uninoculated control.
Figure 4.1: Extracts from roots 5 days after inoculation. HPLC 280 nm profiles of extracts from roots of plants grown with light/dark for 5 days after inoculation with (A) control (F media) and (B) *R. l. bv. trifolii* strain ANU843.
4.6.4 Extracts from roots - HPLC profiles 5 days after inoculation

Figure 4.2; Exudates from roots 5 days after inoculation. HPLC 280 nm profiles (trace) and nod gene inducing activity (Miller Units; bar chart) of exudates isolated on cellulose acetate membranes from roots of plants grown with light/dark for 5 days after inoculation with (A) control (F media) and (B) *R. l. bv. trifolii* strain ANU843. Arrows indicated enhanced (▲) or reduced (▼) peaks. Fractions were collected every 1 min and the nod gene inducing activity measured.
§4.4 Results

4.4.4 Extracts from roots - HPLC profiles 3 days after inoculation

Results in Chapter 3 showed an increase in CHS expression in dark grown plants following *Rhizobium* inoculation (§3.4.2). The experiments described here used the same conditions to investigate if the CHS expression led to detectable changes in flavonoid biosynthesis. The effects of *Rhizobium* inoculation on the internal flavonoid pools in plants grown in the dark for 3 days was examined. The HPLC profiles of root extracts of *T. subterraneum* after inoculation with strain ANU843 were compared to the uninoculated controls (Figure 4.3). Inoculation with strain ANU843 (Figure 4.3, panels A and B), led to a new peak (2 min), an enhanced peak (27 min) and a reduced peak (10 min). The response to wounding is discussed below (§4.4.6).

4.4.5 Exudates from roots - 1 and 3 days HPLC and *nod* gene inducing activity

When total unfractionated exudates from 3 day dark grown plants were examined for *nod* gene inducing activity (Table 4.3) there was no significant differences in exudates from *R.l. bv. trifolii* inoculated compared to uninoculated plants. Consistent with this result, the HPLC profiles of exudates of plants exposed to strain ANU843 showed only small differences when compared to uninoculated controls after 3 days growth in the dark (Figure 4.4). However, when specific fractions were tested following HPLC separation the *nod* gene inducing activities of 3 day strain ANU843 inoculated and control plant exudate fractions were considerably different (Figure 4.4). There was a new inducing fraction (13 min) and a reduced inducing fraction (8 min) in the inoculated plant exudates (Figure 4.4). This inducing fraction confirms that there was a masking of this activity in the unfractionated sample which suggests the presence of anti-inducing compounds in the total unfractionated assay (Table 4.3). This new inducing fraction was different to the inducing fraction found after 5 days in light grown plants (Figure 4.2, panel B).

Exudates were also collected from plants 1 day after inoculation (Figure 4.5). The HPLC profiles of the exudates from uninoculated control and strain ANU843 inoculated plant exudates were similar. There was no increased *nod* gene inducing activity in any of the isolated fractions. The difference in intensity between peaks compared to those observed after 3 days (Figure 4.4) probably reflects a low yield of exudate compounds. This finding suggests either the amounts of UV absorbing compounds in the exudates was very low after 1 day or the technique used to collect exudates was not very efficient.
4.4.6 Extracts and exudates from wounded plants

Root extracts (Figure 4.3, panel C) and exudates (Figure 4.4, panel C) were also examined from plants that were wounded. While the control, *Rhizobium* inoculation and wounding HPLC profiles of internal pools of UV absorbing compounds were very similar (Figure 4.3, panel C versus Figure 4.3, panels A and B), the exudate profiles were very different (Figure 4.4, panel C versus Figure 4.4, panels A and B). In the extracts (Figure 4.3, panel C), there was a new peak (2 min), enhanced peaks (6 min, 14 min and 27 min) and a reduced peak (10 min and 17 min). The similarities in the *Rhizobium* inoculated and wounded treatments were the new peak (2 min) and the reduced peaks (10 min and 27 min). In the exudates the appearance of the HPLC 280 nm profile was very different to those found in untreated controls and following *Rhizobium* inoculation with all peaks reduced except for the peak at 30 min (Figure 4.4, panel C). In the exudates the *nod* gene inducing activity of the fractions was similar to the uninoculated control and the new *nod* gene inducing activity detected after *Rhizobium* inoculation was not present (Figure 4.4, panel C).

4.4.7 Analysis of new exuded *nod* gene inducing compound 3 days after inoculation

The total exudate from the 3 day *R. l. bv. trifolii* strain ANU843 inoculated plants was separated into fractions using a preparative column, and the fraction containing the new *nod* gene inducing activity run on an HPLC at 280 nm (Figure 4.6, panel A), 328 nm (Figure 4.6, panel B). Fractions were collected and the *nod* gene inducing activity determined (Figure 4.6, panel C). Analysis of this peak indicated that the compound could be DHF. This was concluded based on the following results. (a) The retention time of an authentic DHF standard was identical to the inducer (Figure 4.6, panel D), (b) both DHF and the inducer have a $\lambda_{\text{max}}$ of 328 nm, (c) both the inducer and DHF have a similar ratio of peak heights at 328 nm to 280 nm, and (d) when the root exudate sample was spiked with authentic DHF, the DHF co-eluted with the inducer in the sample. DHF is a known *nod* gene inducing compound in *T. repens* (Redmond et al. 1986), but this is the first report of this compound in *T. subterraneum*.

4.5 Discussion

Flavonoid molecules exuded into the rhizosphere have been shown to induce *Rhizobium nod* genes in a number of different legume-*Rhizobium* systems (Peters et al. 1986; Redmond et al. 1986). Using the cellulose acetate membrane method to collect root exudates (Recourt et al. 1991), we show a detectable change in the spectrum of *nod* gene inducing compounds in the exudates of *T. subterraneum* inoculated with *R. l. bv. trifolii* at day 3 in dark grown plants and at day 5 in
Figure 4.3: Extracts from roots 3 days after inoculation and wounding. HPLC 280 nm profiles of extracts from roots of plants grown in the dark for 3 days with (A) control (F media), (B) R. l. bv. trifolii strain ANU843 and (C) wounding treatments. Arrows indicated enhanced (△) or reduced (▽) peaks.
Figure 4.4; Exudates from roots 3 days after inoculation and wounding treatments. HPLC 280 nm profiles (trace) and nod gene inducing activity (Miller Units; bar chart) of exudates isolated on cellulose acetate membranes from roots of plants grown in the dark for 3 days with (A) control (F media), (B) R. l. bv. trifolii strain ANU843 and (C) wounding treatments. Arrows indicated enhanced (▲) or reduced (▼) peaks. Fractions were collected every 1 min and the nod gene inducing activity measured.
Figure 4.5; Exudates from roots 1 day after inoculation. HPLC 280 nm profiles (trace) and nod gene inducing activity (Miller Units; bar chart) of exudates isolated on membranes from roots of plants grown in the dark for 1 day after inoculation with (A) control (F media) and (B) *R. l. bv. trifolii* strain ANU843. Fractions were collected every 1 min and the nod gene inducing activity measured.
Figure 4.6: Inducing peak compound correlates with 4’,7-dihydroxyflavone. Exudate fractions containing the nod gene inducing activity were further separated on a preparative column and the HPLC profiles determined at (A) 280 nm and (B) 328 nm, and compared to (C) nod gene inducing activity of half min fractions, and (D) HPLC 328 nm profile of a commercial DHF standard.
light grown plants (Table 4.3 and Figures 4.3 and 4.5). Other reports are consistent with this finding (Recourt et al. 1991; Schmidt et al. 1991, 1994; Dakora et al. 1993ab). In this study the new compound exuded by dark grown roots 3 days after *R. l. bv. trifolii* strain ANU843 inoculation appears to be DHF, which is a known potent inducer of *nod* genes (Djordjevic et al. 1987b).

