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REGULATION OF EXPRESSION OF THE GLUTAMINE SYNTHETASE *GLN-α* GENE OF PHASEOLUS VULGARIS L.

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Declaration.

I declare that all the work in this thesis, except where specifically stated, was original research performed by myself under the supervision of Dr J V Cullimore at the Department of Biological Sciences, University of Warwick. None of this work has previously been submitted for any degree. All sources of information have been acknowledged by means of reference.

Summary.

The expression of the glutamine synthetase α subunit gene (*gln-α*) is known to be high in young tissues such as root tips, plumules developing nodules and developing leaves. An experiment using nodules were Fix- and began to senesce prematurely, suggested that *gln-α* and a phenylalanine ammonia-lyase gene (PAL 1) were co-induced. This phenomenon was further studied in various systems including nodules derived with *Rhizobium* mutants, cell cultures treated with fungal elicitors, wounded hypocotyls and leaves infected with compatible and incompatible strains of *Pseudomonas phaseolicola*. Expression of these two genes and in some cases the glutamine synthetase ß subunit gene (*gln-ß*) was observed to be related although experiments with short periods between samples suggested that the expression of the two glutamine synthetase genes was delayed with respect to the expression of the PAL 1 gene, the peak of expression occuring a few hours later. Work on cell cultures suggests that the stimulus for the induction of the two glutamine synthetase genes is possibly a product of the phenylpropanoid pathway although other stimuli present in both stress conditions and in young tissues may induce either of the two glutamine synthetase genes.

Abbreviations

A	adenine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CAB	chlorophyll ab binding protein
cAMP	cyclic AMP
cDNA	complementary DNA
CHS	chalcone synthase
CIAP	calf intestinal alkaline phosphatase
cm	centimetre
cv	cultivar
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EF1-α	elongation factor one-α subunit
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
GDH	glutamate dehydrogenase
GOGAT	glutamine-oxoglutamate amido transferase (glutamate synthase)
GS	glutamine synthetase

GSH	reduced glutathione
HPLC	high performance liquid chromatography
kD	kilo daltons
Km	Michaelis constant
M	molar
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NH_4^+	ammonium
PAL	phenylalanine ammonia-lyase
PEG	polyethylene glycol
PIPES	piperazine-N,N'-bis(2-ethanesulphonic acid)
PVP	polyvinylpyridone
P. vulgaris	*Phaseolus vulgaris* L.
RNA	ribonucleic acid
RNase	Ribonuclease
RUBISCO	ribulose biphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
T	thymine
tRNA	transfer RNA
TMV	tobacco mosaic virus

TRIS	2-amino-2(hydroxymethyl)-1,3-propandiol
UV	ultraviolet light
v/v	volume to volume
w/v	weight to volume

Chapter 1

Literature review

1.1 Nitrogen assimilation

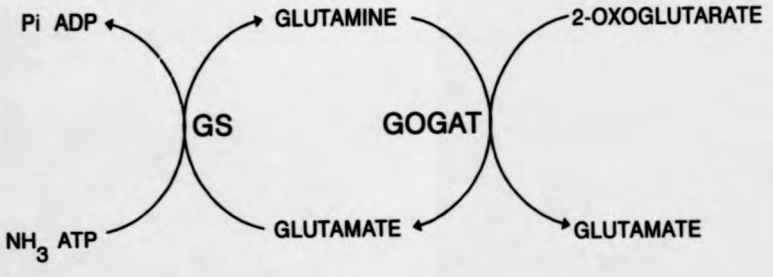
Commonly the limiting factor for plant growth in many soils is the availability of fixed nitrogen. The plant can assimilate nitrogen from a variety of inorganic forms such as nitrate, ammonium and, in the case of plants capable of forming a symbiotic relationship with a nitrogen fixing micro-organism, free nitrogen gas. Nitrate absorption is probably facilitated by the presence of a transport protein (Jackson *et al* 1973). Nitrate is then reduced to nitrite by the action of nitrate reductase and then to ammonium by nitrite reductase (see Beevers and Hageman 1980, Kleinhofs and Warner 1990 for reviews). All inorganic nitrogen compounds taken up by the plant are converted into ammonium before they are assimilated into organic combination. Prior to 1970 it was assumed that glutamate dehydrogenase (GDH) (E.C.1.4.1.2-4) was responsible for the assimilation of this ammonium into glutamate by the following reaction,

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADPH} \xrightarrow{\text{GDH}} \text{glutamate} + H_2O + NADP^+$$

In 1970 an alternative route for the assimilation of ammonium in *Klebsiella aerogenes* was proposed by Tempest *et al* in which two enzymes were involved glutamine synthetase (GS) (E.C.6.3.1.2.) and pyridine nucleotide dependent glutamate synthase (GOGAT) (E.C.1.4.1.13) in a cyclic reaction shown in figure 1.1.

Much experimental evidence for the GS/GOGAT cycle as the main pathway for primary ammonium assimilation has since been observed in many systems including both prokaryotic (see Ginsberg and Stadtman 1973 and Stacey *et al* 1979) and higher plant systems (Dougall 1974, Anderson and Done 1977, Meeks *et al* 1978, Wallsgrove *et al* 1979, Rhodes *et al* 1980). In addition, mutants obtained which are deficient in either chloroplastic GS (Wallsgrove *et al* 1987) or GOGAT (Somerville and Ogren 1980) suggest that the GS/GOGAT cycle is responsible for "reassimilating" the ammonium released by photorespiration as both mutants are lethal under photorespiratory conditions. It has been demonstrated that GDH is capable of assimilating ammonium but only under non-physiological conditions (Davies and Teixeira

Figure 1.1: The GS/GOGAT cycle for the assimilation of ammonia.


Pi ADP
GLUTAMINE
2-OXOGLUTARATE
GS
GOGAT
NH3 ATP
GLUTAMATE
GLUTAMATE

1975) and it is now thought that GDH in plants plays a catabolic role (see Lea *et al* 1990, for a review).

1.2 Glutamine synthetase structure and function

1.2.1 Prokaryotic glutamine synthetase

The majority of the work carried out on the prokaryotic GS enzyme has been performed using enteric bacteria such as *Escherichia coli*. Electron microscopy of the purified enzyme suggested that the enzyme is dodecameric with two rings of six subunits arranged in a parallel planar fashion (Valentine *et al* 1968). This suggestion was confirmed by the X-ray diffraction pattern of *Salmonella typhimurium* GS obtained by Almassy *et al* (1986) which also suggested that the active sites of the enzyme are at the interface of each adjacent subunit within each planar ring. The molecular weight for bacterial GS is *circa* 620 kD with identical subunits of approximately 52 kD. This structure appears to represent the general prokaryotic GS enzyme, however, another form has been isolated from various symbiotic bacteria. This second form has been isolated from various *Rhizobiaceae* (Darrow and Knotts 1977, Fuchs and Keister 1980) and the actinomycete *Frankia* sp strain Cpl1 (Edmands *et al* 1987) and is octameric resembling eukaryotic GS in its structure and regulation (see section 1.2.2).

Regulation of the general prokaryotic GS enzyme activity occurs by two methods; firstly it is regulated by allosteric interactions with feedback inhibitors such as amino acids, various other end products of nitrogen assimilation and ADP and AMP (see Reitzer and Magasanik 1987, for a review). Inhibition of GS by ADP and AMP indicates a dependence on the energy state of the cell (Atkinson 1968). Secondly the enzyme is subject to covalent modifications via adenylation of the subunits which produces a distinct form of the enzyme which has an increased sensitivity to feedback inhibition (Shapiro *et al* 1967). These two forms of regulation appear to hold true for the majority of prokaryotic GS enzymes studied to date. The GS enzyme from cyanobacteria deviates from this regulatory pattern in that it is not adenylated (Orr and Haselkorn 1982) even when expressed in *E. coli* (Fisher *et al* 1981). Control of the cyanobacterial enzyme activity is by feedback inhibition (Orr and Haselkorn 1981).

1.2.2 Eukaryotic glutamine synthetase

In fungi the GS enzyme is smaller than the typical prokaryotic enzyme having a M_r of around 390 kD although the subunit size is similar at approximately 47 kD, suggesting an octameric enzyme. The enzyme is made up of two weakly bound tetramers separable by treatment with

glutamine and ammonium, this treatment rendering the enzyme inactive (Sims *et al* 1974, Palacios 1976).

The GS enzyme of plants has been studied in many species including soybean (McParland *et al* 1976), rice (Hirel and Gadal 1980, Kanamori and Matsumoto 1972) and pea (Kingdon 1974, O'Neil and Joy 1975), in all of these examples the M_r is approximately 370 kD and the enzyme is octameric with subunit sizes of *circa* 47 kD. The structure of the soybean enzyme has been shown, by electron microscopy, to be two parallel stacked tetrameric rings analogous to the structure of the prokaryotic dodecameric enzyme (McParland *et al* 1976) and it is assumed (due to similar subunit and isoenzyme sizes) that this structure is true for all higher plant GS. It has been postulated that the structure is such that of the four available active sites per tetramer only two are catalytic the remaining two possibly have a regulatory role (Eisenberg *et al* 1987).

There is no evidence that adenylation of the plant GS enzyme occurs (Kingdon 1974). *In vitro* experiments have suggested that control of activity is achieved by feedback inhibition of the enzyme (Kanamori and Matsumoto 1972, Kingdon 1974, O'Neil and Joy 1975, McParland *et al* 1976, Hirel and Gadal 1980), this is mechanistically similar to the control of the cyanobacterial enzyme. *In vivo* it has been suggested that control of GS activity, at least for the pea leaf enzyme, may be regulated by the

energy status of the cell (O'Neal and Joy 1975) in addition to the possibility of feedback inhibition.

Mammalian GS has the same octameric structure as the higher plant enzyme and its activity is regulated by similar metabolic factors (see Meister 1974, for a review).

1.3 Glutamine synthetase isoenzymes

In contrast with the majority of prokaryotes, higher plants have a variety of isoenzymes of GS which are distinct from each other. For example, in soybean two forms of GS have been separated from etiolated hypocotyls by ion-exchange chromatography. Both forms have a similar molecular weight (around 365 kD) but have differing kinetic characteristics (Stasiewicz and Dunham 1979). The cellular location of GS has been studied in a number of plants initially by cell fractionation (Mann *et al* 1979, Wallsgrove *et al* 1979, Suzuki *et al* 1981) and more recently by immunocytolocalization (Hirel *et al* 1982, Botella *et al* 1988, Brangeon *et al* 1989). Using these techniques it has been found that GS is located in the cytosol and/or the chloroplast depending on the species and organ studied. For example, in *Phaseolus vulgaris* GS has been located in the cytosol in every organ studied but the plastid form is limited to the

green tissue and the nodule (Suzuki *et al* 1981, Datta *et al* 1991). In barley a similar pattern has been observed with a chloroplastic form present in the leaves and a cytosolic form present in a variety of other organs (Mann *et al* 1979). In tomato leaves only a chloroplastic isoenzyme was located, probably present in the stroma (Botella *et al* 1988). This situation is mirrored in soybean where only a stromal chloroplastic form was found in mature leaves and a cytosolic form was found in the roots and nodules (Brangeon *et al* 1989). Similarly in spinach leaves only the chloroplastic isoenzyme is present and has been located specifically to the parenchymatous cells of the cortex (Hirel *et al* 1982, Ericson 1985). In pumpkin it was found that white cotyledons contained only the cytosolic form of GS but as the cotyledons greened the chloroplastic GS became more abundant to eventually become the dominant form of GS (Nishimura *et al* 1982). McNally *et al* (1983) surveyed the leaf GS enzymes of a number of higher plants and found that the plant species could be divided into a number of classes, plants that have no cytosolic GS such as spinach; plants that have no chloroplastic GS represented by a number of achlorophyllous parasitic plants; those which have a much higher chloroplastic than cytosolic GS activity such as *P. vulgaris* and finally those which have approximately equal chloroplastic and cytosolic GS activities such as maize.

In rice there are three separate and distinct isoenzymes of GS one in the roots termed GS_r and two in the leaves, one of which is chloroplastic (GS_2) the other present in the cytosol (GS_1) (Hirel and Gadal 1980). In addition Hirel *et al* (1984) showed that the different GS isoenzymes have different immunoreactivities to a GS antibody suggesting that there is more than one form of cytosolic GS present in different organs. Indeed it is possible to find several cytosolic GS isoenzymes in one organ. Cullimore *et al* (1982) showed that three isoenzyme activities could be separated from *P. vulgaris* nodules which differed in charge but not size. One of these isoenzymes was shown to be closely related or identical to the root isoenzyme while the other appeared to be nodule specific (Cullimore *et al* 1983, Cullimore and Miflin 1984).

In *P. vulgaris*, using two dimensional gel electrophoresis, the GS enzymes present in a number of organs were found to be made up of a number of different polypeptides, GS enzyme extracted from leaf tissue was found to contain six different polypeptides α, ß, a, b, c and d, the last four polypeptides (a, b, c and d) make up the chloroplastic isoenzyme (*gln*-δ). The root enzyme contained the α and ß polypeptides found in the leaf enzyme as did the nodule enzyme which also contained an additional, nodule specific polypeptide γ (Lara *et al* 1984). In the nodule the α, ß and γ polypeptides are

present from days ten to thirteen after inoculation with *Rhizobium leguminosarum bv phaseoli* whereas only ß and γ polypeptides are found at later stages of development. The γ polypeptide was found to be present, albeit at lower levels, in ineffective nodules suggesting that nitrogen fixation is not strictly necessary for the presence of the γ polypeptides (Padilla *et al* 1987). In studies on later stage nodules, Bennett and Cullimore (1989) found, using ion exchange HPLC, that there were four separable GS activities present in nodules of *Phaseolus vulgaris*. A minor activity was provided by an isoenzyme present in plastids, the other three activity peaks related to various forms of cytosolic GS made up of different proportions of γ and ß polypeptides. This demonstrated that the cytosolic GS isoenzyme could assemble as both homo-octameric and hetero-octameric isoenzymes. The increase in GS activity during the course of nodulation could be ascribed to the appearance of the γ containing and to a lesser extent the ∂ containing forms of the enzyme (Lara *et al* 1983, Bennett and Cullimore 1989). Using native polyacrylamide gel electrophoresis the cytosolic isoenzymes from the later stage nodules have been shown to be composed of every possible combination of ß and γ polypeptides (Cai and Wong 1989). Work on *P. vulgaris* roots suggested that the α polypeptide is the most abundant polypeptide from embryo tissue to day five after sowing when the ß

polypeptide becomes dominant (Ortega *et al* 1986). The observation that the α polypeptide is abundant in embryonic tissue was confirmed by Bennett and Cullimore (1989) who found that imbibed seeds contain α_8 and to a lesser extent $ß_8$ isoenzymes which become replaced by an isoenzyme containing both subunitsd after day 2 post germination. This also suggests that the production of GS polypeptides is initially separated either temporally or spatially within the seed.

1.4 Glutamine synthetase genes and their regulation

1.4.1 Prokaryotic *gs* genes

Genes encoding glutamine synthetase have been isolated and sequenced from a wide variety of prokaryotes the majority of which contain one GS gene termed *glnA*. Examples of these organisms include *E. coli* (Pahel *et al* 1982, Columbo and Villafranca 1986), *Salmonella typhimurium* (Jansen *et al* 1986), *Bacillus subtilus* (Fisher *et al* 1984, Strauch *et al* 1988), *Thiobacillus ferrooxidans* (Barros *et al* 1985, Rawlings *et al* 1987), *Methylococcus capsulatus* (Bath) (Cardy and Murrell 1990), *Clostridium acetobutylicum* (Jannsen *et al* 1988), *Streptomycetes coelicolor* (Wray and Fisher 1988) and

Nostoc 7120 (Fisher *et al* 1981). In addition some prokaryotes carry two or possibly three GS genes, examples include, *Bradyrhizobium japonicum* (Carlson *et al* 1985), *Rhizobium leguminosarum* (Filser *et al* 1986), *Agrobacterium tumefaciens* (Rossbach *et al* 1988) and *Frankia sp.* strain CpI1 (Rochefort and Benson 1990).