The results presented in this Chapter show that *Rhizobium* inoculation, light and a combination of light and *Rhizobium* inoculation affect the composition of exudates, including the *nod* gene inducing compounds (Figures 4.3 and 4.5). Uninoculated (sterile) roots have been shown to exude *nod* gene inducing flavonoids (Redmond et al. 1986; Maxwell et al. 1989; Graham 1990) that were synthesized just prior to exudation (Maxwell and Phillips 1990) and not derived from sloughing off of root cap cells during root growth (Maxwell and Phillips 1990) nor the mobilization of pre-existing pools of glycosylated flavonoids (Graham 1990). These flavonoid *nod* gene inducing compounds exuded into the rhizosphere are developmentally regulated (Graham 1990; Maxwell et al. 1989; Maxwell and Phillips 1990) and their composition is affected by environmental conditions (Graham 1990). The *nod* gene inducing compounds are exuded from the region just behind the growing root tip (Djordjevic et al. 1987b; Peters and Long 1988) which coincides with the zone on the plant root most susceptible to *Rhizobium* infection (Bhuvaneswari et al. 1981). Light affects the distribution of flavonoids within root tissues (Graham 1990) and CuCl₂ treatment has been shown to affect the distribution of flavonoids in the root exudates (Maxwell and Phillips 1990). *Rhizobium* inoculation also alters the composition of flavonoids in the exudates (Maxwell et al. 1989; Recourt et al. 1991; Dakora et al. 1993ab; Schmidt et al. 1991,1994), although some of these results are inconsistent. For example, Zaat et al. (1989) describe 7 inducers of *R. l. bv. viciae* nod genes from sterile uninoculated *V. sativa* exudates while Recourt et al. (1991) using the same material describe only 2 inducers in uninoculated root exudates and 8 new *nod* gene inducing compounds in inoculated root exudates. This inconsistency in the results may be an artefact of the growth conditions, but illustrates the complexity in assessing the exudate response of the plant to various treatments and combinations of treatments. Whether the *Rhizobium* induced changes in the exudates are involved in signalling in the early events of the interaction is still unclear, but our findings suggest that the reported changes may occur too late to be involved in the early pre-infection signalling process.

Results in Chapter 3 showed *CHS* was induced within 6 hour of *R. l. bv. trifolii* inoculation using dark grown seedlings (§3.4.2). Similar *Rhizobium* inoculation induced *CHS* expression has been reported in other systems (Estabrook and Sengupta-Gopalan 1989; Recourt et al. 1991; Stokkermans et al. 1992; Jung-hans et al. 1993). This early expression of the *CHS* gene(s) has been correlated with the increased amounts of flavonoid molecules in the exudates (Recourt et al. 1991), and in *G. max* an inhibitor of flavonoid biosynthesis has been stated to inhibit this exudation of flavonoids (no data shown, Schmidt et al. 1994). This
Changes in the root exudates following Rhizobium inoculation

§4.5 raises the question of whether in our system there is a correlation between the time of *R. l.* bv. *trifolii* induced *CHS* gene expression and the time at which a *nod* gene inducing change in the exudates were detectable.

Plants grown over 5 days in a day/night cycle showed a clear change in *nod* gene inducing exudates following inoculation (Table 4.3 and Figure 4.2). This result was consistent with the report of Recourt et al. (1991), although in *T. subterraneum* there were not as many new compounds exuded and the changes were not as marked. Similarly, when the detection technique was extended to plants grown for 3 days in dark growth conditions (excluding light), a change in the *nod* gene inducing patterns were still detectable following *R. l.* bv. *trifolii* inoculation (Figure 4.4). The differences between the *nod* gene inducing activities found in total exudates (Table 4.3) and fractions (Figure 4.4) may be accounted for by the presence of anti-inducers in the total exudates (Djordjevic et al. 1987b). When the exudates were examined from the 1 day samples, and closer to the time where induced *CHS* expression is first detectable, no major changes in *nod* gene inducing activity were observed (Figure 4.5). One possible explanation is that the exudation of new flavonoid compounds is localized to the infected root hair cells at the time of the interaction and that an accumulation of the *nod* gene inducing compounds with increasing numbers of infected cells is not clearly detectable until 3 days after inoculation. Further, induced *CHS* was detectable over at least 36 h (§3.4.2) and there may be a slow increase in the detectable amounts of *nod* gene inducing compounds. Therefore, these results do not establish a link between the observed *CHS* expression and the exudation of new *nod* gene inducing flavonoids. However, the observation by Schmidt et al. (1994) that an inhibitor of flavonoid biosynthesis inhibits exudation is strong evidence of a correlation between the gene expression and exudation. The rate of flavonoid accumulation has been shown to have a considerable lag behind gene expression. Chappel and Hahlbrock (1984) showed half maximum *CHS* mRNA occurred in *P. hortense* cell suspension approximately 5 h after UV exposure and that flavonoid glycoside achieved half maximum levels after approximately 20 h. In the plate culture system used in this thesis the accumulation of flavonoids might be expected to be slower than a suspension culture as not all cells are responding at the same time and the number of cells responding is considerably less.

For the *nod* gene inducing compounds in the exudates to have a role in signalling between plant and *Rhizobium* that induce their expression, the inducing compounds would need to be exuded within hours of inoculation as the events in infection proceed very rapidly (within hours; Djordjevic and Weinman 1991). One day after inoculation under dark growth conditions, root hair curling has occurred and the infection threads initiated (Huang et al. 1988; §3.4.1). Therefore, a detectable change in the *nod* gene inducing compounds might be expected, but was not apparent (Figure 4.5). Interestingly, Schmidt et al. (1991,1994) show increased *nod* gene inducing compounds in *G. max* root exudates 10 and 18 hr after very high dose *Bradyrhizobium* inoculation respectively. Schmidt et al. (1991)
§4.5 Discussion

The flavonoid compounds derived from the seed coat may be the nod gene inducing compounds involved in the early signalling and infection events (Hungaria and Phillips 1993). The seed coats are known sources of nod gene inducing compounds (Phillips 1992), and removal of some of these flavonoids has been shown to delay nodulation (Hungaria and Phillips 1993). The seed nod gene inducing compounds are synthesised during seed development and are not the result of post germination synthesis (Phillips 1992). Given the inability to detect changes in the nod gene inducing compounds until some time after inoculation (Figures 4.3, 4.5 and 4.6) and the likely contribution of the seed coat nod gene inducers, it is possible that the changes in nod gene inducers detected after inoculation may not be associated with the early signalling processes, but they may have some other function. For example, Vogt et al. (1994) found that the flavonoid kaempferol had a role in both pollen germination and defence, and that these functions were separated by time. The CHS expression occurring upon pollination produced new kaempferol too late for these molecules to have a function in pollen germination. It was concluded that the pre-existing kaempferol was involved in germination and the new kaempferol had a role as part of the plant’s defence response. Similarly, the new flavonoids resulting from the induction of the phenylpropanoid pathway following inoculation may have a role as part of the plant’s induced defence response.
Chapter 5

General Discussion

5.1 Introduction

The aim of this study was to examine the regulation of the CHS multigene family in *T. subterraneum* with a view to understanding transcriptional control of this gene family. The interaction between the host plant *T. subterraneum* and its symbiont *R. l. bv. trifolii* was chosen as a model system in which to examine CHS expression because the flavonoids that are synthesized as a result of CHS enzymatic activity have been shown to be involved in the infection of legumes by *Rhizobium* (§1.5.7). The *Rhizobium*-legume interaction has been well characterized so that the kinetics of gene(s) expression can be placed into a context of the developing symbiosis. Then gene expression patterns can be related to specific steps in the infection process and perhaps provide some insight into the regulation of CHS gene expression and the possible functions of the resulting flavonoids. CHS induction resulting from wounding was used as a contrasting treatment to *Rhizobium* infection. The wounding process has been proposed to share some similarities with the *Rhizobium* infection process and it leads to a rapid induction of CHS as part of the plant’s induced defence response (§1.5.5).

The key findings reported in this thesis are set out in Table 5.1. This thesis showed that the *T. subterraneum* CHS genes are expressed in the root tissues in the very early stages (within 6 h) of the *Rhizobium*-legume interaction and that CHS5 is the major induced CHS gene following both inoculation and wounding treatment. Flavonoid compounds within and exuded from these plants were examined in order to correlate the CHS gene expression with the production of specific flavonoid compounds. The results obtained suggest that CHS expression (Chapter 3) may be associated with the exudation of a new flavonoid compound into the rhizosphere (Chapter 4), although this compound was not detected until some time after the observed CHS gene induction. The wounding treatment however, resulted in different end products even though CHS5 was induced by both *Rhizobium* inoculation and wounding treatment (Chapters 3 and 4). These results link a defined stimulus with the induction of a specific gene and the
exudation of a specific compound.

The remaining sections of this chapter examine the results presented in this thesis in the context of the involvement of CHS and flavonoids in the legume-Rhizobium interaction (§5.2). This is followed by an examination of possible modes of perception and signal transduction that result in plant gene expression (§5.3) and then an analysis of some of the possible regulatory mechanisms that may account for the observed induction of CHS in T. subterraneum (§5.4).

5.2 CHS in the legume-Rhizobium symbiosis

The interaction between the plant host and the Rhizobium is characterized by identifiable steps in the infection process leading to the formation of the successful nitrogen fixing nodule (§1.5.7). The analyses described in this thesis examined the activity of the CHS genes in the very early stages of this interaction (between 6 h and 36 h after inoculation). This period would have included the attachment of the Rhizobium to the root hairs, infection thread formation and the beginning of cortical cell divisions (Huang et al. 1988; Djordjevic and Weinman 1991; §3.4.1; Figure 1.6A). The results presented in this thesis show CHS is induced in the very early stages of this interaction, within 6 h after inoculation (§3.4.2), and support the view that the induction of the phenylpropanoid pathway is involved in the early infection events of the interaction. The consequences of the gene expression and the resulting flavonoids however, remains to be determined. The following discussion examines the possible involvement of this gene expression and the resulting flavonoids in the early infection events following Rhizobium inoculation.

5.2.1 Flavonoids in the early pre-infection events

The results presented in Chapter 3 show CHS5 is rapidly induced by the wild type R. l. bv. trifolii strain ANU843 within 6 h (§3.4.2) and in Chapter 4 that the new nod gene inducer DHF is not detectable until 3 days after inoculation (§4.4.5, §4.4.7). The CHS expression and the reported late detection of new flavonoids in the exudates (Recourt et al. 1991; Dakora et al. 1993ab; Schmidt et al. 1991, 1994) suggests the new nod gene inducing flavonoids are exuded too late to have a role in the early pre-infection events since root hair curling and infection thread formation have occurred by this time (§4.5). The flavonoids in the seed coat and uninoculated root washes are most likely the flavonoid inducing compounds responsible for the pre-infection induction of nod genes. However, in the field where bacterial numbers and fitness may be limiting (§1.5.7) the exudation of new nod gene inducing flavonoids may enhance the legume's competitive advantage by attracting more Rhizobium and increasing the possibility of successful nodulation and nitrogen fixation (§4.5).
Table 5.1: Summary of major findings reported in this thesis.

<table>
<thead>
<tr>
<th>chapter</th>
<th>summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td>• Microscopically visible root hair responses to inoculation are detectable within 10 h,</td>
</tr>
<tr>
<td></td>
<td>• wild type <em>Rhizobium</em> inoculation induces <em>CHS</em> within 6 h,</td>
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<tr>
<td></td>
<td>• the <em>nod</em> genes are necessary for <em>CHS</em> induction within 6 h,</td>
</tr>
<tr>
<td></td>
<td>• <em>nodDABC</em> are not sufficient to induce <em>CHS</em>,</td>
</tr>
<tr>
<td></td>
<td>• a <em>Rhizobium</em> with different host range genes does not induce <em>CHS</em>,</td>
</tr>
<tr>
<td></td>
<td>• wounding treatment induces <em>CHS</em> within 2 h,</td>
</tr>
<tr>
<td></td>
<td>• water saturated n-butanol extracts of <em>Rhizobium</em> cultures which contain LOSs did not induce <em>CHS</em> even though there was sufficient</td>
</tr>
<tr>
<td></td>
<td>LOSs to cause extensive root hair curling,</td>
</tr>
<tr>
<td></td>
<td>• <em>CHS5</em> was the major induced <em>CHS</em> gene after inoculation and wounding,</td>
</tr>
<tr>
<td></td>
<td>• conserved regions were identified within the <em>CHS5</em> promotor compared to <em>G. max Gmchs7</em> which has similar expression patterns.</td>
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Chapter 4  
• Minor differences were detected in pools (extracts) of UV absorbing compounds within *Rhizobium* inoculated and wounded roots 3 and 5 days after treatment,  
  • 1 day (dark) after *Rhizobium* inoculation and compared to uninoculated controls there were no differences detected in either HPLC profiles of exudates or the *nod* gene inducing activity of the exudate fractions,  
  • 3 day (dark) after *Rhizobium* inoculation and compared to uninoculated controls there were differences detected in both the HPLC profiles of exudates and the *nod* gene inducing activity of exudate fractions,  
  • the new 3 day (dark) exudate *nod* gene inducing activity was correlated with DHF,  
  • 3 day (dark) after wounding treatment compared to unwounded control there were differences detected in exudate HPLC profiles but not in the *nod* gene inducing activity of exudate fractions,  
  • 5 day (light/dark) after *Rhizobium* inoculation and compared to uninoculated controls there were differences detected in HPLC profiles of exudates and the *nod* gene inducing activity of exudate fractions with a new *nod* gene inducing compound that was different to the 3 day compound.
It has been shown that flavonoids derived from the plant (and most likely from the seed coat and uninoculated root washes) have a role in determining specificity in the plant’s interaction with \((Brady)\)Rhizobium (Spaink et al. 1987; Rolfe et al. 1989). The legumes \(T. \ repens\), \(V. \ sativa\), \(M. \ sativa\), \(G. \ max\) and other legumes produce flavonoids which have been shown to have specific \(nod\) gene inducing activity with compatible \((Brady)\)Rhizobium strains (Redmond et al. 1986; Peters et al. 1986; Spaink et al. 1987; Zaat et al. 1987, 1988; Bassam et al. 1988). For example, \(T. \ repens\) produces DHF as the major \(nod\) gene inducing compound (Redmond et al. 1986; Djordjevic et al. 1987b) while \(M. \ sativa\) produces luteolin (Peters et al. 1986). Therefore it is clear that flavonoids may contribute to the host specificity (Rolfe et al. 1989).

### 5.2.2 CHS induction occurs during successful and unsuccessful infections

The techniques described in this thesis to detect both the \(CHS\) mRNA and the exuded flavonoids relies on an accumulation of mRNA and flavonoids from a population of cells within the root that are responding to the \(Rhizobium\) inoculation. These techniques do not distinguish between the rare infections of root hairs that lead to a successful infection (where a nodule is formed) and the majority of infections where the infection thread is unsuccessful (Nutman 1959). Given the relative rarity of a successful interaction and the apparent lack of correlation between the time of a successful infection and the induction of \(CHS\) gene expression (6 h; §3.4.1; §3.4.2) and flavonoid exudation (3 day; §4.4.5), it is possible that these changes are caused by unsuccessful interactions.

This hypothesis is in contrast to the results of Estabrook and Sengupta-Gopalan (1991). They examined the induction of \(CHS\) in the supernodulating \(G. \ max\) mutant nts382 which yields 40% more successful infections (nodules) than the wild type even though the number of infections in both nts382 and the wild type is the same (Gresshoff et al. 1988). \(CHS\) was found to be induced following inoculation of both the nts382 and the wild type plants, with slightly higher levels of expression in the nts382 plants. The increase in \(CHS\) mRNA was concluded to reflect the increased number of successful infections on nts382. If the signal transduction pathway leading to \(CHS\) induction is triggered by infection thread initiation (“perturbation”; §3.5), then similar levels of \(CHS\) expression might be expected in nts382 and the wild type because there are the same number of infections. The increased levels of \(CHS\) mRNA in nts382 roots might be accounted for by the increased number of successful infections through increased numbers of plant cells being penetrated (“perturbed”) by the growing infection threads. This result therefore suggests that \(CHS\) induction occurs in successful infections. Until temporal studies of \(CHS\) accumulation in situ (either by hybridization or using transgenic plants with the appropriate promoter reporter constructs) are carried out it is not possible to exclude the possibility that \(CHS\) is also expressed
in unsuccessful infections. There are examples in the literature that provide some evidence to suggest that CHS expression may also be involved in unsuccessful infections. For example, CHS has been shown to accumulate in *M. sativa* cells containing aborted infection threads and in the surrounding cells (Vasse et al. 1993).

### 5.2.3 Defence response in regulating infection

If the CHS expression observed following *Rhizobium* inoculation is part of the plant’s induced defence response (Vance 1983; Djordjevic et al. 1987a; §3.5) then a successfully infecting *Rhizobium* must exhibit resistance or suppress the plant’s defence response. This might be achieved by a specific mechanism or combination of mechanisms. For example, either (1) by a limitation of the induction or extent of the plant’s defence response at the site(s) of *Rhizobium* infection, or (2) by some active mechanism in the *Rhizobium* that evades, suppresses or provides immunity to the effects of the plant’s defence response, or (3) by the *Rhizobium* overcoming the plant’s defence response. Each of these possibilities requires biochemical and organismal specificity thereby providing a mechanism(s) for basic compatibility (host specificity; reviewed Gabriel and Rolfe 1990). For a successful infection involving a biotrophic organism it is important that the induced defence response does not lead to the death of the host cell. To further complicate the analysis there is some evidence to suggest that the plant’s defence response can be triggered and not result in cell death or damage (Hamdan and Dixon 1986; Jakobek and Lindgren 1993).