Transcriptional control of *gln*-A in *E. coli* where the GS gene is part of an operon (*glnALG*) that contains three genes and three promoters (Pahel *et al* 1982). The three genes are the GS structural gene (*glnA*) and two regulatory genes *glnL* and *glnG* (*ntrB* and *ntrC*), the *glnA* gene is situated upstream of the two regulatory genes (Backman *et al* 1981, Chen *et al* 1982, Pahel *et al* 1982). Two promoters (*glnAP1* and *glnAP2*) are situated upstream of the *glnA* gene, the remaining one (*glnLP*) lies between the *glnA* and *glnLG* genes. The individual promoters are activated differentially: *glnAP1* is stimulated by catabolite activating protein charged with cAMP and is repressed by NR_1 (the product of *glnG*). *GlnAP1* is responsible for the low level expression of *glnA*. The second promoter *glnAP2* requires NR_1, σ^{60} and nitrogen depravation for full activity, this promoter is responsible for the high level expression of GS and NR_1 seen under conditions of low nitrogen availability. The third promoter is repressed by high concentrations of NR_1 and is responsible for maintaining low levels of NR_1

under conditions where *glnAP1* is active (see Reitzer and Magasanik 1985, 1986 for reviews).

The cyanobacterium *Nostoc* Pcc 7120 also contains a single GS gene (*glnA*), however, this gene is not part of an operon, but, its transcription is regulated by two promoters. One of these promoters resembles the *E. coli* consensus promoter and is responsible for low level constitutive expression of the *glnA* gene. The other promoter has significant similarity to *Nostoc nif* promoters and is responsible for transcription of *glnA* under nitrogen limiting conditions (Tumer *et al* 1983).

Prokaryotic organisms with multiple GS genes show a different pattern of regulation. Changes in GS abundance are not as a result of differential expression of the same gene, but are due to a switch in the GS gene transcribed. The *glnA* gene encoding GS1 (the dodecameric form) is transcribed constitutively whilst *glnII* is activated in response to nitrogen limitation (Carlson *et al* 1987, Rossbach *et al* 1988, Rochefort and Benson 1990). There is some evidence for the presence of three GS genes in the *Rhizobiaceae* (by complementation of an *E. coli* mutant) although the third GS gene may be cryptic *in vivo* (Filser *et al* 1986, Rossbach *et al* 1988).

The degree of sequence homology between bacterial GS genes is surprisingly low with conservation mainly concentrated around the regions encoding the active site of the enzyme (Jannsen *et al* 1988). Indeed the GS gene

of the gram positive organism *Streptomyces coelicolor* shows a greater sequence identity to that of gram negative bacteria than to other gram positive bacteria (Wray and Fisher 1988). The *glnII* genes of symbiotic bacteria species show a higher similarity to the GS genes of eukaryotes than to other prokaryotic GS genes. Lateral gene transfer between the plant host and the bacteria has been proposed to account for this sequence similarity (Carlson and Chelm 1986). However, further examination of the sequence homology indicated that the bacterial *glnII* genes have a similar identity to mammalian GS genes than to those of higher plants. This suggests that any transfer occurred prior to the split between prokaryotes and eukaryotes (Shatters and Cain 1989, Pesole *et al* 1991).

1.4.2 Lower eukaryotic GS genes

In the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* it has been shown that induction of GS activity is due to *de novo* protein synthesis (Quinto *et al* 1977, Mitchell and Magasanik 1983). The structural GS gene from many lower eukaryotes have been cloned, for example, the gene from *Saccharomyces cerevisiae* (Gonzalez *et al* 1985, Benjamin *et al* 1989) and the regulation of expression of this is at the level of transcription. The status of three metabolites affect GS expression. The

first depends on the relative levels of glutamine and glutamate; under conditions of high relative concentrations of glutamine the gene is repressed, while under low relative concentrations the gene is activated. A second regulatory circuit activates the gene when amino acids become limiting and a third induces the gene when purine levels become limiting (Benjamin *et al* 1989).

1.4.3 Vertebrate genes

Several cDNA's for GS have been cloned from mammals including Chinese hamster (Haywood *et al* 1986), human (Gibbs *et al* 1988) and rat (van de Zande *et al* 1988). In addition the cloning of a GS gene from murine 3T3-L1 adipocytes has been reported (Bhandari *et al* 1988) although further investigation has shown that this is probably a retroposon (Bhandari *et al* 1991) as it lacks introns, is flanked by 10 base pair direct repeats and its 5' flanking region has identity an intron containing gene cloned by Kuo *et al* (1989) from mouse. There is a suggestion that GS is encoded by a multigene family in elasmobranchs where it is likely that the cytosolic and mitochondrial GS subunits (from brain and liver tissue respectively) are coded by different genes (Smith *et al* 1987).

1.4.4 Higher plant GS genes

GS from higher plants appears, in general, to be encoded by multigene families. Examples of such families are found in *Phaseolus vulgaris* (Cullimore *et al* 1984), *Medicago sativa* (Donn *et al* 1984), Pea (Tingey *et al* 1987), *Nicotiana plumbaginifolia* (Tingey and Coruzzi 1987), *Oryza sativa* (Sakamoto *et al* 1989) and *Arabidopsis thaliana* (Peterman and Goodman 1991). Only one exception to this representation of GS by multigene families has been found; in *Sinapis alba* it appears, by Southern hybridisation, that GS is encoded by only one gene which encodes the chloroplastic enzyme (Hopfner *et al* 1991). This data correlates with the observation that only chloroplastic GS enzyme is seen in mustard (Schmidt and Mohr 1989, Hopfner *et al* 1991).

In *Phaseolus vulgaris* the multigene family is made up of four expressed genes, three of which encode cytosolic polypeptides (Cullimore *et al* 1984, Gebhardt *et al* 1986, Bennett *et al* 1989) and one which encodes a polypeptide with a pre-sequence allowing transport of the precursor polypeptide into the chloroplast (Lightfoot *et al* 1988). The first GS cDNA (pN-1a) isolated was obtained from a nodule cDNA library and appeared to show nodule specificity of expression as it hybridised most strongly to polyA RNA from nodules (Cullimore *et al*

1984), this clone was truncated and the full length cDNA (pN-1b) was later isolated by Bennett *et al* (1989). Two other distinct full-length cDNA clones (pR-1 and pR-2) were isolated from a root cDNA library (Gebhardt *et al* 1986). S1 nuclease protection assays showed that R-1 message appeared to be present in all organs studied (nodules, roots and leaves) while R-2 mRNA was present in roots and leaves but was not seen in nodules (Gebhardt *et al* 1986). Using an *in vitro* transcription/translation system these cDNA's generated products which co-migrated with distinct subunits, previously separated by Lara *et al* (1984) from plant tissue. The product of the R-1 cDNA co-migrated with the ß subunit, the product of the R-2 cDNA with the α subunit and the product of the cDNA from nodules co-migrated with the γ subunit (Bennett *et al* 1989). The cDNA's were renamed pcGS-α1 (pR-2), pcGS-ß1 (pR-1) and pcGS-γ1 (pN1b) and the chloroplastic GS encoding cDNA was named pcGS-∂1 (Lightfoot *et al* 1988). The genes relating to these cDNA's were named after the bacterial nomenclature *gln-α*, *gln-ß*, *gln-γ* and *gln-∂*. In addition to these four expressed genes, a fifth gene (*gln-ε*) has been cloned which is linked to the *gln-γ* gene, however no expression of this gene has been observed and it is therefore thought to be silent (Forde *et al* 1989).

Bennett *et al* (1989) showed that *gln-γ* message was not specific to nodules but could be detected in stems, petioles and green cotyledons. It has since been shown

that its mRNA appears in cotyledons two days post germination (Swarup *et al* 1990) but this induction of *gln-γ* transcription did not appear to relate to an increase in γ polypeptides in this tissue. In the nodules the expression of the *gln-γ* gene occurs prior to the onset of nitrogen fixation and appears to be under developmental control, although, the expression appears to be enhanced under nitrogen fixing conditions. It is not affected by the addition of exogenous ammonium, although, the presence of *rhizobia* is necessary for expression (Cock *et al* 1990). The gene for the γ subunit (*gln-γ*) has been cloned and the promoter sequences used to study expression of the gene in transgenic *Lotus corniculatus* plants (Forde *et al* 1989). This work showed that *gln-γ* is highly expressed in the infected cells of the nodule and that extremely low levels of expression were present in the roots and shoots of the transgenic plants. Nuclear factors, which interact with the promoter of *gln-γ*, have been isolated and these appear to belong to a conserved group of factors which interact with AT-rich regions of promoters from diverse plant genes (Forde *et al* 1990).

The full length cDNA for *gln-∂*, isolated from a leaf cDNA library, encodes a polypeptide with a cleavable pre-sequence enabling transport into the chloroplast. The presequence can be processed at one of several sites perhaps accounting for the variety of mature products

seen in leaf extracts (Lightfoot *et al* 1988). Lightfoot *et al* (1988) showed expression of *gln-∂* not only in mature leaves but also, at a reduced level, in roots and nodules. Cock *et al* (1991) have shown that the *gln-∂* gene is transcribed from two different start sites during leaf development, mRNA from both of these start sites is present at a low abundance in the dark. The two transcripts accumulate differentially during leaf development being induced by light. Photorespiration appears not to have a direct effect on the expression of *gln-∂* but probably has an indirect effect via physiological changes, such as pH, as there is a gradual increase in *gln-∂* message under photorespiratory conditions (Cock *et al* 1991). The gene encoding the ∂ subunit (*gln-∂*) has been cloned and the transcription start sites determined (Cock *et al* 1992). The 5' flanking sequences were fused to a reporter gene and used to study expression in transgenic tobacco. GUS activity was observed both in green tissue and root apices (Cock *et al* 1992).

Gebhardt *et al* (1986) showed that *gln-ß* message was present in roots, leaves and nodules. At first it was assumed that *gln-ß* was a housekeeping enzyme present ubiquitously in the plant, however, other evidence now suggests that this gene shows specific expression patterns during development (Cock *et al* 1990). The abundance of *gln-ß* mRNA during normal nodule development

is constant (Bennett *et al* 1989), however, during development of Fix$^-$ nodules *gln-ß* message rises while *gln-γ* is not induced (Cock *et al* 1990). The gene coding for the ß subunit (*gln-ß*) has been cloned (Turton *et al* 1988) and its expression studied using transformed plants (as for *gln-γ*). Interestingly, although the level of *gln-ß* mRNA is known to remain constant during nodule development its spatial distribution in the nodule changes dramatically, in young nodules the promoter is active in the infected and cortical regions of the nodule while in mature nodules expression is only seen in the vascular tissue (Forde *et al* 1989).

The cDNA for *gln-α* was cloned by Gebhardt *et al* (1986) using a root cDNA library. The expression of *gln-α* is associated with young and embryonic tissue. High levels of *gln-α* mRNA have been observed in young roots (Ortega *et al* 1986), early stages of nodule development (Bennett *et al* 1989), in seed germination from dry seed to day 2 (Swarup *et al* 1990) and early in leaf development (Cock *et al* 1991). Other work has suggested that *gln-α* has another role, besides that in early development, preliminary work by Cock and Cullimore and work presented in this thesis suggest that *gln-α* may be important during stress responses of the plant particularly when the phenylpropanoid pathway is induced. Preliminary evidence for this was observed during an experiment where *P. vulgaris* root systems were grown

under a argon/oxygen atmosphere. In these conditions the nodules are not fixing nitrogen and senesce early (Cock *et al* 1990). It was observed that *gln-α* was co-induced with a phenylalanine ammonia-lyase gene (PAL 1) (Cock and Cullimore unpublished data). A partial clone for *gln-α* has been isolated and work is in hand to study the expression of the gene using reporter gene fusions in transformed plants (Watson and Cullimore unpublished data).

In other legumes there are differences in the expression of the multigene family particularly with respect to induction in nodules. For example, pea does not contain a gene which is specifically enhanced in nodules, instead, all three genes encoding cytosolic subunits are induced during nodule development. This induction is also seen in Fix^- nodules demonstrating that nitrogen fixation is not necessary for the induction observed (Walker and Coruzzi 1989). In soybean there is some controversy regarding the presence of a GS gene with a nodule enhanced expression pattern Sengupta-Gopalan and Pitas (1986) showed, by *in vitro* translation of nodule mRNA, that a nodule specific form of GS appeared concomitantly or just after the onset of nitrogenase activity although nitrogenase activity was not necessary for the induction of the gene. In contrast Hirel *et al* (1987) showed any increased expression of GS genes in the nodules could be mimicked in the root by ammonium

treatment. Also in soybean there was no increased expression of the GS genes if the nodules were Fix^-. Studies on the expression of a cytosolic GS promoter from soybean when fused to a GUS gene and transformed into *Lotus corniculatus* showed a non nodule enhanced expression pattern with high expression present throughout the central region of the nodule, in the root apices and in the vascular tissue of the mature root (Miao *et al* 1991). Alfalfa does have a nodule enhanced gene (Dunn *et al* 1988).

All other GS genes encoding chloroplastic subunits are transcribed with a transit pre-sequence, show light regulation and show an indirect response to photorespiratory conditions (Tingey *et al* 1988, Edwards and Coruzzi 1989, Edwards *et al* 1990, Hopfner 1991, Peterman and Goodman 1991).

There is no evidence for ammonium induction of GS in *Phaseolus vulgaris* in contrast to soybean (Hirel *et al* 1987, Miao *et al* 1991) and rice (Zozaki *et al* 1991) where ammonium induction has been demonstrated for genes encoding cytosolic subunits.

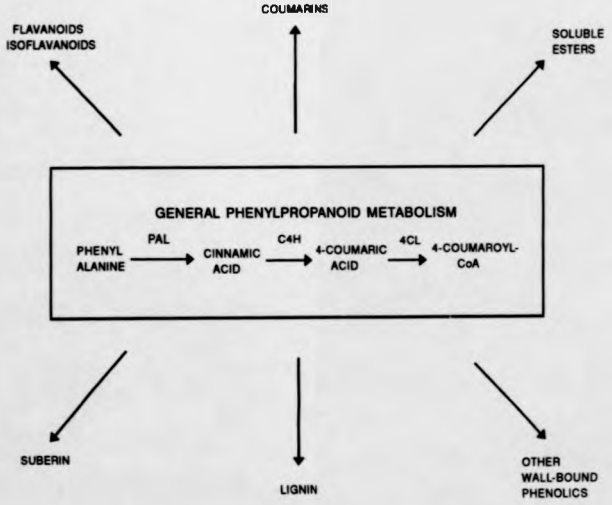
1.5 Production of ammonium in higher plants

In addition to nitrogen absorbed by the plant from the environment or derived from a symbiotic association

with *Rhizobium sp.* or *Frankia sp.* (see section 1.1) plants also have to recycle ammonium released by several metabolic pathways within the plant. Of these pathways the most important in terms of ammonium flux is the photorespiratory nitrogen cycle which liberates ammonium via the ribulose bisphosphate carboxylase/oxygenase RUBISCO oxygenase activity, indeed, it has been estimated that ten-fold more ammonium may be liberated by this pathway than is obtained from primary nitrogen assimilation (Keys *et al* 1978). Barley plants mutated in the chloroplastic GS gene are unable to grow under photorespiratory conditions demonstrating that the chloroplast GS enzyme is essential for the re-assimilation of the ammonium liberated (Wallsgrove *et al* 1987). Other pathways that liberate ammonium include amino acid catalysis and the phenylpropanoid pathway (see Miflin and Lea 1980, Lea *et al* 1990 for reviews). The first enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL)(E.C.4.3.1.5.), liberates ammonium ions during the conversion of phenylalanine to *trans*-cinnamate. The remainder of this pathway converts cinnamate to a wide variety of phenolic compounds including lignins, furanocoumarins, flavanoids and isoflavanoids (figure 1.2). These products have a wide range of functions including a role in cell wall thickening and flower pigmentation as well as providing compounds which act as UV protectants and phytoalexins

Figure 1.2: The central phenylpropanoid pathway with some of the end products.

PAL	phenylalanine ammonia lyase
C4H	cinnamic acid 4-hydroxylase
4CL	4-coumarate CoA ligase



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(see Hahlbrock and Scheel 1989, Dixon and Lamb 1990, for reviews). It is assumed that the ammonium liberated by this pathway is re-assimilated by the GS/GOGAT cycle (Miflin and Lea 1980), although this has not been proven.

1.6 Phenylalanine ammonia-lyase structure and function

Phenylalanine ammonia-lyase is the first enzyme of the phenylpropanoid pathway converting phenylalanine to *trans*-cinnamate with the release of ammonium (see below). In monocotyledonous plants the enzyme has an additional tyrosine ammonia lyase activity which is not present in dicotyledonous plants (see Stafford 1974 for a review).

PAL

phenylalanine ------> cinnamate + ammonia

The phenylalanine ammonia-lyase enzyme of higher plants has been demonstrated to be tetrameric in every species studied including; parsley (Zimmerman and Hahlbrock 1975), soybean (Havir 1981), French bean (Bolwell *et al* 1985a, Bolwell *et al* 1985b, da Cunha 1988), buckwheat (Belunis and Hrazdina 1988), tomato (Bernards and Ellis 1991) and loblolly pine (Whelton and Sederoff 1992). The enzyme has a native size of *circa* 280-330 kD and subunit

size of *circa* 74-83 kD although it has been demonstrated in French bean that the subunit size is extremely variable due to instability of the subunits both *in vitro* and *in vivo* (Bolwell *et al* 1985a). The enzyme was originally thought to exhibit negative co-operativity due to the complicated kinetic pattern shown under conditions where the enzyme was induced (Havir 1981, Hanson 1981a). However, this was later explained by the presence of a number of separate isoenzymes displaying normal Michaelis Menten kinetics but each having a different Km for phenylalanine and distinct temporal expression during induction (Bolwell *et al* 1985b, Jorrin and Dixon 1990, Lopez-Valbuena *et al* 1991). Despite being tetrameric the PAL enzyme contains two active sites (Hanson 1981a) each of which contains a dehydroalanine residue (Hanson 1985b). The presence of this residue is not plant specific as the dehydroalanine is correctly formed in PAL expressed in *E. coli* which has no endogenous PAL enzyme (Schulz *et al* 1989). The PAL enzyme is cytosolic although some degree of binding to subcellular membranes has been observed (see Jones 1984 for a review, Shaw *et al* 1990). There are reported to be differences in the degree of glycosylation between PAL enzymes from different species. For example in some species such as potato and maize the enzyme is glycosylated, the potato enzyme being N-glycosylated with mannose residues (Shaw *et al* 1990). Some plants however, such as French bean,

show no evidence that their PAL enzyme is glycosylated *in vivo* (Lawton *et al* 1983).

1.7 Phenylalanine ammonia-lyase isoenzymes

The PAL enzyme is represented by several different isoenzymes each with distinctive kinetic and isoelectric characteristics. Examples of this isoenzyme variability can be seen in: French bean, where five forms of the enzyme have been observed (Bolwell *et al* 1985a, Bolwell *et al* 1985b, Bolwell and Rodgers 1991), alfalfa, where three distinct forms have been isolated (Jorrin and Dixon 1990); Vicia faba, where three isoenzymes have been isolated (Lopez-Vabuena 1991) and potato, where four isoenzymes have been observed (Ishizuka *et al* 1991). The different isoenzymes from these plants display both temporal and spatial differences in the conditions under which they present. For instance, in French bean four isoenzymes, with subunits of *circa* 77kD, are present during the elicitation response to fungal elicitors. Several isoenzymes appear sequentially over a period of time such that forms with a low Km for phenylalanine appear before those with higher Km's (Bolwell *et al* 1985b). In addition the fifth observed French bean isoenzyme, subunit size *circa* 83 kD, appears to be

expressed constitutively in tissues where lignin deposition is likely to occur (Bolwell and Rodgers 1991).

1.8 Phenylalanine ammonia-lyase genes and their regulation.

In all of the species studied to date PAL is represented by a multigene family, examples include French bean (Cramer *et al* 1989), parsley (Kuhn *et al* 1984, Lois *et al* 1989), rice (Minama *et al* 1989), *Arabidopsis* (Ohl *et al* 1990), alfalfa (Gowri *et al* 1991) and potato (Joos and Hahlbrock 1992). Members of these PAL multigene families show differential expression patterns after induction by the developmental and environmental stimuli discussed above. The majority of these multigene families consist of around three members as exemplified by French bean where the three family members are described as PAL 1, *PAL* 2 and *PAL* 3. These three genes show a differential expression pattern. All three genes are expressed after wounding of hypocotyl tissue, PAL 1 and *PAL* 3 are induced in response to fungal infection and PAL 1 and *PAL* 2 are induced by fungal elicitors and illumination of hypocotyls (Cramer *et al* 1989, Liang *et al* 1989b). It has also been observed that *PAL* 2 is expressed in coloured parts of flowers, in tissues where lignification is occuring and just behind

the root and shoot tips where its presence may play a role in the control of polar auxin transport (Bevan *et al* 1989, Liang *et al* 1989a). A more complicated if incompletely studied multigene family has been observed in potato where there are forty to fifty PAL genes per haploid genome, at least ten of which are active. Two genes have been isolated which show differences in their expression patterns; both are expressed at a low level in unstressed plants and both are induced equally after wounding or infection with an incompatible pathogen. However, infection with a compatible pathogen produces a different pattern one gene being expressed later in the response (Joos and Hahlbrock 1992). Studies of isolated PAL gene promoters has suggested that combinations of *cis* elements and trans-acting factors are important in the transcriptional response to different stimuli (Lois *et al* 1989, Ohl *et al* 1990, Leyva *et al* 1992).

The regulation of PAL expression has been extensively studied in many systems with much of the work being performed using suspension cultures. Dramatic induction of PAL activity can be observed in suspension cultures of several plant species when exposed to fungal elicitors (heat released from fungal cell walls) (Dixon *et al* 1981, Hahlbrock *et al* 1981, Lawton *et al* 1983a, Lawton *et al* 1983b, Bolwell *et al* 1985b, Robbins *et al* 1985, Cramer *et al* 1985, Bolwell *et al* 1986, Dalkin *et al* 1990 and Jorrin and Dixon 1990). The response of PAL

activity in cell cultures to fungal elicitor was found to be non-linear having a bi-phasic response curve in relation to elicitor concentration (Dixon *et al* 1981). In addition, the elicitor has been shown to cause stabilisation of PAL activity at high concentrations (Lawton *et al* 1980). This treatment induces many other enzymes including others from the phenylpropanoid pathway such as chalcone synthase (CHS) in French bean (Lawton *et al* 1983a, Cramer *et al* 1985). In parsley CHS is not induced by fungal elicitors as the phytoalexins of this plant produced in response to fungal elicitors are not flavanoid in nature and CHS is the first enzyme of the committed flavanoid pathway (Hahlbrock *et al* 1981). An almost identical induction, to that observed with fungal elicited French bean cultures, can be obtained using other elicitors such as autoclaved RNase A (Dixon and Bendall 1978, Dixon *et al* 1980). The use of elicitors on cell suspension cultures is a convenient way of mimicking the response of the intact plant to a fungal pathogen. PAL can also be elicited by exposure to ultra-violet light (Zimmerman and Hahlbrock 1975), although this light induced production of flavanoids is repressed if the cells are first treated with a fungal elicitor (Hahlbrock *et al* 1981). Other stimuli which can induce PAL activity include the interaction with an incompatible pathogen to produce a hypersensitive response or the much slower response to a compatible pathogen resulting in a disease

response (Fritig *et al* 1973, Borner and Grisebach 1982, Cramer *et al* 1985, Smith *et al* 1988). The induction response of the plant to these varied stimuli was found to be due, at least primarily, to *de novo* transcription of PAL mRNA (Chappel and Hahlbrock 1984, Kuhn *et al* 1984, Edwards *et al* 1985, Cramer *et al* 1985, Lawton and Lamb 1987, Fritzemer *et al* 1987, Lois *et al* 1989, Richey and Belknap 1991). Work on the induction of PAL mRNA also demonstrated other environmental and developmental stimuli that induce PAL. Developmental situations where PAL mRNA is induced include flowering (Bevan *et al* 1989, Liang *et al* 1989a, Ohl *et al* 1990), the root and shoot meristems (Liang *et al* 1989a) and lignification (Bevan *et al* 1989, Liang *et al* 1989a, Ohl *et al* 1990, Lin and Northcote 1990, Gowri *et al* 1991). Environmental cues for the induction of PAL mRNA include wounding (Lawton and Lamb 1987, Bevan *et al* 1989, Tanaka *et al* 1989) and reduced glutathione treatment of French bean suspension cultures (Wingate *et al* 1988) an effect not observed in alfalfa cultures (Dalkin *et al* 1990).

The level of PAL mRNA and activity of the enzyme is under strong control particularly by the product of PAL, cinnamate, which both inhibits the production of PAL enzyme and promotes its inactivation (Dixon *et al* 1980, Shields *et al* 1982, Jorrin and Dixon 1990). This effect of cinnamate can be mimicked by many derivatives and related compounds of cinnamate, the hydrophobic ring, a/ß

double bond and carboxyl group being essential for the inhibition (Sato *et al* 1982). It has been suggested that the cinnamate induced inactivation of PAL occurs by the action of an inactivating system which may be proteinaceous in nature probably acting on the dehydroalanine residue of the active site (Dixon *et al* 1980, Bolwell *et al* 1986, Jorrin *et al* 1990). Jorrin *et al* (1990) also suggested that cinnamate may, in addition to inhibiting PAL at high concentrations, raise PAL activity at low concentrations. The tight regulation of PAL activation can also be observed spatially during the hypersensitivity response to incompatible pathogens where a high PAL induction can be observed in a very localised area around the infection site (Cuypers *et al* 1988, Schmelzer *et al* 1989) as well as during wounding, lignification and in certain coloured flower parts (Bevan *et al* 1989, Liang *et al* 1989a).

The induction of the PAL message during the response to fungal elicitors is extremely rapid, peaking at around three hours post elicitation (Lawton *et al* 1983b, Lawton and Lamb 1987), with a measurable increase by five minutes post elicitation suggesting that the signal transduction pathway is in place prior to elicitation and that there are few steps between activation of a putative receptor and stimulation of gene expression (Lawton and Lamb 1987). It has been demonstrated that the slower

light induction of PAL is mediated by phytochrome in cotyledons (Lecari *et al* 1982).

1.9 Aims of the project

This thesis presents work undertaken to study the expression of one of the glutamine synthetase genes of *Phaseolus vulgaris*. This gene, *gln-α*, had been observed to be possibly co-induced with PAL 1 in nodules senescing due to growth under an argon/oxygen atmosphere, where the nodules are not fixing nitrogen (Cock and Cullimore unpublished data). Chapter three further explores the induction of *gln-α* and PAL 1 in senescent nodules while chapter four studies the expression of the two genes in a variety of other systems where PAL 1 induction is known to occur. Chapter five describes attempts made to obtain a genomic clone for *gln-α*.

Chapter two

Materials and methods

2.1 Plant material

Phaseolus vulgaris cv. Tendergreen was used in all studies unless otherwise specified. Plants for nodulation were grown as described in Chen and Cullimore (1988) except that 1 litre beakers were used for plant growth. The CE3 strain of *Rhizobium leguminosarum bv phaseoli* was used to inoculate the plants unless otherwise stated and sterile distiled water was used to make up the nutrient solution. Plants grown for all other studies performed at Warwick were not inoculated with *R. leguminosarum* and grown in seed trays of vermiculite, all others details were as described. Plumule tissue was dissected from seeds imbibed overnight in running tap water. All plant tissue collected was wrapped in aluminium foil, snap frozen in liquid nitrogen and stored at -80^{o} C before use. Any variations in growth conditions used will be stated.

2.2 Bacterial strains, growth and maintenance

Several *E. coli* strains were used for recombinant DNA manipulation:

TG2	*(lac pro), thi, strA, hsdM⁻, recA⁻, scIC 300:Tn10(tet)/F' traD36, proAB, lacIq, lacZ M15.*
LE392	*e14⁻(mcrA), hsd R514, sys E44, sup F58, lacY1 or -(lacIZY)6, galK2, galT22, metB1, trpR55*
P2392	LE392 (P2 lysogen)
K803	*e14⁻(mcr), mcrB1, hsdS3, galK2, galT22, SupE44, metB1*

All *E. coli* strains were grown on LB media except stocks of TG2 which were grown on minimal media at 37°C and stored at 4°C. Long term storage of strains was as described by Maniatis *et al* (1982). LB, minimal media and any antibiotics used were prepared as described by Maniatis *et al* (1982) with the exception of ampicillin which was used at twice the recommended concentration (100 ug/ml).

Rhizobia leguminosarum bv phaseoli strains used were as described in chapter three. These were grown on a modified 2x TY media (16g/l bactotryptone, 10g/l yeast extract, 10mM $CaCl_2$) at 30°C, when large ammounts of *Rhizobia* were required the bacteria were grown on slopes.

All other conditions were identical to those used for *E. coli*.

2.3 Chemicals

All reagents used were of the highest possible purity and were obtained from BDH Chemicals Ltd., Sigma chemical Co. Ltd., Fisons PLC. and FSA lab. systems. Sephadex was obtained from Pharmacia Fine Chemicals Co.. Radiolabelled nucleotides were obtained from Amersham PLC.

2.4 Enzymes

All DNA restriction and modification enzymes were obtained from Gibco BRL, Amersham PLC., or Pharmacia Fine Chemicals Co. unless listed below: *Xmn*I was obtained from New England BioLabs. Inc., *Lsp*I was obtained from NBL. and CIAP. was obtained from Boehringer Mannheim.

Rnase A and RNase T1 were obtained from Boeringer Mannheim.

2.5 General nucleic acid manipulations

2.5.1 Gel electrophoresis.

Agarose gel electrophoresis was performed essentially as described in Maniatis *et al* (1982) using a percentage of agarose best suited to the fragments to be separated. The agarose was dissolved in 1X TAE buffer (50X stock, 2M TRIS-Base, 0.05M EDTA and 57.1ml/l glacial acetic acid) or 1X TBE buffer (10X stock, 0.89M Tris-Base, 0.89M boric acid and 0.02M EDTA) by boiling. The agarose solution was cooled to around 60oC, ethidium bromide was added to a final concentration of 0.5ug/ml and the gel was cast in a suitable mould for the gel tank to be used. When set the comb was removed and the gel submerged in the relevant buffer (1X TAE or TBE) and the samples were applied to the wells using loading buffer (6X buffer, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol and 15% w/v Ficoll type 400) after which the gel was electrophoresed at a constant voltage suitable for the gel tank. DNA was visualised after electrophoresis on an UV transilluminator.

Denaturing polyacrylamide gel electrophoresis was performed as described by Smith (1980) using 6% 20:1

acrylamide:bisacrylamide, 8M urea in 1X TBE. Gels were run at 40W constant power and fixed in 10% acetic acid, 10% methanol before drying under a vacuum on a drier.