#### 5.2.3.1 A role for an HR limiting the extent of nodulation

Limitation or control of the nodule number probably occurs at several stages. It is clear that few root hairs are infected and only few infections are successful at triggering nodule formation. In addition, subsequent infections are limited after nodules have formed by the autoregulatory response and therefore the number of nodules that form are considerably less than the potential (reviewed Hirsch 1992). The development of nodules in the absence of *Rhizobium* (Caetano-Anolles et al. 1991) shows that the development of the nodule structure can be uncoupled from the infection process (Long 1989). Plants may achieve autoregulation through more than one mechanism, and Vasse et al. (1993) have shown a role for the plant’s defence response. Vasse et al. (1993) concluded that the number of infection threads resulting in termination increased as a function of time and that these aborted infection threads were located in plant cells that showed a classical HR response normally found in an incompatible interactions between plants and pathogens. They proposed that the HR in the cells containing the infection thread tips is one of the plant’s mechanisms for limiting further nodule formation. This phenomenon was apparent 7 days after inoculation in a proportion of infected cells and may represent a late onset mechanism of infection regulation. This is
unlikely to be the only mechanism of limiting infection as it was not apparent in all of the late infection threads. Further, the result of HR is cell death which does not occur in all infection threads as some may be merely “dormant” (Vincent 1974). If mature nodules are removed from plants the autoregulation response is depressed, and these “dormant” infection threads proceed to mature nodules (Nutman 1959).

Interestingly, differential induction of defence genes has been observed in cell suspension cultures exposed to elicitors. Ellis et al. (1989) found *P. vulgaris CHS* was rapidly induced by an elicitor and that a different *CHS* gene of the multigene family was induced some time later. By analogy, *Rhizobium* infection may trigger several mechanisms for gene activation, one of which is a delayed induction of the defence response leading to abortion of late infection threads by an HR. Alternatively, increased levels of LOSs in the rhizosphere resulting from LOS accumulation and increased *Rhizobium* numbers producing LOS (from induced *nod* genes) may prime the sensitivity of the root hairs so that they respond to infection by cell death (HR). There is increasing evidence that the quantity of LOS produced by the strain may influence its ability to infect a particular host (McKay and Djordjevic 1993).

Other examples of the plant HR to *Rhizobium* have been described, but in each instance they have involved *Rhizobium* mutants. For example, Djordjevic et al. (1988) showed that inoculation of *Macroptilium atropurpureum* with a *Rhizobium* NGR234 adenine auxotroph mutant resulted in rapid cell collapse. The accumulation of electron dense staining compounds at the site of aborted infection was apparent in electron micrographs (also discussed §3.5). Puhler et al. (1991) inoculated *M. sativa* with a *R. meliloti* exo mutant and found cells containing aborted infections threads and the surrounding cells had accumulated phenolic compounds in their cell walls.

A further examination of the kinetics and location of *CHS* expression in a characterized legume-*Rhizobium* system is necessary to determine the involvement of the *CHS* gene(s) in this process. Conclusive evidence showing the plant using the defence response to limit *Rhizobium* infection remains to be established, but the available evidence suggests this may be at least one of the mechanisms operating (Vasse et al. 1993).

### 5.2.3.2 *Rhizobium* evasion or suppression of the plant’s defence response

The second possibility is that the *Rhizobium* suppresses (limits) or evades (overcomes) the plant’s defence response. The mechanism(s) are likely to be complex and occur at different stages of the infection process so that pathogens, such as the closely related *Pseudomonas* (Werner 1992), are unlikely to be able to mimic the *Rhizobium* strategy over evolutionary time.

*Rhizobium* lipo-polysaccharide (LPS) mutants generally exhibit defects in the infection process with aborted infections, and any nodules that are induced are incompletely developed (the *Ndv*– phenotype). It is thought that LPS might be
involved in evasion or suppression of the plant’s defence response (Djordjevic et al. 1987a). This response varies between plant hosts suggesting some additional complexity (Noel 1991). Evidence for roles of specific LPS structures in the later stages of nodule development and in mature symbiosomes have been presented (van den Bosch et al. 1989). In the infection process, the bacterial LPS forms a continuous layer outside the outer membrane of the *Rhizobium*. This LPS could be involved in recognition and/or disguise of the infecting *Rhizobium* thereby sustaining the infection (Long 1989). However, there is no accounting for the apparent contradiction that LPS is a known elicitor of the plant defence response (Goodman 1980), unless the *Rhizobium* changes/modifies the LPS so that it is no longer recognized by the plant. Consistent with this, Reuhs et al. (1994) have shown that *R. fredii* responds to the exuded flavonoid apigenin by modifying the LPS O-antigen and other polysaccharides. Djordjevic et al. (1987a) proposed the LPS might have a protective role masking elicitors of the defence response or preempting the host defence response. There is some support for this proposal. For example, Yang et al. (1992) showed enhanced CHS expression in nodule tissues containing a *Rhizobium* unable to synthesize the O-antigen containing LPS 1 compared to nodule tissues containing the wild type bacteria. The LPS might also serve a function on the bacterial cell surface to prevent access of plant defence products (such as phenolics and degrading enzymes) to the *Rhizobium* (Nikaido and Vaara 1985; Noel 1991). The abundance of LPS may be necessary to suppress the effects of the plant’s defence response allowing normal infection, so that reduced levels of LPS make the *Rhizobium* susceptible to the plant defence products thereby limiting infection. This conclusion would account for the observation that the mutant *R. leguminosarum* strain CFN42 which produces reduced levels of the wild type LPS but has an Ndv⁻ phenotype like other LPS mutants which do not produce LPS (Cava et al. 1989).

Exopolysaccharide (EPS) mutants also exhibit defects in the infection process with aborted infections, and any nodules that are induced are incompletely developed (Exo⁻ phenotype). The EPS may have a similar role to the LPS. However, the roles and mechanisms of both the LPS and EPS action and other oligosaccharides and polysaccharides (the capsular polysaccharides and the β-1,2-glucans) remain inconclusive (reviewed Rolfe et al. 1989). Further analysis is necessary to determine their roles in the infection process and establish their roles, if any, in “controlling” the plant’s defence response(s). The use of transgenic plants containing the *Rhizobium* induced CHS5 linked to a reporter gene will provide a new tool for this analysis. An examination of the kinetics of CHS5 induction using a range of mutants may give new insight into the roles of these compounds.

The involvement of plant secreted lectins in the *Rhizobium*-legume interaction is unclear. There is some evidence for lectin mediated attachment of *Rhizobium* to root hairs (reviewed Lugtenberg et al. 1991). This may be a specific recognition event (Dazzo and Hubbell 1975; Kijne et al. 1985) that has a role in mediating the plants response to the interaction. The involvement and role of lectins in these
interaction events remains speculative (reviewed Rolfe et al. 1989). It has been proposed that lectins titrate bacterial surface molecules which would otherwise trigger the plant defence response making the *Rhizobium* more "palatable" to the plant (Djordjevic et al. 1987a).

The LOS molecule coded for by the *Rhizobium nod* genes (§1.5.7) may also have a role in modulating the plant’s defence response. Inoculation of *T. subterraneum* with the wild type *R. l. bv. trifolii* strain ANU843 containing the pSym plasmid and a full compliment of the *nod* genes induced CHS, while inoculation with the pSym" strain ANU845 without the *nod* genes did not induce CHS (§3.4.2). To examine the possibility that the LOS molecule(s) associated with the *nod* genes induce CHS, LOSs were isolated and applied to plants in sufficient quantities to cause root hair curling (§3.4.4). This did not induce CHS suggesting the LOS molecule(s) alone were not the factor triggering the induction of CHS and leaves open the possibility that LOSs might modulate the plant’s defence response. Stacey et al. (1993) suggest the host specific nodulation genes (and possibly the common *nod* genes) may be analogous to avirulence genes which change the LOS so that it is not recognized by a plant resistance gene product. This may account for the observed genotype specific nodulation in some interactions (Lewis-Henderson and Djordjevic 1991; reviewed Stacey et al. 1993).