Polyacrylamide gels for the separation of small DNA restriction fragments was performed essentially as described in Maniatis *et al* (1982) using the mini-protean gel system (Biorad). 10% gels were used being prepared from a 30% stock solution of acrylamide (29:1 acrylamide:N,*N*'-methylene bisacrylamide) in 1X TBE buffer. Ammonium persulphate and TEMED were added as described and the gels were polymerised in the plates and casting apparatus supplied with the gel system. The samples were loaded in loading buffer as described for agarose gels and electrophoresed at 40mA constant current. The gels were then removed from the plates and stained in 0.5ug/ml ethidium bromide in 1X TBE for 15 minutes followed by two changes of water for 15 minutes each to destain the gel. The bands were then visualised on a UV transilluminator.

RNA agarose gel electrophoresis was performed as described in Maniatis using an 1.5% agarose gel made up with 1X MOPS buffer (10X stock, 0.4M MOPS pH7, 100mM Na acetate, 5mM EDTA) and containing 2.2M filtered formaldehyde. RNA samples were prepared by mixing samples with 4X denaturing buffer (1X MOPS, 20% v/v filtered formaldehyde, 66% v/v deionised formamide), the samples were then heated to 55^{o}C for 15 minutes. RNA

loading buffer (10X stock 50% v/v glycerol, 1mM EDTA, 0.4% w/v bromophenol blue, 0.4% w/v xylene cyanol) was then added and the samples were loaded on the gel. the gel was then electrophoresed at 150V until the bromophenol blue had travelled 10cm into the gel. The gel was then stained and destained with ethidium bromide to visualise the ribosomal RNA as follows. The gel was stained by immersion in 250ml of 10% w/v glycine for 20 mins followed by the addition of 100ul of 10 mg/ml ethidium bromide for 10 mins. The gel was then destained in two 15 min washes of distilled water and photographed on a UV transilluminator.

2.5.2 Purification of DNA from gels.

DNA fragments were purified from agarose gels using the dialysis tube method of Maniatis *et al* (1982). The bands were visualised on a transilluminator as before and the required bands were excised using a razor blade. The excised blocks of agarose were placed into dialysis tubing (pre-boiled in 2% w/v $NaHCO_3$, 1mM Na_2EDTA followed by 1mM Na_2EDTA for ten minutes each) closed at one end with a dialysis clip. The tubing was then filled with the relevant buffer (1X TBE or TAE). The majority of the buffer was then removed by gentle squeezing such that there was enough buffer to ensure even contact with the

gel slice. The bag was then closed (without allowing air bubbles to remain in the tubing) with a second dialysis clip and the bag was immersed in the relevant buffer in a gel tank. The bag was then electrophoresed to extract the DNA from the gel slice, following electrophoresis the DNA was released from the bag wall by a brief period of reverse polarity electrophoresis. The buffer was then removed from the bag and subjected to phenol/choroform extraction followed by ethanol precipitation to recover the DNA.

DNA fragments were isolated from polyacrylamide gels by the method of Maniatis *et al* (1982). Gel slices containing the desired fragment was removed as above and then macerated in an eppendorf tube. 1 volume of elution buffer (0.5M NH_4acetate, 1mM EDTA pH8) was then added and the fragment were eluted passively overnight with agitation at 37^{o}C. The polyacrylamide was then removed by centifugation in a microfuge and the DNA was recovered and purified by two rounds of ethanol precipitation.

2.5.1 Labelling of nucleic acids.

RNA probes were produced by *in vitro* transcription as described by Krieg and Melton (1987). The transcriptions were performed with a bacteriophage RNA polymerase (100u/ml) using a linearised plasmid vector

(100ug/ml) as a template in transcription buffer (40mM TRIS pH7.5, 6mM MgCl2, 2mM spermidine-HCl, 10mM DTT, 100ug/ml BSA, 500uM rATP, rCTP, rGTP, 1u/ul RNasin, 500um [α-^{32}P]rUTP). This mixture was incubated at 37^{o}C for 1 hour after which RNase free DNase is added to a final concentration of 20ug/ml, the reaction is then incubated at 37^{o}C for 10 minutes. The reaction mix was then denatured by the addition of formamide dyes and heating to 80^{o}C for 10 minutes. The probe was then separated from the unincorporated nucleotides on a denaturing polyacrylamide gel. The probe was recovered from the gel by passive elution at 60^{o}C.

DNA probes were produced by multi priming as described by Feinburg and Vogelstein (1983). 20-50ng of boiled restricted DNA was mixed with OLB buffer (5x stock: 25mM MgCl2, 5mM ß-mercaptoethanol, dATP, dGTP, dGTP @ 2mM, 1M HEPES pH6.6, 1mg/ml random hexamer oligonucleotides, 250mM TRIS-Cl pH8), 25% w/v BSA, 60uCi ^{32}P-CTP, 10U Klenow fragment of DNA polymerase I. This mixture was incubated at room temperature for 4 hours. After the incubation the probe was separated from the unincorperated nucleotides on a G-25 sephadex column using TEN buffer (0.1M NaCl, 1mM EDTA pH8, 10mM TRIS-Cl pH8) with 0.1% w/v SDS.

2.5.4 Autoradiography

The signal from ^{32}P labelled probe sequences, hybridised to target sequences on blots and in gels, was visualised by exposing the signal to X-ray film (Fuji) in a cassette using an intensifying screen at -70°C. A standard overnight exposure time was used (unless otherwise stated) after which the film was processed in a manual minilab X-ray processor (X-ograph Ltd.) under safe light illumination autoradiography with Kodak LX-24 X-ray developer for 1-5 mins followed by a water rinse and fixation in Kodak LX-40 solution for 5 mins followed by prolonged rinsing in water.

2.6 DNA manipulations

2.6.1 Restriction analysis

DNA was incubated with the appropriate restriction enzymes using an approximate 4-5 fold excess using the buffers and conditions recommended by the manufacturer. If it was necessary to digest the DNA with a combination of restriction enzymes either conditions were selected under which both restriction enzymes were active or the

reaction conditions were adjusted as necessary between separate digests.

2.6.2 Preparation of DNA size markers

Markers for gel electrophoresis of DNA were prepared in a number of ways: lambda DNA was restricted with *Hind*III and *Eco*RI for use on agarose minigels, or pBR322 was restricted with *Hae*III for use on acrylamide minigels. Preprepared markers (1kb ladder Gibco BRL) were also used on agarose minigels. Radiolabelled markers for Southern blots were prepared by filling in the 5' overhangs present on *Acc*I digested lambda DNA with [α-32P]dCTP directly after digestion in the restriction buffer with the addition of the Klenow fragment of DNA polymerase I Maniatis *et al* (1982) to the reaction mix. The labelled fragments were then purified by two rounds of ethanol precipitation.

2.6.3 DNA modifications

All DNA modifications were performed using the manufacturers conditions unless specified below.

2.6.3.1 Removal of 5'phosphate from DNA

Calf intestinal alkaline phosphatase (CIAP) was used to remove the 5' terminal phosphate groups from plasmid vectors as follows: 1ug of linearised vector was incubated with 1/10 volume of CIAP salts (10mM $MgCl_2$, 1mM $ZnCl_2$), 0.1 volumes of CIAP buffer (0.5M NaOH, 20% glycerol pH 9.4) and six units of CIAP. This reaction mixture was then incubated at 37^{o}C for thirty minutes followed by phenol/chloroform extraction and ethanol precipitation to inactivate and remove the CIAP and to recover the DNA.

2.6.3.2 DNA ligation

A method modified from Rusche and Howard-Flanders (1985) was used to maximise the efficiency of ligation. Ten units of T4 DNA ligase was mixed with the linearised vector/insert mixture, 1ul of 10X super ligase buffer (250mM TRIS acetate pH 7.5, 50mM $MgCl_2$, 10mM hexamine cobaltic chloride and 1mM ATP), 1ul of 2.5 mM DTT and made up to 10ul with sterile distiled water. This reaction mix was then incubated at 16^{o}C overnight before direct use in transformation of *E. coli*.

2.6.4 Transformation of *E. coli*

2.6.4.1 Preparation of competent cells

1.5mls of a 5ml overnight culture of *E. coli* TG2 was used to inoculate 250mls of 2X TY media which was then grown to mid log phase (estimated at an optical density of 0.3 (A_{600}) at 37°C with vigorous agitation (300 rpm). The cells were then chilled on ice for 5 mins after which they were pelleted by centifugation at 6000 rpm for 5 mins at 4°C. The supernatant was removed and the cells returned to ice where they were resuspended in 100ml of ice cold TfbI (30mM KCH_3COO, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% v/v glycerol, pH5.8) and incubated for 5 mins after which the cells were pelleted as before. The cells were then gently resuspended in 10ml of TfbII (10mM PIPES pH 6.5, 75mM $CaCl_2$, 10mM $RbCl_2$, and 15% v/v glycerol) and incubated on ice for 15 mins after which the cells were aliquoted into prechilled eppendorf tubes and snap frozen in liquid nitrogen before storage at -70°C.

2.6.4.2 Transformation of *E. coli*

Pre-prepared competent cells (see above) were thawed at room temperature and incubated on ice for 10 mins, DNA was then added to a maximum of 2/5 total volume and a maximum concentration of 100ng/100ul of cells. After the addition of DNA the cells left on ice for a further 30mins, heat shocked at 42°C for 90 secs and returned to the ice for 5 mins. After this incubation 900ul of 2XTY broth was added to the mix before incubation at 37°C for 1 hour with agitation. After this time had elapsed various aliquots of the transformation were spread onto selective agar plates and incubated overnight at 37°C.

2.6.5 Isolation of plasmid DNA

2.6.5.1 Small scale preparations.

Plasmid DNA was isolated from small scale cultures by the alkaline lysis method essentially as described in Maniatis *et al* (1982). Bacteria were harvested from 1.5 mls of LB media (10 g/l w/v Bactotryptone, 5g/l w/v Bacto yeast extract, 10 g/l w/v NaCl adjusted to pH 7.5 with NaOH) by centifugation in a microfuge. The bacterial

pellet was resuspended in 100ul of resuspention buffer (50mM glucose, 10mM EDTA, 25mM TRIS-Cl pH8) and then incubated for 5 minutes at room temperature. 200ul of alkaline SDS (0.2M NaOH, 1% w/v SDS) was then added followed by gentle mixing (by inversion) and incubation on ice for 5 minutes. 150ul of ice cold K acetate solution (3M Potassium 5M acetate) was then added followed by gentle mixing and a further 5 minute incubation on ice. The preparation was then centrifuged for 5 minutes in a microfuge and the plasmid DNA was precipitated with an equal volume of isopropanol. After centifugation the pellet was washed with 70% ethanol and dried before resuspension in SDW.

2.6.5.2 Large scale preparations

Large scale preparation of plasmid DNA was carried out as follows: *E.coli* were grown up in 500ml of T broth (solution A 100ml of: 0.17M KH_2PO_4, 0.72M K_2HPO_4, and solution B: 24g bacto yeast extract, 12g bacto tryptone, 4ml of glycerol made up to 900ml with water, autoclaved separately and then mixed) and harvested by centifugation in a bench centrifuge at 500 rpm for 15mins. The resulting pellet was resuspended in 6ml of buffer (25mM TRIS-HCl pH 7.5, 10mM EDTA, 15% w/v sucrose and 2mg/ml lysozyme) and incubated on ice for 20mins. 12 ml of alkaline SDS (0.2M NaOH, 1% w/v SDS) was then added to the bacterial suspension and after gentle mixing the

resulting mixture was incubated on ice for a further 10mins. 7.5 ml of 3M Na-acetate (pH 4.6) was then added to the mixture, gently mixed and incubated on ice for 20mins followed by centifugation at 15,000 x g for 20 mins at 4°C. The supernatant was removed and 50ul of RNase A (1mg/ml) was added followed by incubation at 37°C for 20 mins. The sample was then phenol/chloroform extracted and the plasmid DNA was precipitated by ethanol precipitation with two volumes of cold ethanol. The resulting pellet was resuspended in 1.6ml of sterile distiled water to which 0.4ml of 4m NaCl was added. After mixing, 2ml of 13% w/v PEG 6000 was added, mixed and incubated on ice for 1 hour. The samples were then centrifuged at 6000 xg for 10mins at 4°C followed by a wash with 70% v/v ethanol and a further spin at 10,000 x g for 10 mins at 4°C. The pellet was then dissolved in 500ul of TE buffer.

2.6.6 Isolation of plant genomic DNA.

Plant genomic DNA was extracted by the method of Dellaporta. 5g of root tissue was ground in liquid nitrogen, as the ground tissue began to thaw 15mls of extraction buffer (100mM TRIS pH8, 50mM EDTA, 500mM NaCl, 10mM BMe) was added and the extract was incubated at 65°C

for 10 minutes. K acetate was then added to a final concentration of 1M followed by vigorous mixing and incubation on ice for 20 minutes. The extract was then centrifuged at 13K for 15 minutes at 4°C, filtered though muslin and precipitated using a half volume of isopropanol. After drying the pellet was resuspended in 700ul of TE (50/10) and spun in a microfuge for 10 minutes, the supernatent was then reprecipitated using Na acetate and isopropanol. This pellet was washed twice with 70% ethanol, dried and redissolved in 300ul of TE (50/10) before the sample was treated with RNase A. After this treatment the sample was subjected to two rounds of phenol/chloroform extraction followed by precipitation with NH_4 acetate and ethanol. The resulting pellet was resuspended in 200ul of TE before quantification.

2.6.7 Southern blotting of DNA and hybridisation with radiolabelled probes

Southern transfer of genomic and cloned DNA was essentially performed as described in Maniatis *et al* (1982) with the following differences: Hybond-N™ (Amersham) nylon filters were used and the DNA was covalently bound to them by 5mins of UV irradiation on a

transilluminator followed by vacuum baking of the filter. The blots were then hybridised as described by Maniatis *et al* (1982) except that the hybridisation solution consisted of 5 x SSPE (0.9M NaCl, 50mM Na_2HPO_4, 5mM EDTA pH 7.7), 5 X Denhardts (0.1% w/v Ficoll, 0.1% w/v PVP, 0.1% w/v BSA), 0.2% w/v SDS, 50% v/v de-ionised formamide and 100ug/ml denatured sheared herring sperm DNA. A RNA probe was used (see section 2.5.2) and the hybridisation temperature was 45°C. Washes were performed at both low and high stringencies (2 x SSC, 0.1% w/v SDS at 65°C or 0.1 x SSC, 0.1% w/v SDS at 65°C respectively). The filters were then subjected to autoradiography.

2.6.8 Colony and plaque hybridisations.

Filters were prepared from plates by lifting plaques or colonies onto pre-wetted (sterile distiled water followed by 1M NaCl) nylon filters followed by denaturation by placing the filters (plaque/colony side up) onto whatman soaked in 0.5M NaOH, 1.5M NaCl for five minutes followed by two similar treatments using 1M TRIS pH7, 3m NaCl in order to neutralise the NaOH. The filters were then vacuum baked at 80°C for 1 hour. The filters were then treated identically to the Southern blots except that for NA probes the formamide was omitted from the hybridisation solutions and the hybridisation steps took place at 65°C.

2.6.9 Double stranded sequencing of plasmid DNA

Sequencing of double stranded DNA was performed using the Sequenase^{R} kit (United States Biochemicals, Cleveland USA) by the method of Sanger *et al* (1977) with a primer for the T7 promoter of pGEM vectors. As the sequence required was close to the primer the reaction time was cut down to thirty seconds and the labelling mix was used at a lower concentration (1/5 normal).

2.6.10 Sequence analysis

All sequence analysis was performed on the Daresbury computer using various programmes (Devereax *et al* 1984).