There are reports showing LOS induction of CHS in *M. sativa* (Savoure et al. 1994) and exudation of increased levels of flavonoids in *G. max* by isolated LOSs at concentrations of M"7 (Schmidt et al. 1994). The quantification of LOSs was not possible for the experiments described in §3.4.4, so it is possible that the discrepancy reflects the use of too low concentrations of LOS in these experiments. However, it is not known what concentrations of LOS are physiologically relevant and the addition of 10-7 M LOS is 5–6 orders of magnitude higher than needed to elicit a root hair curling response (Truchet et al. 1991). Therefore, at these high concentrations, LOS(s) in the rhizospere may act as elicitors. The LOSs and chitin both have β(1–4)glucosamine backbones (Figure 1.6A) and chitin oligomers between 3 and 6 units in length are elicitors of the plant’s defence response (Barber et al. 1989). Plants may also possess an oligosaccharide-dependent communication system that controls the defence response (Ryan 1988). The ability of chitosan (acetylated chitin) to induce the defence response has required high concentrations (Kohle et al. 1984; Conrath et al. 1989) and at these concentrations chitosan may act by cell toxification similar to abiotic elicitors (Ebel and Scheel 1992). It seems possible that the LOSs at high concentrations may also have the same eliciting effect as chitin. It is also possible that increased levels of LOS results in increased amounts of LOS embedded in the plant cell membrane. The conformation of the LOS fatty acyl chain is sufficiently “twisted” to cause disruption to the membrane if it is present at high concentrations and this may also be the trigger for the plant’s defence response.
5.2.3.3 *Rhizobium* overcoming the defence response

The third possibility is that the *Rhizobium* overcomes the plant’s defence responses by degrading, inactivating or developing resistance to the flavonoids. Flavonoid degradation has been reported in *Eubacterium oxidoreducens* (Krumholtz et al. 1986), *Pseudomonas putida* (Schultz et al. 1974) and *Brady/Rhizobium* species (Rao et al. 1991; Rao and Cooper 1994). These breakdown products can serve as the sole carbon source for some *Rhizobium* strains (Parke and Ornstom 1984). Inactivation of flavonoids may also occur by sulphanation (Koizumi et al. 1990) and glycosylation (Rao and Weisner 1981). These findings show that flavonoid compounds can be degraded or inactivated and this might form one of the mechanisms by which *Rhizobium* avoids the toxic effects of flavonoids exuded from roots into the rhizosphere and within the infection thread. This conclusion is supported by analogy to the infection of *P. sativum* by the Pad+/− *N. haematococca* which is either able to metabolize pisatin and infect (compatible) or unable to metabolize pisatin and unable to infect (incompatible; §1.5.5). The *Rhizobium* may also have mechanisms for extruding flavonoids thereby overcoming their toxic effects. *R. meliloti* has been shown to take up the flavonoid luteolin (Hubac et al. 1993) and in the presence of low temperatures and metabolic inhibitors the rate of luteolin uptake was shown to increased with a decrease in the rate of active luteolin extrusion (Hubac et al. 1994). Together these findings suggested luteolin may be accumulated in the membrane and extruded to avoid its toxic effects (Hubac et al. 1994).

The *Rhizobium* may overcome the defence response by developing a specific resistance to the host flavonoids. For example, *B. japonicum* has also been shown to have an induced resistance to the isoflavonoid glyceollin (Parniske et al. 1991).

### 5.2.4 Localizing CHS expression

The results presented in this thesis show *CHS5* is the major induced *CHS* gene following *Rhizobium* inoculation (§3.4.5). It is possible that the *CHS5* gene is expressed in the root hair cells in which the *Rhizobium* infections occur. To determine the patterns of *CHS* gene expression in a root hair and surrounding cells of a successful and unsuccessful infection following *Rhizobium* inoculation it will be necessary to examine the distribution of *CHS* mRNA in those cells. This is possible using the *in situ* hybridization technique and with transgenic plants containing a *CHS* promotor-reporter gene construct. These techniques have the potential to provide insight into the role(s) of *CHS* expression and flavonoids in the stages of the infection process and determine the possible involvement of other *CHS* genes. To date using *in situ* localization, *CHS* has been shown to occur in the uninfected zone of a nodule meristem (Yang et al. 1992) and within cells adjacent to aborted infections (Vasse et al. 1993). *CHS* has not however been examined in the early stages of infection, and the results presented in this thesis indicate that localization should be examined within 12 h of inoculation.
5.2.5 **CHS expression after the early infection events**

From *in situ* experiments it is apparent that *CHS* expression has a role in the latter stages of nodule development. Yang et al. (1992) found *CHS* expression in meristematic tissues which do not contain *Rhizobium*. Transgenic tobacco plants containing a bean *CHS* promoter GUS fusion also expressed in the root tip meristematic tissues (Schmid et al. 1990). The function of *CHS* expression or the flavonoids that may result from *CHS* expression in meristematic tissues has not been determined. It has been proposed that the flavonoids act as polar auxin transport inhibitors (Jacobs and Rubery 1988; Hirsch 1992). Application of the auxin transport inhibitors *N*-(*1*-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid to roots induced nodule-like structures (Hirsch et al. 1989; §3.5). However, there is as yet no direct evidence that the flavonoids resulting from the *CHS* expression or the existing flavonoid pools are the auxin transport inhibitors involved in the cell divisions leading to nodules.

Wingender et al. (1989) found waves of *CHS* expression 9, 16 and 28 days after *Bradyrhizobium* inoculation. This finding suggests that *CHS* expression may be transiently controlled through the course of the interaction and that the level of *CHS* at a particular time point may not reflect the true state of flavonoid function. This could also account for the apparent differences found in the reported results. For example, Wingender et al. (1989) and Grosskopf et al. (1993) found no differences in *CHS* mRNA levels between *Bradyrhizobium* inoculated and uninoculated root tissue, and Grosskopf et al. (1993) and Yang et al. (1992) found *Rhizobium* mutants with a similar phenotype behaved differently.

Inoculation with ineffective (Fix−) and defective *Rhizobium* has been shown to induce *CHS* some days after the early infection events. For example, Grosskopf et al. (1993) found the (Fix−) bacteria which formed nodules in a similar way to the wild type for 14 days and then senesced, induced higher *CHS* levels 16 and 18 days after inoculation than the wild type. They concluded that the mutant lacked some mechanism for avoiding the plant’s defence response and therefore that the *CHS* induction was a plant defence response to the ineffective bacteria. Yang et al. (1992) found increased *CHS* expression and impaired nodule development in nodules formed by a β(1−2)glucan mutant and an LPS 1 mutant. Parniske et al. (1990) found increased levels of the glyceollin and evidence of an HR in nodules containing genotype/strain-specific incompatible interactions between *G. soja* and *B. japonicum*. In each instance the plant limited nodule development by inducing the plant’s defence response.

### 5.3 Perception and signal transduction

The induction of the same *CHS* gene observed following wild type *Rhizobium* inoculation and wounding treatments (Chapter 3, §3.4.5) suggests that the perception (recognition) and signal transduction mechanisms within the plant leading
§5.3 Perception and signal transduction


to the induction of this gene may share some similarities. The use of *Rhizobium* strains that produced different LOSs also showed that the correct compliment of *nod* genes was necessary to be recognized by the plant as a compatible *Rhizobium* (§3.4.2). However, since different compounds are exuded by the plant following inoculation and wounding treatments (Chapter 4, §4.4.5) this suggests that there are some differences in the outcomes.

The mechanisms of perceiving microbes have been best characterized in plant-pathogen interactions. A range of models of specific recognition in plant-microbe interactions have been proposed (reviewed Gabriel and Rolfe 1990) and there are some examples of perception triggers. The application of fungal elicitors from *P. megasperma* f. sp. *glycinea* and *P. infestans* to cultured cells of *G. max*, *S. tuberosum* and *P. hortense* showed that the different plants responded to different components in the elicitor preparations (Scheel et al. 1989). This finding suggests that the mode of perception of each plant to the elicitors was different and that there are specific targets for elicitor perception (Dixon 1986; Scheel and Parker 1990; Ebel and Scheel 1992). The characterization of elicitor molecules and the identification of plant resistance genes (Martin et al. 1993) and self-incompatibility genes (Nasrallah et al. 1994) which have membrane bound, receptor structure or catalytic domains suggests that at least some of the targets are specific receptor-like binding sites on the plasma membrane. For example, the tomato *Pto* CD186 gene which compliments susceptibility to the *P. syringae* *avrPto* gene, codes for a serine/threonine protein kinase (Martin et al. 1993). It has been proposed that the *Pto* kinase interacts with a transmembrane receptor that binds directly the *avrPto* gene product (Lamb 1994). The protein kinase activity then effects the signal transduction leading to induction of the defence genes.