2.7 RNA manipulations

2.7.1 Isolation of plant tissue RNA.

Plant RNA was extracted by a method modified from that of Logemann *et al*, tissue was homogenised in extraction buffer (8M guanidine hydrochloride, 20mM MES, 20 mM EDTA, 50mM B-mercaptoethanol pH7) and subjected to two rounds of phenol extraction followed by ethanol precipitation with 0.7 volumes of ethanol and 0.2 volumes of 1M acetic acid. The precipitate was then resuspended in water prior to lithium chloride treatment (2M final concentration) overnight. RNA was recovered by centifugation before being washed with sodium acetate (3M pH 5.2). The remaining salt was removed by washing with 70% v/v ethanol washing prior to drying and resuspension in sterile distiled water. In samples where high levels of polysaccharide caused a low yield of RNA either a low ethanol/low salt (25% v/v ethanol, 0.35 M ammonium acetate) precipitation was included (to remove polysaccaride) before the LiCl precipitation or an alternative extraction buffer consisting of 200mM TRIS-HCl pH 9.0, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$ and 25 mM EGTA (Jackson and Larkins 1976) was used in place of the standard extraction buffer.

2.7.2 Quantification of total RNA

Total RNA was quantified using a scanning spectrophotometer between 340 and 220 nm (Maniatis *et al* 1982). The concentration of the RNA was then adjusted as appropriate and stored either as an ethanol precipitate at -20°C (if used for RNase protection) or as an aqueous solution at -70°C (if used for Northern blots).

2.7.3 RNA blotting and hybridisation

Total RNA samples were size fractionated on a formaldehyde agarose gel (Maniatis *et al* 1982). One extra track was loaded with total RNA and removed from the gel with a razor blade after running, to be stained and used as markers. Northern transfer was performed asdescribed in Maniatis *et al* (1982) using a Gene Screen^{tm} membrane nylon membrane (Du Pont). The blot was pretreated in hybridisation buffer [50% formamide, 1X P buffer (1% w/v BSA, 1% w/v PVP, 1% w/v Ficoll, 250mM TRIS-HCl pH 7.5, 0.5% w/v sodium pyrophosphate and 5% w/v SDS), 10% w/v dextran sulphate, and 100 ug/ml denatured sheared salmon sperm DNA] in an airtight bag overnight at 42°C Probe RNA was denatured (by heating to 85°C in 40mM PIPES pH6.4, 1mM EDTA, 0.4mM NaCl, 80% v/v deionised formamide) and added to the hybridisation solution. The

bag was then resealed, and incubated overnight at 37°C. The probe was then removed and the blot was then washed three times in 0.2 X SSC, 0.1% w/v SDS at 60°C (45 mins/wash). Probe signal was then visualised by direct autoradiography.

2.7.4 RNase protection assays to determine mRNA abundance

RNase protection assay's were performed on 2ug of total RNA essentially as described by Krieg and Melton (1987) with the following modifications. The RNase digestion was performed using 150ul of the digestion buffer which contained 3.4ug/ul of RNase T1. The digestion step was then followed by a proteinase K digestion with 0.5% SDS. The final ethanol precipitation was altered by reducing the amount of tRNA added to 5ug, the amount of ethanol to 320 ul and by adding 8 ul of 7M NH_4-acetate. The precipitate obtained from this ethanol precipitation was then dried under vacuum before being dissolved in formamide loading buffer and run on an denaturing acrylamide gel. The gel was then fixed in 10% v/v methanol, 10% v/v acetic acid before drying and visualisation of the signal by direct autoradiography.

2.7.5 Densitometer scans of RNase protection assays.

The autoradiographs from the RNase protection assays were scanned using a Pharmacia LKB Ultroscan XL enhanced laser densitiometer.

2.7.6 *In situ* hybridisation.

Probes for *in situ* hybridisations were prepared by *in vitro* transcription of templates prepared from the 3' flanking regions of the relevant cDNA's. An exception to this was the ENOD2 probe which was prepared using the entire cDNA as a template followed by partial degradation of the probe using NaOH. *in situ* hybridisdations were performed by the method of de Billy *et al* (1991) briefly described below.

2.7.6.1 Fixation and microtomy.

Individual nodules were excised from the plant and immediately fixed in 2% *p*-formaldehyde and 0.05% glutaraldehyde in 0.1M $NaPO_4$ buffer (pH 7.2). After fixation the nodules were rinsed twice in buffer and subjected to one of two sectioning protocols. In the first, nodules were dehydrated in a graded ethanol series

and a graded xylene series before being embedded in paraplast. Section were taken at a thickness of 7um and were deposited on poly-L-lysine coated slides, the paraplast was removed with xylene washes before the sections were rehydrated. The rehydrated sections were rinsed twice with distiled water. In the second protocol the fixed nodules were sectioned to a thickness of 80um and then rinsed twice with phosphate buffer and distiled water.

2.7.6.2 Pretreatments.

The sections were successively treated with 0.2M HCl (20 minutes at 20°C), rinsed with 2xSSC (20x stock 3M NaCl, 0.3MNa_3 citrate), digested with proteinase K(1ug/ml in 10mM TRIS pH8, 5mM EDTA) for 30 minutes at 37°C followed by three rinses in distiled water. The sections were then treated with 0.25% v/v acetic anhydride in 0.1M triethanolamine pH8 for 10 minutes at 20°C. The thin sections were then dehydrated in a graded ethanol series and air dried whilst the thick sections were pre-hybridised in hybridisation solution (without dextran sulphate).

2.7.6.3 Hybridisation

The RNA probes were denatured in 50% v/v formamide, 10mM DTT by heating at 80°C for 1 minute, followed by rapid cooling on ice before being added to the hybridisation solution (50% v/v formamide, 10% w/v dextran sulphate, 0.5mg/ml yeast tRNA, 1mg/ml polyA, 10mM DTT, 1X Denhardts solution, 300mM NaCl, 20mM TRIS pH 7.5, 2mM EDTA). Thin sections were hybridised in a moist chamber for 16 hours at 42°C, the thick sections were hybridised in eppendorf tubes under the same conditions. After hybridisation the sections were washed in 4X SSC, 5mM DDT for 15 minutes at 20°C, followed by incubation in Rnase buffer (50 ug/ml RNase A, 0.5M NaCl, 10mM TRIS pH8, 1mM EDTA, 10mM DTT) for 30 minutes at 37°C. The sections were then washed three times in the same buffer without the RNase A (30 minutes at 37°C, followed by three washes in 0.2X SSC, 1mM DTT (40 minutes at 63°C). The sections were then dehydrated in a graded ethanol series and air dried. The thick sections were placed on poly-L-lysine coated slides prior to drying.

2.7.6.4 Autoradiography.

Slides were coated with Kodak NTB_2 nuclear emulsion (diluted 1:1 with distilled water), dried in obscurity for

16 hours at 20°C and then left for exposure at 4°C. The sections were then developed in Kodak dektol (diluted 1:1) with distiled water for two minutes at 15°C followed by fixing in Agfa agefix (diluted 1:9 with distiled water) for 3 minutes at 15°C and rinsed in distiled water. After staining with toluidine blue (0.02% w/v) for 20 seconds the section were observed by bright field and dark field microscopy.

2.8 Protein manipulations.

2.8.1 Preparation of crude plant protein extracts.

Crude plant protein extracts were obtained as described in Bennett *et al* (1989). Approximately 0.5g of tissue was extracted in buffer (10mM TRIS-Cl pH 7.5, 5mM glutamic acid, 10mM Mg acetate, 1mM DTT, 0.1% v/v Triton X-100, 0.02% w/v PMSF) at 4°C. The homogenate was then centrifuged for 3 minutes in a microcentifuge and filtered through a 0.2um filter.

2.8.2 HPLC chromatography

Extracted GS isoenzymes were separated by HPLC ion exchange chromatography using a DEAE TSK 5PW column 75mm long with an internal diameter of 7.5mm with a flow rate of 0.5 ml/min, eluting the isoenzymes on a KCl gradient (0-0.4M) and collecting samples every minute (Bennett and Cullimore 1989). These fractions were assayed for GS activity individually (using the transferase assay, section 2.8.3), the activity was then adjusted for the column loading to give an activity per mg of soluble protein loaded on the column.

2.8.3 Glutamine synthetase assays.

Glutamine synthetase transferase assays were performed as described in Cullimore and Sims (1980). Enzyme preparations were incubated with assay mixture (100uM TRIS acetate pH 6.4, 100uM L-glutamine, 60uM hydroxylamine, 0.5uM ADP, 1uM MnCl2, 20uM Na arsenate). The mix was incubated at 37oC for varying periods of time before the reaction was stopped with the addition of an excess of stop mix ($FeCl_3$ 26g/l, trichloroacetic acid 40g/l) and the product measured at OD_{500}.

2.8.4 Measurement of total protein

Total protein concentrations were measured by the method of Bradford (1976), using Biorad reagents, a Bovine γ globulin standard curve following the manufacturers instructions.

2.8.5 Acetylene reduction assay.

Acetylene reduction assays were performed as described in Lara *et al* (1983). Nodules with attached roots were incubated in a sealed flask with Acetylene. Ethylene production was measured on a gas chromatograph (Pye series 104 oven fitted with a Kathometer detector).

Chapter three

The expression of *gln-α* and PAL 1 in normal and senescing root nodules

3.1 Introduction

The root nodule is the specialised organ for nitrogen fixation in leguminous plants such as *Phaseolus vulgaris* (French bean). Nodules are formed after a complex interaction of factors between the plant and the rhizobial symbiont and can take two forms: indeterminate, nodules found in temperate legumes such as pea and alfalfa or determinate nodules, found in tropical legumes such as French bean and soybean. Determinate nodules have a meristem which is only active in the immature nodule. Cell division occurs in all planes resulting in a spherical mature nodule containing cells which are all at an equivalent developmental stage. A different growth pattern is seen in indeterminate nodules which maintain an active persistent meristem, usually at the tip of the nodule. This results in an elongated nodule which contains tissues at various stages of development from meristematic to senescent (see Robertson and Farnden 1980, Vance 1990, for reviews).

In *Phaseolus vulgaris* the nodule contains a variety of GS isoenzymes dominated, at least in the mature nodule, by γ subunits (Cullimore *et al* 1983, Lara *et al* 1983, Lara *et al* 1984) encoded by *gln-γ* (Bennett *et al* 1989). The induction of expression of *gln-γ* occurs

before the onset of nitrogen fixation, the expression becomes measurable at around eight days post inoculation peaking at around twelve days. This expression pattern is similar to that shown by leghaemoglobin and to that shown by *gln-∂* but is in contrast to that shown by *gln-α* and *gln-ß*. *Gln-ß* is expressed throughout nodule development whilst *gln-α* is expressed early in nodule development then decreasing after around ten days (Bennett *et al* 1989). The expression patterns of the GS genes were found to be altered in nodules incubated under an argon/oxygen atmosphere (80% AR:20% O_2) in such a way that the levels of *gln-γ*, *gln-ß* and *gln-∂* were all reduced whilst the expression of *gln-α* showed a sustained induction after thirteen days (Cock *et al* 1990). It was then noticed that this expression pattern correlated with that observed for PAL 1 and that the nodules under this treatment appeared to be senescing prematurely (Cock and Cullimore unpublished data).

It was decided to further study this phenomenon to determine whether this co-induction of *gln-α* and PAL 1 is purely a function of nodule growth under the argon/oxygen atmosphere or as a result of the senescence induced under these conditions. To determine this plants were inoculated with a Fix- strain of *Rhizobium* that induces early senescence of the nodule (Noel pers comm). The expression patterns of several genes was studied, using the RNase protection technique allowing a specific study

of individual gene family members, in order to observe any co-induction of PAL 1 and *gln-α*. In addition, attempts were made to locate both the cellular location of the message, using *in situ* hybridisation techniques, and changes in the GS isoenzyme content of the nodules using ion exchange HPLC.

3.2 Growth and nodulation of *Phaseolus vulgaris* plants

Plants were grown and nodulated essentially as described in section 2.1 except that in addition to the wild type *Rhizobium leguminosarum bv phaseoli* (CE3) (Noel *et al* 1984) strain a separate set of plants was inoculated with a Rhizobial mutant CE119 which was known to cause the nodules to senesce early (Noel unpublished data). Seven pots were set up, one for each time point, which were taken at three day intervals starting at day ten and concluding at day twenty eight. The rhizobial strains used (see table 3.1) were checked, by measuring the levels of nitrogen fixation of nodules derived with the strains, using the acetylene reductase assay described in section 2.8.6 of the materials and methods. In addition to nodules derived using the two rhizobial strains used in the experiment one other was used for the acetylene reductase assay. This strain, CE116, produces nodules which fix nitrogen at a much lower efficiency

Table 3.1: A list of *Rhizobium legumuinosarum bv phaseoli* strains used for nodule experiments.

Rhizobial strain	Description	Reference
CE3	CFN42 *str*-1 (Sym $^{+}$)	Noel *et al* (1984)
CE116	Mutant isolated after transfer of pJB4JI to the strain	Noel *et al* (1984)
CE119	Mutant isolated after transfer of pJB4JI to the strain	Noel (unpublished)

(Fix$^{\pm}$) than the wild type derived nodule (Noel *et al* 1984). The results of the acetylene reduction suggested that the identity of the rhizobial strains was correct (figure 3.1),the root systems inoculated with strain CE116 produced *circa* 5% of the amount of product produced by the wild type derived nodules whilst the roots inoculated with strain CE119 produced no product (within the limitations of the assay (< 5 umoles of C_2H_2 per minute per plant).

Both the aerial portions of the plants and the nodules showed visible differences between plants inoculated with the wild type CE3 rhizobia and the mutant CE119 strain. The plants with the wild type inoculum showed normal growth with healthy leaves and red nodules whilst the plants with the mutant inoculum had chlorotic leaves and smaller nodules which turned green as the time course progressed, indicating early senescence (figure 3.2).

3.3 Studies on the expression of PAL 1 and gln-a mRNA in the nodules

Total RNA was extracted from the harvested nodules and a small quantity was run on a 1.5% formaldehyde gel to check for errors in quantitation and for degradation of the RNA (figure 3.3). This RNA was used for RNase

Figure 3.1: The acetylene reduction activity of various *Rhizobium leguminosarum bv. Phaseoli* strains.

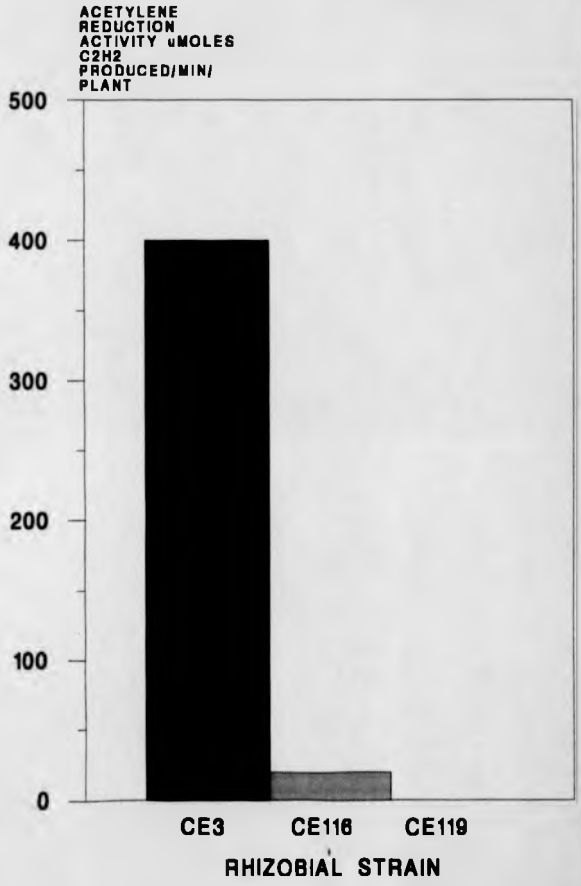
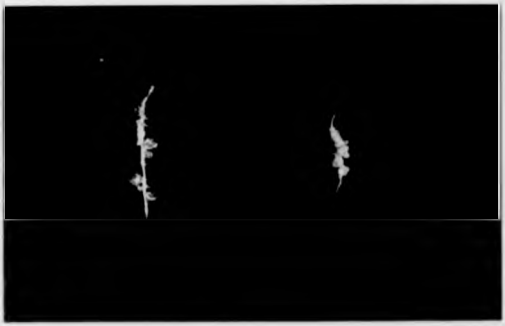

ACETYLENE
REDUCTION
ACTIVITY uMOLES
C2H2
PRODUCED/MIN/
PLANT
500
400
300
200
100
0
CE3
CE116
CE119
RHIZOBIAL STRAIN

Figure 3.2: *Phaseolus vulgaris* plants and root systems at 28 days post-inoculation with the CE3 strain of *Rhizobium leguminosarum bv. Phaseoli*, or the CE119 strain of *Rhizobium leguminosarum bv. Phaseoli*. A. The aerial portion of the plant, the plant on the right was inoculated with the CE119 mutant whilst the plant on the left was inoculated with the wild type CE3 strain. B. Small segments of the root systems showing nodules with CE119 derived nodules on the left and CE3 derived nodules on the right.

A

B



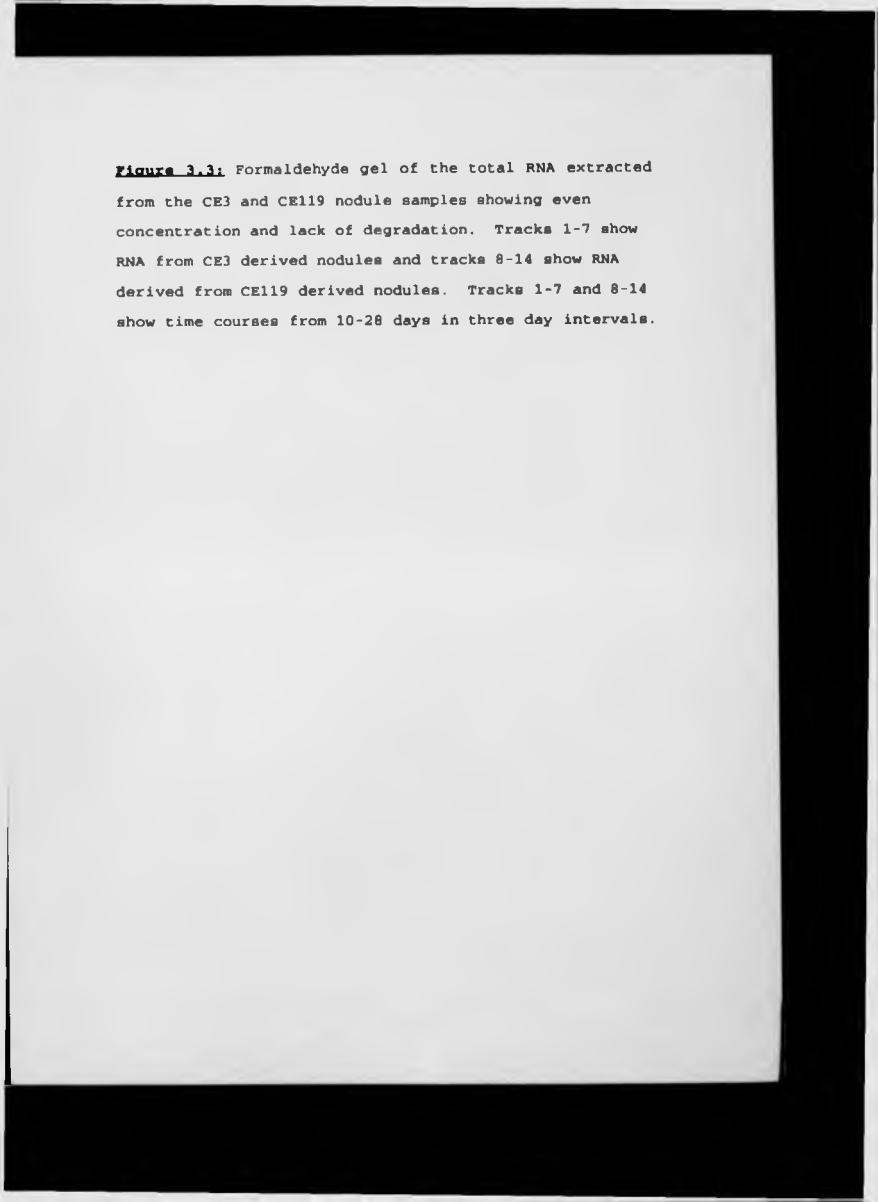
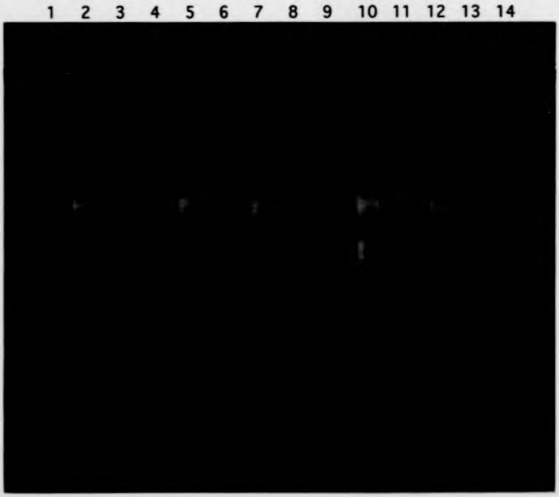


Figure 3.3: Formaldehyde gel of the total RNA extracted from the CE3 and CE119 nodule samples showing even concentration and lack of degradation. Tracks 1-7 show RNA from CE3 derived nodules and tracks 8-14 show RNA derived from CE119 derived nodules. Tracks 1-7 and 8-14 show time courses from 10-28 days in three day intervals.


1 2 3 4 5 6 7 8 9 10 11 12 13 14

protection assays, the results of which are shown in figure 3.4. The autoradiographs were scanned using a densitometer and results are therefore also shown graphically as a percentage of the maximum expression for each gene in figure 3.5. With the wild type derived nodules the mRNA's studied showed differential expression patterns. *Gln-γ*, which has previously been demonstrated to be nodule enhanced (Bennett *et al* 1989) showed an induction peaking at around day 22 and then decreasing. Note that unfortunately one of the time points for *gln-γ* was lost (13 days) during the assay procedure. *Gln-ß* also showed an earlier induction than that observed for *gln-γ* although the levels of message then stabilised for the remainder of the time course. The *gln-α* message abundance was high early in the time course but decreased falling to a low but apparently stable level after day sixteen. This expression pattern was similar to that for PAL 1 although the PAL 1 message reduced to an undetectable level by day sixteen. The levels of elongation factor 1-α subunit (*EF1-α*) remained relatively constant from days 13 to 28 although there is a possible reduction in the abundance of the message throughout the time course.

The expression patterns of the genes in the nodules containing CE119 is radically different. *Gln-γ* shows a premature reduction in the levels of mRNA whilst *gln-α* and PAL 1 expression is induced co-ordinately at around

Figure 3.4: A measure of the abundance of message for the following genes: *gln-α*, *gln-ß*, *gln-γ*, PAL 1 and EF1-α using the RNase protection assay on samples of CE3 and CE119 nodules. Tracks 1-7 represent a time course of 10, 13, 16, 19, 22, 25 and 28 days respectively. A represents RNA from CE3 derived nodules and B represents RNA from CE119 derived nodules.

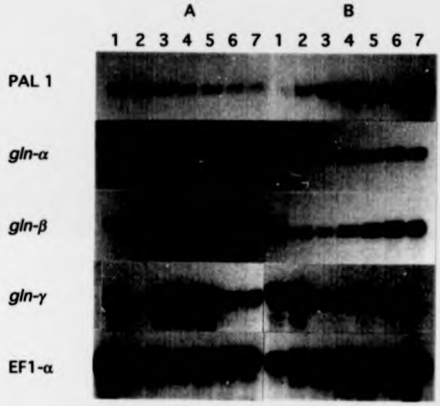
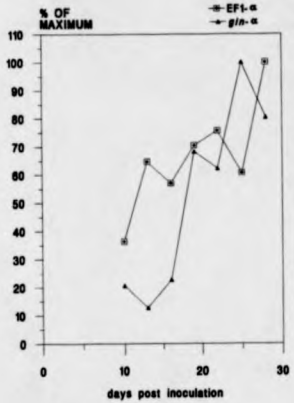

A
B
1 2 3 4 5 6 7 1 2 3 4 5 6 7
PAL 1
gln-α
gln-β
gln-γ
EF1-α

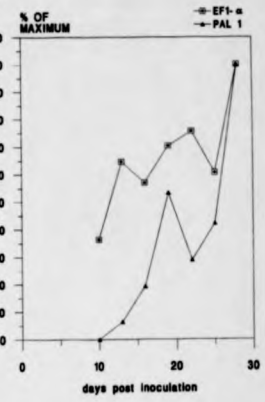
Figure 3.5: Graphical representations of the scanning densitometer analysis of the nodule RNase protection assays plotted with EF1-α.

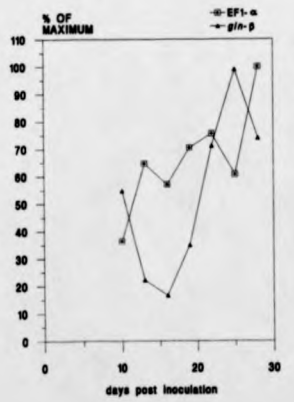
A CE119 derived nodules

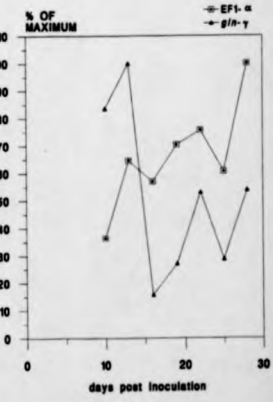
B CE3 derived nodules

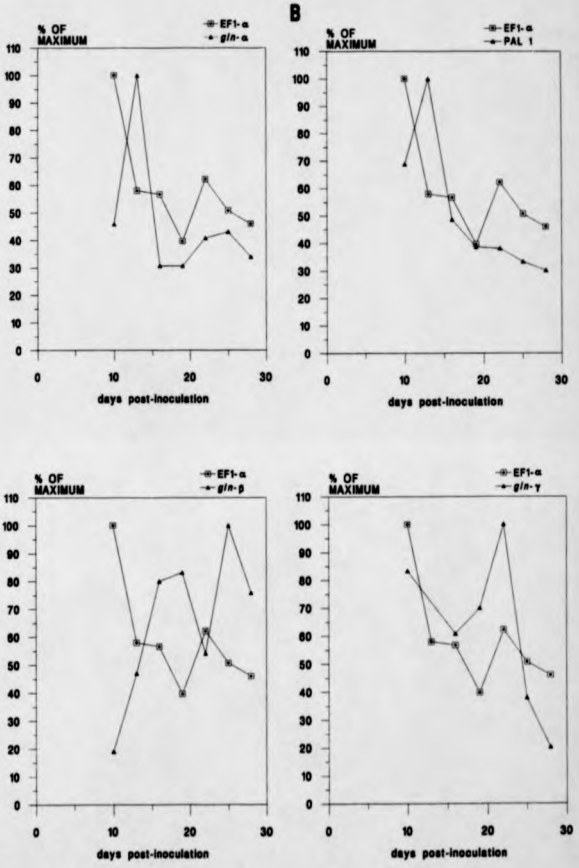
A


% OF MAXIMUM
EF1-α
gln-α
days post inoculation


% OF MAXIMUM
EF1-α
PAL 1
days post inoculation


% OF MAXIMUM
EF1-α
gln-β
days post inoculation


% OF MAXIMUM
EF1-α
gln-γ
days post inoculation


B
% OF MAXIMUM
EF1-α
gln-α
PAL 1
gln-β
gln-γ
days post-inoculation
0
10
20
30
100
90
80
70
60
50
40
30
20
10
110

day sixteen, although the PAL 1 message becomes more abundant. The expression of *gln-ß* is reduced compared to the level seen in the wild type nodules but the message does appear to be induced in a similar fashion to *gln-α* and PAL. *EF1-α* mRNA shows an increase in abundance over the time course with a level of expression similar to that observed in the wild type derived nodules between days 13-25.

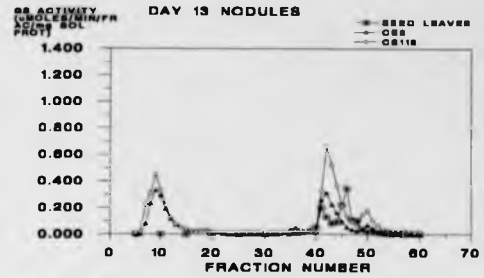
3.4 Studies on the differences in isoenzyme content of normal and senescent nodules

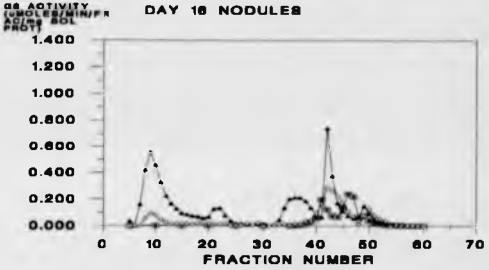
Total soluble protein was extracted from the nodule tissue at all of the time points except the ten day harvest. The extracted protein was separated on an ion exchange HPLC and the fractions assayed for GS transferase activity (for details see materials and methods section 2.8.3). Plumule extracts were also run on the column as a control as it is known that this tissue contains only α_8 and $ß_8$ isoenzymes which run as a double peak eluting in fractions 46 and 41 respectively (Bennett and Cullimore 1989). It is also known that the γ rich isoenzymes are eluted in the void volume of the column and that the plastid GS enzyme (∂) is eluted after the cytosolic forms, at *circa* fraction 50, (Bennett and Cullimore 1989). The results of the HPLC analysis of in

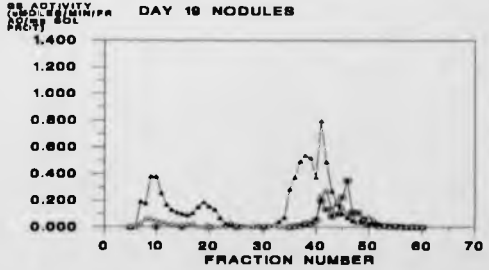
the nodule samples are shown in figure 3.6. The pattern of GS isoenzymes in the wild type nodules is as described by Bennett and Cullimore (1989). The subunit composition of each of the isoenzyme peaks was analysed by Bennett and Cullimore (1989) using 2 dimensional electrophoresis.

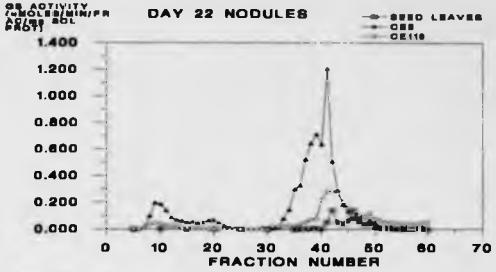
The pattern of GS activity seen in the older mutant derived nodules is radically different to that seen with the wild type derived nodules. At day thirteen there is little or no difference between the pattern of activity between the mutant derived and wild type derived nodules except that the mutant derived nodules showed a higher activity level in the α/ß containing isoenzymes. However by day sixteen the level of activity of the γ containing isoenzymes has fallen dramatically and continues to fall as the time course progresses until it is barely detectable by day twenty two and absent by day twenty eight. The other cytosolic GS peak also behaves differently in the mutant as compared to the wild type derived nodules. In the mutant derived nodules the peak widens towards the later α_8 position demonstrating an enrichment of α subunits in the cytosolic isoenzyme wheras in the wild type derived nodules the peak shifts toward the ß8 position. The plastidic isoenzyme appears to behave in a similar fashion in both the wild type and mutant derived nodules.