Receptor-like binding sites have been proposed as the mechanism by which the plant perceives the *Rhizobium* (model proposed by Hirsch 1992). This model proposes the LOSs bind to specific receptors and thereby triggers the plant responses. However, the application of isolated LOSs in sufficient quantities to cause root hair curling failed to induce *CHS* (§3.4.4) suggesting that LOS by itself is not enough to trigger the perception by the plant that leads to *CHS* expression (see above discussion §5.2.3). Although the LOSs is not by itself sufficient to triggering perception, the LOS may be a necessary component to enable the *Rhizobium* to proceed to a stage of infection that enables the *CHS* triggering perception to take place (“perturbation”; discussed above §3.5). There is some evidence to suggest that flavonoids exuded from the plant modify the *Rhizobium* associated polysaccharides (Reuhs et al. 1994) which may have specific effects on the perception of the *Rhizobium* by the plant. The *Rhizobium* polysaccharides may also function by evading/suppressing the perception (discussed above, §5.2.3).

Following perception of the *Rhizobium* the plant must then communicate that perception to the other parts of the cell and the other tissues in order to coordinate
a suitable response. The signal transduction mechanisms in plants are not well understood. However, there are several mechanisms which may be relevant to CHS induction following *Rhizobium* infection which have been examined in plant defence responses (reviewed Scheel and Parker 1990) and by analogy to animal systems (Johannes et al. 1991).

Ca\(^{2+}\) has been shown to affect the elicitor responsiveness in cell suspension cultures (Kurosaki et al. 1987; Stab and Ebel 1987; Scheel et al. 1989) and defence gene activation (Ebel and Scheel 1992). The external levels of Ca\(^{2+}\) have been shown to effect the transcription rates of elicitor induced plant defence genes (Dietrich et al. 1990). Direct measurement of Ca\(^{2+}\) fluxes showed rapid uptake of Ca\(^{2+}\) (Scheel et al. 1991). Interestingly the Ca\(^{2+}\) binding *nodO* has been shown to be necessary to achieve rapid *Rhizobium* induced nodulation (Economou et al. 1990). Ca\(^{2+}\) is unlikely to be the sole component of a signal transduction pathway as gene activation requires the further operation of proteins to effect transcription. Phosphoinositides have been shown to affect Ca\(^{2+}\) levels in animals (Berridge and Irvine 1989) and have been shown to have a role in defence gene activation in plants (reviewed Ebel and Scheel 1992; discussed further below §5.4.4). The involvement of phosphoinositides is extremely complex and poorly understood, although the tight regulation of Ca\(^{2+}\) and the important role that these compounds play in animals indicates they may have a central part in the signal transduction mechanism involving Ca\(^{2+}\) in plants.

Ca\(^{2+}\) dependent phosphorylation has been proposed as a possible mechanism of activating specific transcriptional factors by analogy to animal systems (Ebel and Scheel 1992). Elicitor treatment has been shown to alter the phosphorylation patterns of proteins in cell suspension cultures (Grab et al. 1989; Dietrich et al. 1990) and limiting external Ca\(^{2+}\) reduced this phosphorylation (Dietrich et al. 1990). The inhibition of protein kinases by inhibitors was also shown to limit phosphorylation (and PAL activity) following elicitor treatment (Grosskopf et al. 1990; Felix et al. 1991; MacKintosh et al. 1994). These results provide strong evidence for the involvement of Ca\(^{2+}\) dependent protein kinases in the signal transduction pathway leading to transcriptional activation of the defence genes. Interestingly, the binding of two *trans* factors to the H box *cis* element in defence genes is modulated by phosphorylation (Yu et al. 1993).

There is some evidence to suggest following perception of different elicitors the signal transduction pathways in different plants share some similarities as well as similar transcriptional activation mechanisms. For example, *G. max* responds to \(\beta\)-glucan elicitors by producing the flavonoid phytoalexin glyceollin while *P. crispum* responds to a protein elicitor by accumulating furanocoumarins (Ebel and Scheel 1992). The finding that the *G. max* *chs1* promoter was able to respond to elicitation in both *G. max* and *P. crispum* protoplasts (Wingender et al. 1990) indicates a common signal transduction mechanism and shared *cis* elements for responding to the elicitation stimulus (discussed below §5.4.1).

The perception and signal transduction may conceivably be combined by spe-
5.4 Transcriptional regulation of the phenylpropanoid pathway

The above discussion examined the possible roles of flavonoids and some of the mechanisms triggering the development of a symbiotic interaction between T. subterraneum and R. l. bv. trifolii. This analysis confirms that the flavonoids have a range of possible functions throughout the infection and nodule development processes. The results presented in this thesis show that the phenylpropanoid pathway is rapidly induced following Rhizobium inoculation and wounding treatment (§3.4.2 and §3.4.3). The plant recognizes the inoculation and wounding treatment (perception), communicates that recognition (signal transduction) and responds to the stimulus. In very simple terms, this response involves an interaction between a regulatory gene product(s) (trans factor(s)) and the phenylpropanoid pathway structural gene cis element(s) (§1.3.1). Activation of gene expression is only one of the responses. For example, there is some evidence of post-transcriptional regulation over the rates of accumulation of phenylpropanoid pathway enzymes (Lawton et al. 1980, 1983a).

5.4.1 5’ cis regulatory elements

Regulatory trans factors bind to cis elements in the promoters of structural genes and influence the expression of the structural genes (§1.3.1). Transcriptional factors and enhancers are composed of modular components so that a relatively small number of different factors can in various combinations be used to regulate complex patterns of gene expression (Tjian and Maniatis 1994). The assembly of the factors necessary to initiate transcription from the TATA cis element provides an example of the complexity of the mechanisms involved in gene transcription (reviewed Tjian and Maniatis 1994). Analysis of the T. subterraneum CHS5 promotor sequence revealed four regions of conserved sequence that may be associated with regulatory DNA binding proteins (§3.4.6). An analysis of other phenylpropanoid pathway genes has identified cis elements common to all these genes and correlated these elements with UV light and elicitor inducibility (Lois et al. 1989). Other regions have also been identified but they do not appear to be easily identifiable sequences (Schulze-Lefert et al. 1989b). However, a characterization and understanding of the mechanisms by which the trans factors bind these and other, as yet unidentified motifs within the phenylpropanoid pathway genes remains to be determined. The mechanisms involved in influencing transcription
are complex. Transcription has been shown to be affected by positively acting factors (Schulze-Lefert et al. 1989ab), synergism between elements (Schulze-Lefert et al. 1989b), interaction between trans factors and the need for multiple factors (Grotewold et al. 1994). There are also negatively acting elements and factors (silencers; Lawton et al. 1991; Arias et al. 1993). For example, Holdsworth and Laties (1989) have identified a nuclear factor that binds to the promotor of the D. carota extensin gene involved in the wounding response. Upon wounding the amounts of this binding factor decrease enabling increased levels of transcription.

The G box occurs in the CHS5 promotor (region II; §3.4.6) as well as a range of other light induced and stress regulated CHS genes (reviewed Dangl 1992). The sequences flanking the G box core affect the specificity of protein binding (Block et al. 1990; Williams et al. 1992) and differentiate between at least two families of G box binding proteins (Williams et al. 1992). According to the rules set out by Williams et al. (1992) the CHS5 G box is a class II element and would form type B complexes. Similar binding activities would be predicted for the S. tuberosum wound induced WIN2 (Stanford et al. 1988) and the majority of CHS genes (Williams et al. 1992). There appears to be some plasticity in the G box recognition sequence and binding activities implicating multiple G box binding factors that possess overlapping specificities (Oeda et al. 1991). The G box sequence has also been implicated in organ specific regulation in P. hybrida (van der Meer et al. 1990) and A. majus (Sommer et al. 1988) and light induction in A. majus (Lipphardt et al. 1988; Staiger et al. 1989). Further characterization of G box binding factors and their heterodimeric companions is necessary to elucidate the precise mechanisms of G box function and regulation.

The sequence 5'-CCAiC1AAC~CC-3' which also appears in the CHS5 promotor (region III; §3.4.6) has been correlated with elicitor and light responsiveness (Lois et al. 1989) and the region IV sequence is also present in a range of other stress induced (Chang and Meyerowitz 1986; Samac et al. 1990) and wound induced genes (Siebertz et al. 1989; Figure 5.1). The presence of these three regions within the CHS5 promotor that have been correlated with stress, elicitors and wounding does not provide a simple explanation for the mechanism(s) leading to CHS5 induction. Further experimentation delineating each of these regions and the possible involvement between these regions is necessary.

The H box 5'-CCTACC(N)7CT(N)4A-3' which occurs in the P. vulgaris chs15 promotor and has been correlated with induction of the defence genes following elicitors and stress treatments (reviewed Yu et al. 1993) does not appear in the CHS5 promotor. The absence of the H box in the CHS5 promotor suggests the induction of this gene by Rhizobium and wounding treatment utilises a different signal transduction pathway. Alternatively, the involvement of the H box in gene activation by these treatments may represent an artifact of the cell suspension and protoplast systems used to define the cis elements. Experiments in planta show the H box to be involved in binding a Myb like transcriptional activator in flowers and not roots, stems or leaves (Sablowski et al. 1994). This finding highlights the
Figure S1: Alignment of the CHS region IV with other stress and wound induced promoters. Alignments of the T. subterreanum CHS promoter region IV against (A) induced S. intermedica CHS and (F) A. italicura CHS. The bold '1' represents the same nucleotide in each T. subterreanum CHS sequence. The NUCALN alignments and numbering (MCG package) of the various promoters were made: k-tuple size = 3, window = 20 and a gap penalty = 2.
need to define and examine the factors and elements involved in gene regulation in a system that reflects the regulation occurring in planta. Although suspension cultures and protoplasts enable easy manipulation they probably do not reflect the true state of regulation in planta as they assume maintenance of a specific response and that all cells respond in the same way.

5.4.2 Differential regulation

The presence of multiple copies of CHS genes in T. subterraneum and other plants that encode near identical CHS enzymes suggests that either very subtle functional divergence or multiplicity of expression as a result of the variable regulatory regions within the promotors. The only evidence of functional divergence arises from the production of 5-deoxychalcones rather than 5-hydroxychalcones and this can be accounted for by a co-acting reductase enzyme (§1.4.3). There is however considerable evidence to suggest that differential regulation is achieved through regulating expression patterns of different CHS gene copies (Ellis et al. 1989; Koes et al. 1989b; Harker et al. 1990; Junghans et al. 1993).

The finding that CHS5 was the predominant induced CHS gene over a background of other copies after inoculation and wounding treatment (§3.4.5) supports the possibility that there is differential regulation of the CHS genes in T. subterraneum. These findings are consistent with other reports. For example, Harker et al. (1990) showed P. sativum CHS1 and CHS3 were expressed in petal and root tissues while CHS2 was only expressed in root tissues and in P. vulgaris a class of CHS genes detected by hybridization accounted for most of the observed induced CHS following elicitation (Ryder et al. 1987). Differential regulation of CHS has also been reported in G. max (Wingender et al. 1989; Estabrook and Sengupta-Gopalan 1991), M. sativa (Junghans et al. 1993) and P. hybrida (Koes et al. 1989b).

5.4.3 Coordinate regulation

The analysis of the exudates 3 days after Rhizobium inoculation showed a new flavonoid compound in the exudates which the data indicated was DHF (§4.4.5 and §4.4.7). DHF is a 5-deoxyflavonoid that is synthesized by CHS co-acting with a reductase and NADPH (§1.4.3). Welle and Grisebach (1989) showed the parallel induction of the reductase gene and CHS following P. megasperma f. sp. glycinea inoculation. It may be hypothesised that a similar coordinate induction of CHS5 and the reductase occur in T. subterraneum for the production of DHF.

5.4.4 myb and myc like elements

myb and myc like elements have been shown to be involved in the regulation of the phenylpropanoid pathway (§1.3.1). These regulatory genes are tightly controlled in animals and perturbation of this control has drastic consequences on
cell growth and differentiation (reviewed Shen-Ong 1990; Marcu et al. 1992). In animals, multiple protein isoforms of Myb (and Myc) are found with alternative splicing and initiation sites that may act alone or in concert with other regulatory gene products enabling complex and precise regulation of gene expression (reviewed Shen-Ong 1990). Multiple proteins are probably also present in plants enabling the same complex and precise regulation (Marocco et al. 1989).

The involvement of myb and myc like genes in the regulation of the phenylpropanoid pathway following Rhizobium inoculation and wounding treatment has not been shown. However, it seems likely that such gene products (or like gene products) may be involved in either or both the infection/wounding process and nodule development. For example, the CHS5 promotor (Howles 1992) which is activated by these treatments (§3.4.5) contains the Myb binding consensus \( \overline{GAC\overline{G}} \) at positions \(-397\) and \(-563\); Table 1.1) which has been shown to bind plant Myb like proteins (Urao et al. 1993) and the binding site 5'-CCAACC-3' for the Myb homologous P gene encoded protein (at position \(-183\); Grotewold et al. 1994). The CHS5 promotor also contains the Myc binding consensus sequence (G box). It is perhaps possible to speculate that some of the signal transduction mechanisms proposed to be involved in responding to Rhizobium may also be involved in regulating myc expression. For example, myc has been shown to be induced by LPS (Kelley et al. 1983; §5.2.3), and platelet-derived growth factor has been shown to activate myc as well as phospholipase C. Phospholipase C cleaves phosphatidylinositol to inositol triphosphate which in turn stimulates intracellular \( \text{Ca}^{2+} \) and protein kinase C (reviewed Hokin 1985; discussed above §5.3). The finding that platelet-derived growth factor mediated myc induction is linked with protein kinase C desensitization implicates a protein kinase C pathway in the platelet-derived growth factor effect on myc transcription (Coughlin et al. 1985). Similar pathways have been suggested to have a role in the response to Rhizobium inoculation and wounding (discussed above §5.3).

The expression of myc and its involvement in activating CHS5 transcription seems possible as the Myc binding G box (region II) has been implicated in transcriptional activation of stress induced CHS genes (reviewed Dangl 1992; discussed above §5.4.1). The apparent conflict between the Myc binding consensus 5'-CAGGTG-3' and the R gene product binding consensus 5'-CAGGTG-3' (Roth et al. 1991; Goff et al. 1992) suggests some plasticity in the motif, although the latter site has direct homology to the K-E2 enhancer motif (Sen and Baltimore 1986) which also binds proteins with myc homologous regions and may therefore represent a different family of trans factors. Similar variation in the cis sequences have been shown for the binding factor TAF-1 (Guiltinan et al. 1990; Oeda et al. 1991) which binds to the motifs 5'-GTACGTGGC-3' and the G box 5'-GCCACGTGGC-3' (Oeda et al. 1991). Our understanding of the mechanisms involved in transcription regulation are still naive because the proteins and genes involved in these processes have only recently been identified and there is as yet no universal plant in vitro transcription system to analyse transcription, so
that firm data relies on the production of multiple independent transgenic plants (Dangl 1992). However, the involvement of myb and myc like genes in at least some of the regulation of the phenylpropanoid pathway is certain.

myb and myc like genes most probably occur in families in plants (Marocco et al. 1989) with patterns of expression determining some of the regulation (Ludwig et al. 1989; Ludwig and Wessler 1990; Goff et al. 1990). For example, in Z. mays differential expression of the B gene (a myb homolog) has been shown to result from differences in the promotor sequence and anthocyanin accumulation was directly related to the levels of B gene expression (Radicella et al. 1992). However, a further characterization of cis elements that bind myb and myc like gene products is necessary as it is evident from animal studies that general and specific regulatory factors interact and bind the promotor sequences to activate and suppress gene expression in a complex manner. This is well illustrated by reference to animal studies. For example, in animals Myc has been shown to interact with the transcriptional factor Max (Blackwood and Eiseman 1991) to activate transcription (Amati et al. 1993) and that the factor Mxi1 inhibits Myc activation by competitively binding Max, preventing Myc-Max heterodimer activator factors and binding to Myc-Max target sites (Zervos et al. 1993). The Mad factor has been shown to bind Max forming heterodimers which are transcriptional repressors (Ayer et al. 1993). It is tempting to speculate a similar system may occur in plants as the analyses of G box binding has implicated the involvement of heterodimers in G box binding (Williams et al. 1992) and a multiplicity of proteins with G box binding activity have also been shown to interact with other proteins (DeLisle and Ferl 1990; Guiltinan et al. 1990; Oeda et al. 1991).