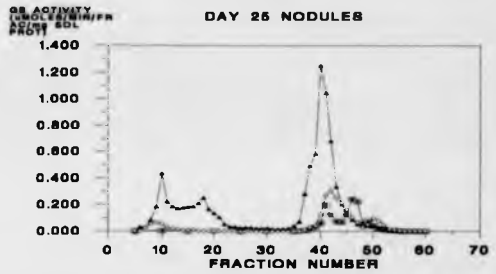
Figure 3.6: Graphical representation of the HPLC ion exchange chromatography of the GS isoenzyme activities present in crude extracts from CE3 and CE119 nodule samples:

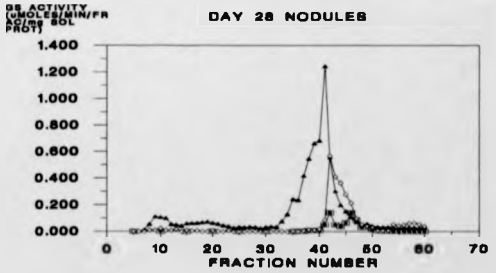

GS ACTIVITY
(UMOLES/MIN/FR
ACTIVE SOL
PROT)
DAY 13 NODULES
SEED LEAVES
GS2
GS1t1
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER


GS ACTIVITY
(UMOLES/MIN/FR
ACTIVE SOL
PROT)
DAY 16 NODULES
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER


GS ACTIVITY
(UMOLES/MIN/FR
ACTIVE SOL
PROT)
DAY 19 NODULES
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER


GS ACTIVITY (UMOLES/MIN/FR AC/mg SOL PROT)
DAY 22 NODULES
SEED LEAVES
GS8
GE110
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER


GS ACTIVITY (UMOLES/MIN/FR AC/mg SOL PROT)
DAY 25 NODULES
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER


GS ACTIVITY (UMOLES/MIN/FR AC/mg SOL PROT)
DAY 28 NODULES
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
FRACTION NUMBER

3.5 *In situ* hybridisation of wild type and mutant derived nodules.

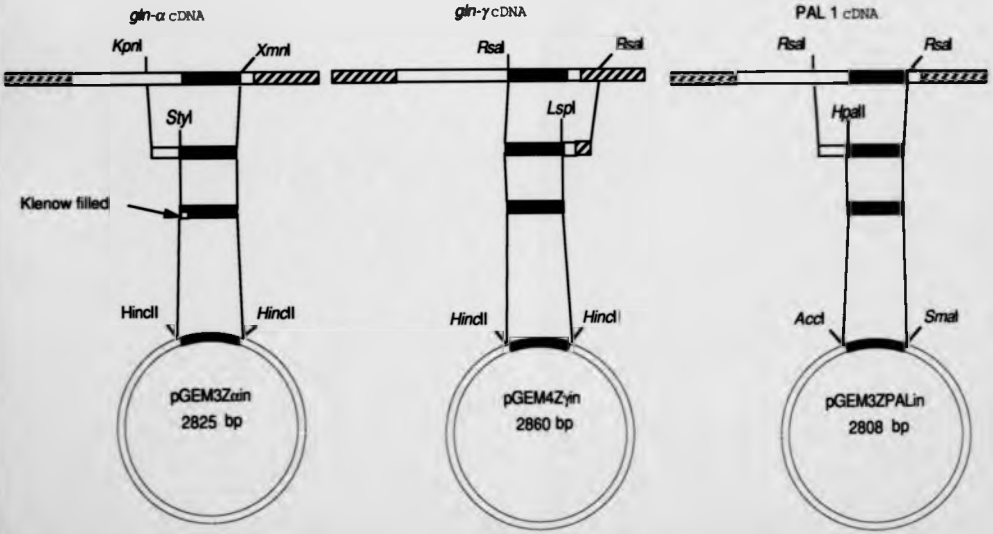
The expression of the PAL 1 and *gln-α* genes in the mutant and wild type derived nodules was studied further using *in situ* hybridisation to ascertain whether the message for the two genes was present in the same cell types. This would add further evidence towards the hypothesis that the two genes are co-induced in the mutant nodules.

3.5.1 Preparation of probe templates for *in situ* hybridisations.

For this study probes were required which would specifically recognise GS family members at relatively low stringencies (when compared stringencies employed into RNase protection assays). These probes were designed to be around 80-100 base pairs in length, as this was suggested to be the ideal length of probe for *in situ* hybridisaton (G Truchet pers comm). These probes were prepared by sub-cloning fragments from the 3' untranslated regions of the cDNA's for the required genes. The 3' untranslated region was chosen as this region carries the lowest homologies between the family members. These fragments were cloned into pGEM vectors such that both sense and antisense riboprobes could be produced from the T7 and SP6 promoters present in these

vectors (see figure 3.7 for the cloning scheme). Three probe templates were prepared *gln-α*, *gln-γ* and PAL 1. The *gln-α* template was produced by liberating and gel isolating a 850bp *KpnI/XmnI* restriction fragment (see figure 3.8A), this fragment was then cleaved with *StyI* and the 5' overhangs were subsequently filled in using the Klenow fragment of DNA polymerase I. These fragments were ligated into pGEM-3Z vector (*HincII* digested and gel purified) (see figure 3.8B). Only the required of the two *StyI* generated fragments of 78bp and 772bp could be ligated into the linearised vector although the correct fragment could be present in either orientation. The *gln-γ* template was generated in a similar fashion. A 1496bp *RsaI* fragment from the *gln-γ* cDNA was gel isolated and then cut with *LspI* liberating a 101bp and 1395bp fragments. These were then ligated into pGEM-4Z (*SmaI/AccI* digested and gel purified). Both fragments could be cloned into these sites but plasmids containing the two fragment were easily distinguished by restriction analysis. The template for the PAL 1 probe was produced by releasing a 429bp *RsaI* fragment which was gel purified, a 85bp fragment was liberated using *HpaI* and the mix of large and small fragments was ligated into *SmaI/Acc I* digested pGEM-3Z vector. In all of the above clonings the identity of the insert was checked both by restriction analysis of plasmid DNA obtained from the colonies with vector inserts (pGEM vectors give

Figure 3.7: The cloning scheme to produce short, gene specific transcription probes for use in *in situ* hybridisation analysis of CE3 and CE119 nodules.


gln-α cDNA
KpnI
XmnI
StyI
Klenow filled
HincII
HincII
pGEM3Zαin
2825 bp
gln-γ cDNA
RsaI
RsaI
LspI
HincII
HincII
pGEM4Zγin
2860 bp
PAL 1 cDNA
RsaI
RsaI
HpaII
AccI
SmaI
pGEM3ZPALin
2808 bp

blue/white colour selection) (see figure 3.8C for an example) and by double stranded sequencing of the putative positives (see figure 3.8D for an example). After cloning, problems were encountered in transcribing probes using the SP6 promoter, this was overcome by recloning the fragments. The fragments were isolated as *Hind*III/*Eco* RI fragments (*Hind*III and *Eco*RI cut in the vector polylinker) and then ligating the fragments released into an alternative *Hind*III/*Eco*RI digested GEM vector. Vectors were chosen such that the polylinker was in the opposite orientation to that of the original clone (ie pGEM3Z --> pGEM4Z and vice versa) such that both antisense and sense probe could be transcribed from a T7 promoter. All or the templates were linearised using an appropriate restriction endonuclease cutting at the opposite end of the polylinker to the T7 promoter, these templates could then be transcribed to produce probe (figure 3.9). The probes were checked with RNase protection assays using nodule and root RNA. An example of the result of this test can be seen in figure 3.10 where the α antisense recognises and protects a fragment from nodule total RNA but not from root total RNA. RNase protection assays performed on nodule tissue grown in the growth rooms at Toulouse, (where the *in situ* hybridisations were to be performed) suggested that the PAL 1 and *gln*-α messages peaked at day 31 post inoculation (figure 3.11). This indicated that the *in*

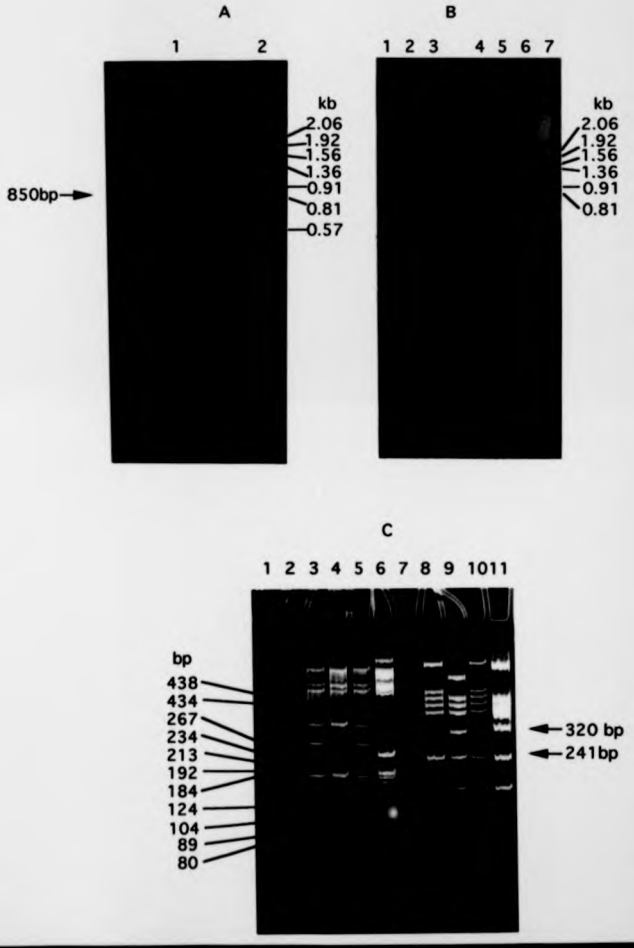
Figure 3.8: The subcloning of an *gln-α* fragment to produce a template for *in situ* hybridisation probes:

A The digest of pcGS-αM3 with *Kpn*I and *Xmn*I run on an 1% agarose gel in preparation for electroelution (track 1).Track 1 represents *Kpn*I/*Xmn*I digested α cDNA. Track 2 represents *Hind*III/*Eco*RI digested lambda DNA run as markers.

B The purified fragment and linearised and purified vector run on a 1% agarose gel. Track 2 represents the purified *Kpn*I/*Xmn*I fragment. Track 4 represents the purified linear vector. Track 7 represents *Hind*III/*Eco*RI digested lambda DNA run as markers.

C Restriction analysis of 5 small scale preparation from transformants with the *Xmn*I/*Sty*I fragment using *Dde*I and *Mbo*II digests run on a 10% acrylamide gel. Track 1 represents pBR322 digested with *Hae*III (used as markers), tracks 2-6 and 7-11 represents digested DNA from putative positive transformants. Tracks 1-6 represent *Mbo*II digests and tracks 7-11 represent *Dde*I digests. Tracks 6 and 11 represent a putative positive clone.

D Result of the double stranded sequencing of the putative positive clone seen in C. The insert is shown in bold type and the vector in normal type.


A
1
2
kb
2.06
1.92
1.56
1.36
0.91
0.81
0.57
850bp
B
1 2 3 4 5 6 7
kb
2.06
1.92
1.56
1.36
0.91
0.81
C
1 2 3 4 5 6 7 8 9 1011
bp
438
434
267
234
213
192
184
124
104
89
80
320 bp
241bp

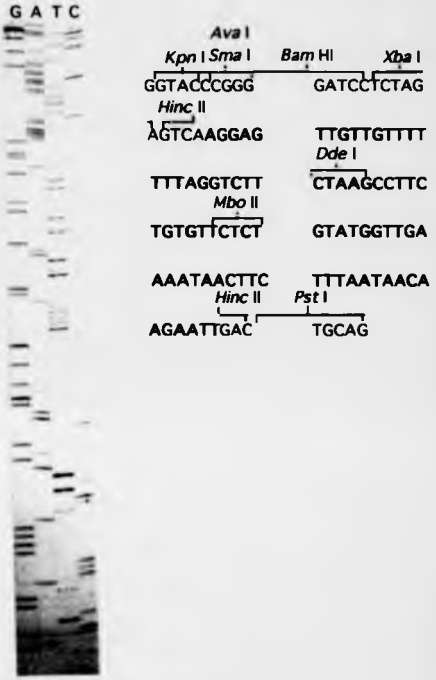

G A T C
Ava I
Kpn I Sma I
Bam HI
Xba I
GGTACCCGGG
GATCCTCTAG
Hinc II
AGTCAAGGAG
TTGTTGTTTT
Dde I
TTTAGGTCTT
CTAAGCCTTC
Mbo II
TGTGTTCTCT
GTATGGTTGA
AAATAACTTC
TTTAATAACA
Hinc II
Pst I
AGAATTGAC
TGCAG

Figure 3.9: Probe templates for *in situ* hybridisations transcribed as sense and antisense. Tracks 1 and 2 represent sense *gln-α* probe. Tracks 3 and 4 represent antisense *gln-α* probe. Tracks 5 and 6 represent sense *gln-γ* probe. Tracks 7 and 8 represent antisense *gln-γ* probe. Tracks 9 and 10 represent sense PAL 1 probe. Tracks 11 and 12 represent antisense PAL 1 probe.

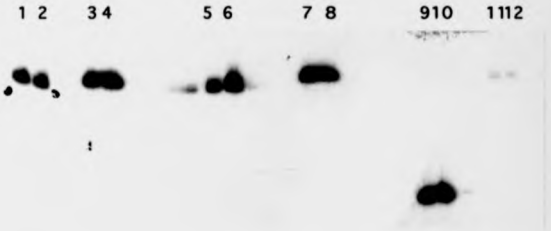

1 2
3 4
5 6
7 8
910
1112

Figure 3.10: RNase protection assay on CE119 derived nodule and root total RNA to check the specificity of the *gln-α* sense and antisense probes for *in situ* hybridisations. A represents antisense probe hybridisations and B represents sense hybridisations. Track 1 contains undigested probe, 1 and 2 contain day 19 CE119 derived nodule total RNA. Track 4 contains root tip RNA.


A
B
1 2 3 4
1 2 3 4

Figure 3.11: A measure of the abundance of message for the following genes: *gln-α*, PAL 1 and EF1-α using the RNase protection assay on samples of total RNA extracted from CE3 and CE119 derived nodules. Odd tracks represent hybridisations with the *gln-α* probe. Even tracks represent hybridisations with PAL 1 and EF1-α probes. Tracks 1-7 represent CE3 derived nodules and tracks 8-14 CE119 derived nodules. Tracks 1, 2, 7 and 8 represent 15 day samples. 3, 4, 9 and 10 represent 22 day samples. 5, 6, 11 and 12 represent 31 day samples. Tracks 13 and 14 represent hybridisations containing root tip RNA.