5.5 Future directions

An analysis of the regulation of the CHS multigene family has been undertaken in this thesis and the results presented here provide a grounding upon which further research may be based. This analysis has clearly demonstrated that there is differential regulation of the CHS multigene family in T. subterraneum and that the T. subterraneum-R. l. bv. trifolii symbiosis provides a powerful system by which mechanisms of plant gene regulation may be dissected and understood. Molecular biology techniques have been combined with biochemical analysis to dissect this interaction. A further dissection of CHS5 regulation would best be undertaken combining these techniques with cell biology and traditional biochemistry linking more defined stimuli (such as EPS and LPS) with the expression of the gene in the context of the developing Rhizobium-legume symbiosis. A broad analysis of the interaction utilizing these different techniques will provide a spatial and temporal map which is much more powerful and useful than the results from a single discipline approach. The results reported in this thesis provide the direction for at least two approaches for further research. The creation of transgenic plants with the CHS5 promotor linked to a reporter gene and the dissection of the cis
sequence motifs involved in transcriptional regulation of this gene.

The creation of transgenic plants with the CHS5 promotors identified by the analysis described in this thesis will provide a powerful new tool for dissecting the early (and perhaps later) molecular events in the *Rhizobium* infection of the roots and as a reporter system for dissecting the signal transduction mechanisms involved in responding to *Rhizobium* inoculation. Of particular interest will be the sequential distinction between the plant responses to *Rhizobium* inoculation and wounding. The results presented in this thesis show similarities and differences between *Rhizobium* inoculation and wounding treatments which have the potential to highlight key response/regulatory elements involved in perception, signal transduction and finally the expression of genes and enzyme function. The CHS5 promoter transgenic plants will also provide insight into the localization of temporal and spatial patterns of *CHS* expression. The judicious use of *Rhizobium* mutants that do not form successful infections (such as Nod, LPS, EPC, NdV, Fix, etc.) will be particularly useful for dissecting the involvement of CHS5 in the infection (and development) processes as well as providing a marker for the mechanisms that trigger gene expression.

The second approach is a dissection of the functional architecture of the CHS5 promoter. This thesis has identified by sequence similarities regions with potential regulatory functions. A dissection of these regions and other as yet unidentified motifs is necessary using transgenic plants with promotor deletions. Other *in vitro* systems do not provide a reliable assay of function. Following such a characterization the identification of *trans* factors has the potential to provide powerful evidence of transcriptional regulation. The system set out in this thesis has the potential to distinguish between *trans* factors on the basis of function by isolating proteins with binding activity from control, *Rhizobium* inoculated and wounded roots. An identification of the regulatory regions will add to the growing data available highlighting similar regions within the promotors of other phenylpropanoid pathway genes and related pathway genes. This accumulated data should provide some insight into the mechanisms of coordinate regulation of the pathways.

### 5.6 Conclusions

This thesis set out to examine the mechanisms of regulation of the *CHS* multigene family in *T. subterraneum*. This aim has been satisfied by showing that a specific stimulus results in the expression of a particular *CHS* gene copy and leads to the exudation of a specific compound. The identification of expressed members of multigene families has been a substantial challenge that required considerable effort. This has been combined with the significant task of collecting and analyzing root exudates. Combining the specificity of the molecular biology techniques developed in this thesis with these other physiological and (bio)chemical techniques has provided a new insight into the early event associated with *Rhizobium*
infection. The strength of this study lies in the use of consistent and reproducible growth conditions and materials, the use of an in planta assay system and the combination of Rhizobium treatment with uninoculated and wound treated controls. There remains a long way to go before the mechanisms underlying the regulation of this gene family are understood. However, the basis for further investigation has been laid and future work will rely on the results presented in this thesis.
Literature Cited


Its structure and variation in populations and among species, Theor Appl Genet, 63, 337-348.


Beggs, C. J., U. Schneider-Ziebert and E. Wellmann (1986) UV-B radiation and adaptive mechanisms in plants. In Stratosphere Ozone Reduction, Solar Ultraviolet Radiation and Plant Life, R. C. Worrest and M. M. Caldwell (eds),
Springer Verlag, New York, 235-250.


Charest, P. M., L. Brisson and R. K. Ibrahim (1986) Ultrastructural fea-
tures of flavonoid accumulation in leaf cells of *Chrysosplenium americanum*, Protoplasma, 134, 95-101.


Cramer, C. L., T. B. Ryder, J. N. Bell and C. J. Lamb (1985) Rapid switching of plant gene expression induced by fungal elicitors, Science, 227, 1240-
Cressy, L. L. (1968) The increase in phenylalanine ammonia-lyase activity in strawberry leaf disks and its correlation with flavonoid synthesis, Phytochemistry, 7, 441-446.


Dakora, F. D., C. M. Joseph and D. A. Phillips (1993a) Common bean root exudates contain elevated levels of diazein and coumestrol in response to Rhizobium inoculation, Mol Plant Microbe Interact, 6, 665-668.


Djordjevic, M. A., J. W. Redmond, M. Batley and B. G. Rolfe (1987b) Closers secrete specific phenolic compounds which either stimulate or repress nod gene expression in Rhizobium trifolii, EMBO J, 6, 1173-1179.
Literature Cited

Natl Acad Sci (USA), 86, 7370-7373.


Fliegmann, J., G. Schroder, S. Schanz, L. Britsch and J. Schroder (1992) Molecular analysis of chalcone and dihydropinosylvin synthase from Scots pine (Pinus sylvestris), and differential regulation of these and related enzyme activities in stressed plants, Plant Mol Biol, 18, 489-503.


Gianinazzi-Pearson, V., B. Branzanti and S. Gianinazzi (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscl-
ular mycorrhizal fungus by host root exudates and plant flavonoids, Symbiosis, 7, 243-253.


Horvath, B., C. W. B. Bachem, J. Schell and A. Kondorosi (1987) Host-
specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal interacting with the *nodD* gene product, EMBO Journal, 6, 841-848.


Ingham, J. L. (1976) 3,5,4-trihydroxystilbene as a phytoalexin from groundnut (Arachis hypogaea), Phytochemistry, 15, 1791-1793.
Jorgensen, R. (1990) Altered gene expression in plants due to trans interactions...
between homologous genes, TIBTECH, 8, 340-344.
Keen, N. T., A. I. Zaki and J. J. Sims (1972) Biosynthesis of hydroxyphaseollin and related isoflavonoids in disease-resistant soybean hypocotyls, Phytochemistry, 11, 1031-1039.


Krumholtz, L. R., and M. P. Bryant (1986) *Eubacterium oxidoreducens* sp. nov. requiring H$_2$ or formate to degrade gallate, pyrogallol, chlorogluconol and quercitin, Arch Microbiol, 144, 8-14.


tiana anthocyanin production activated by maize regulators R and C1, Science, 258, 1773-1795.


Margna, U. (1977) Control at the level of substrate supply – an alternative in
the regulation of phenylpropanoid accumulation in plant cells, Phytochemistry, 16, 419-426.


McKay, I. A. and M. A. Djordjevic (1993) Production and excretion of Nod metabolites by Rhizobium leguminosarum biovar trifolii are disrupted by the same environmental factors that reduce nodulation in the field, Appl Environ Microbiol, 59, 3385-3392.


Nutman, P. S. (1959) Some observations on root hair infection by nodule bacteria, J Exp Bot, 10, 250-263.


flavonoids via C-ring fission mechanisms, J Bacteriol, 176, in press.


Reihs, B. L., J. S. Kim, A. Badgett and R. W. Carlson (1994) Production of cell associated polysaccharides of Rhizobium fredii USDA205 is modulated by apigenin and host root extract, Mol Plant Microbe Interac, 7, 240-247.


Literature Cited


Smith, G. S. (1988) The role of phosphorous nutrition in interactions of vesicular-arbuscular mycorrhizal fungi with stilbene nematodes and fungi. Phytopathol-


Wagner, G. J. (1979) Content and vacuole/extravacuole distribution of neutral sugars, free amino acids and anthocyanins in protoplasts, Plant Physiol, 64, 88-93.


Werner, D. (1992) Symbiosis of plants and microbes, Chapman and Hall, Lon-