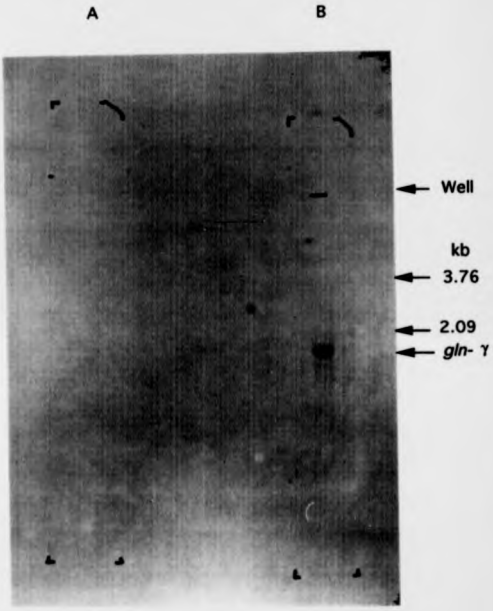

1 2 3 4 5 6 7 8 9 10 11 12 13 14
PAL 1
gln-α
EF1-α

situ experiments should be performed on 31 day tissue in order to obtain the highest signal from the sections. The specificity of the probes was checked using Northern blots with stringencies similar to those to be utilised in the *in situ* hybridisation experiments. The blot used for the *gln-γ* hybidisation is shown in figure 3.12. The message levels for *gln-α* and PAL 1 were too low in the tissue samples for any signal to be detectable on the Northern blots.

3.5.2 *In situ* hybridisation of nodule tissue.

Nodules for the *in situ* studies were grown in the Toulouse laboratories growth rooms where the experiments were carried out. Nodules were harvested at day 31 and immediately fixed, embedded and used for the *in situ* hybridisation studies as described in the materials and methods (see section 2.7.5). The exposed and developed sections were studied using both dark and light field microscopy (see figure 3.13). Silver grains representing hybridisation events were counted in both infected and uninfected cell types of several sections ensuring that an identical area of tissue was quantified in both cases. the results of the counting can be seen in table 3.2. The lack of any significant difference between the number of silver grains present in the treatments indicates that no conclusions can be reached from this experiment. The

Figure 3.12: A measure of the specificity of the *gln-γ* sense and antisense probes by northern hybridisation on total RNA derived from day 19 CE119 derived nodules. A represents a blot hybridised with sense probe and B represents an equivalent blot hybridised with antisense probe.


A
B
Well
kb
3.76
2.09
gln- γ

J: A bright field view of a hybridisation on a CE119 derived nodule section using an antisense *gln-α* probe.

K: A dark field view of the section from J.

L: A bright field view of a hybridisation on a CE119 derived nodule section using an antisense PAL 1 probe.

M: A dark field view of the section from L.

Figure 3.13: Results of the *in situ* experiments.

A: A low power view of a CE3 derived nodule.

B: A diagram of A denoting nodule structure.

a: Stele

b: Epidermis

c: Lightly stained un-infected cells

d: Nodule parenchyma

e: Endodermis

f: Cortex

g: Densly stained infected cells

h: Vascular tissue

i: Central region

C: A bright field view of a hybridisation on an CE3 derived nodule using an antisense ENOD2 probe.

D: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense *gln-γ* probe.

E: A dark field view of the section from D.

F: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense *gln-α* probe

G: A dark field view of the section from F.

H: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense PAL 1 probe

I: A dark field view of the section from H.

Figure 3.13: Results of the *in situ* experiments.

A: A low power view of a CE3 derived nodule.

B: A diagram of A denoting nodule structure.

a: Stele

b: Epidermis

c: Lightly stained un-infected cells

d: Nodule parenchyma

e: Endodermis

f: Cortex

g: Densly stained infected cells

h: Vascular tissue

i: Central region

C: A bright field view of a hybridisation on an CE3 derived nodule using an antisense ENOD2 probe.

D: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense *gln-γ* probe.

E: A dark field view of the section from D.

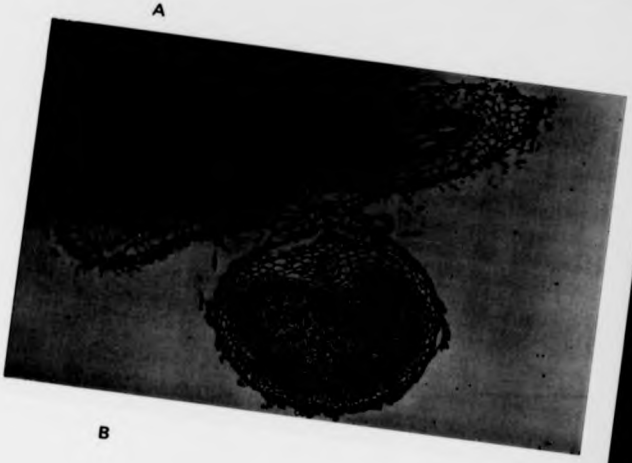
F: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense *gln-α* probe

G: A dark field view of the section from F.

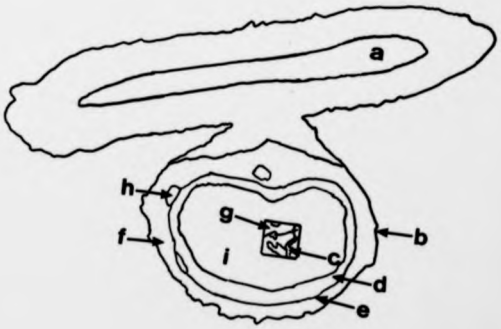
H: A bright field view of a hybridisation on a CE3 derived nodule section using an antisense PAL 1 probe

I: A dark field view of the section from H.

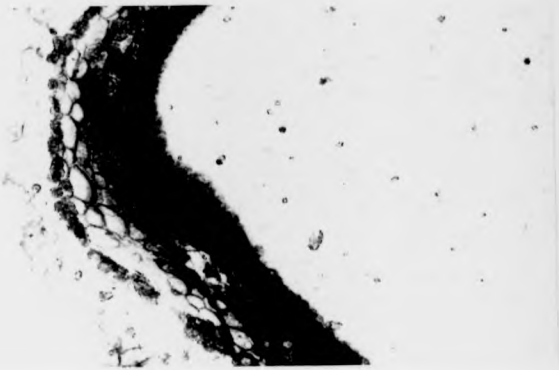
A



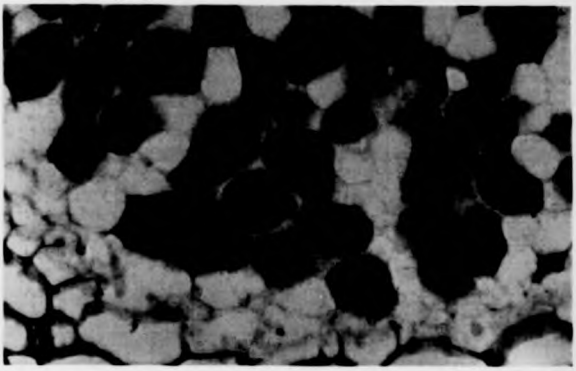
B



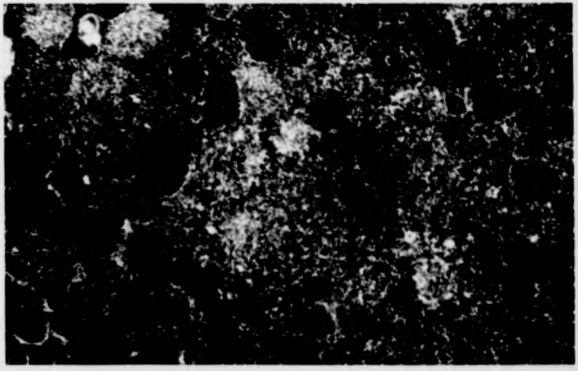
C



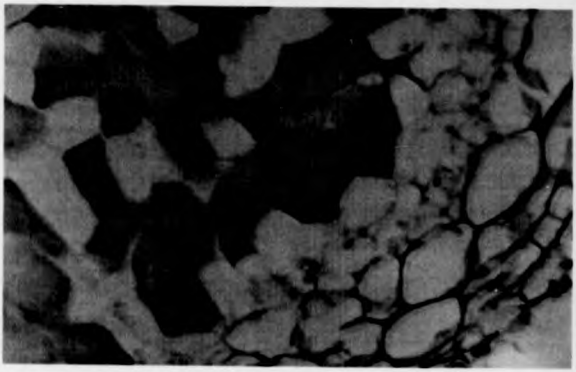
D



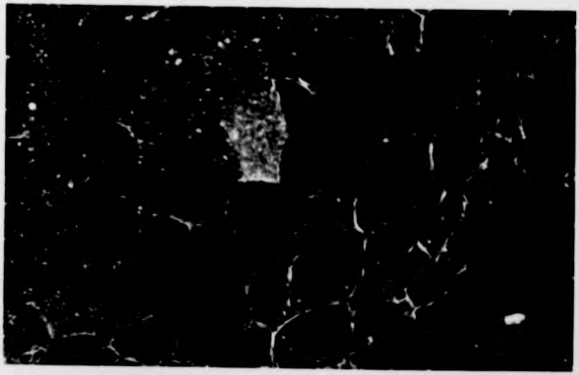
E



F

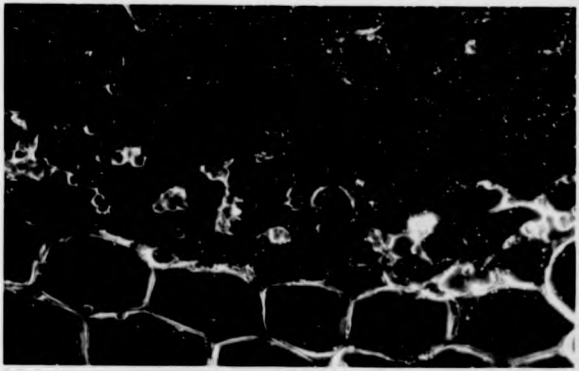


G



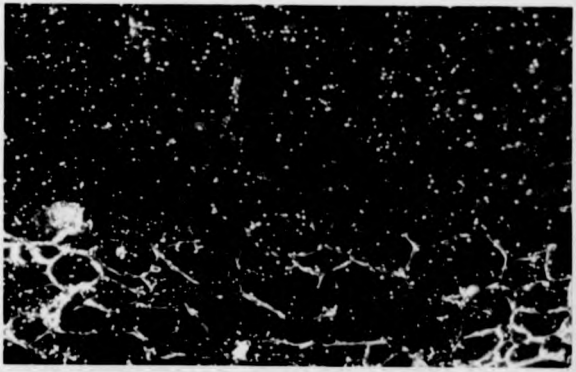
H

I

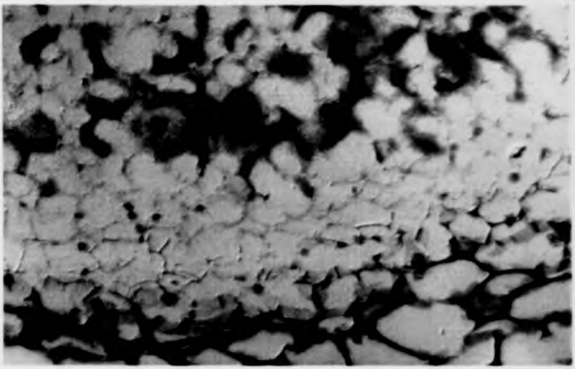


J

K



L



M

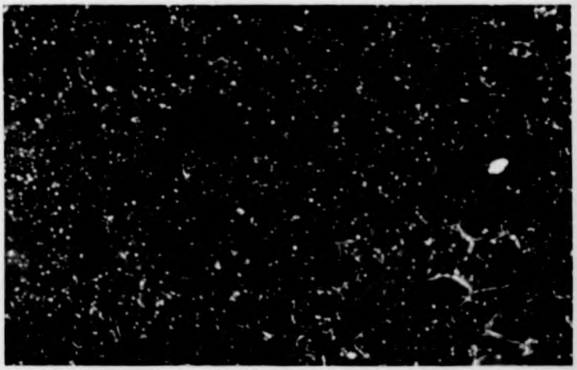


Table 3.2: A table showing quantitation of *in situ* hybridisations for *gln-α* *gln-γ* and PAL 1 probes on sections of CE3 derived nodules.

	gln-γ		*gln*-α		PAL 1	
	antisense	sense	antisense	sense	antisense	sense
infected cells	5.3±1.05	4.7±1.27	13.35±4.53	10.3±2.45	9±1.58	10.1±3.5
uninfected cells	1.8±0.87	2.5±0.92	3.6±1.5	5.5±1.5	5.95±1.24	6.45±1.32

result with the ENOD 2 probe show that this gene is highly expressed in the nodule parenchyma as demonstrated previously in soybean nodules by van de Wiel *et al* (1990). The success of this control treatment suggests that the lack of any significant differences in the remainder of the treatments is not due to the experimental conditions used. In addition, the central region of the CE119 derived nodules shows advanced senescence with breakdown of the cellular structure rendering it impossible to distinguish between infected and uninfected cells. In the wild type derived nodules the difference between the infected and uninfected cells is apparent. The infected cells stain more densely than the uninfected cells, due to the presence of a denser cytoplasm, and thus appear blue.

3.6 Discussion

The results from the Rnase protection assays performed on total RNA extracted from CE3 and CE119 derived nodules confirmed the preliminary experiment performed by Cock and Cullimore in that a coinduction of PAL 1 and *gln-α* was observed in both the early stages of nodule development with the CE3 derived wild type nodules and also in the later time points of the CE119 derived nodule time course. In the later time points of the

CE119 derived nodule time course the nodules were obviously beginning to senescence as denoted by the pronounced green colour of degraded leghaemoglobin in the harvested tissue. The behaviour of *gln-ß* in these samples was also interesting, the expression pattern of this gene is normally radically different to that of *gln-α* as shown in the CE3 derived nodule samples and previously by Bennett *et al* (1989) and Cock *et al* (1990). However, in the CE119 derived nodules the expression pattern of *gln-ß* is completely changed. The gene shows an induction in expression similar to that seen with *gln-α* and PAL 1 although the abundances of the mRNA are always below those observed in the wild type nodules. This induction pattern was not observed in nodules grown under an argon/oxygen atmosphere (Cock *et al* 1990), conditions which also produce Fix$^-$ nodules that senesce early. This indicates that the response of *gln-ß* in the CE119 nodules is likely to be due to the presence of the mutant bacteria rather than to the general conditions produced in these nodules. The expression pattern exhibited by *gln-γ* in the wild type derived nodules is basically similar to that observed previously in wild type nodules (Bennett *et al* 1989, Cock *et al* 1990) although the expression of the gene appears to be maintained albeit at a reduced level in the CE119 derived nodules throughout the length of the time course. This observation is in contrast to the pattern of expression

observed for this gene during the argon/oxygen experiment performed by Cock *et al* (1990) where the level of *gln-γ* mRNA was undetectable after 17 days.

The *EF1-α* expression levels were measured as a control treatment, the levels of this message were unlikely to be regulated by the same developmental and environmental cues as the other genes studied (Aguillar *et al* 1991, Ursin *et al* 1991). The levels of the *EF1-α* message in this experiment did remain relatively constant although certain samples were high, for example, the 10 day time point of the wild type nodules and the 28 day time point of the CE119 derived nodules. The reasons for this variability in the expression pattern of *EF1-α* remain unknown although the high mRNA levels observed with 28 day time point may be due to an inconsistency in the loading of the RNA samples.

The HPLC ion exchange separation of the GS isoenzymes from the nodule samples reinforced the evidence from the RNase protection assays. The GS isoenzymes displayed an enrichment in both α and ß subunits over the CE119 derived time course, although the possibility of α/γ mixtures cannot be ruled out without two dimensional gel electrophoresis data. The behaviour of the other subunits,suggested by ion exchange chromatography, also reflects the abundances of mRNA observed for the different genes. This suggests that the control of expression of GS activity is at the

transcriptional level although an increase in mRNA stability cannot be ruled out.

There could be many reasons for the induction of *gln-α*, *gln-ß* and PAL 1 observed in the CE119 derived nodule time course. Two appear most likely: 1 The induction is in response to the senescence of the plant tissue and has no connection with the mutant *Rhizobia* beyond the fact that the early senescence was in response to infection with the CE119 *Rhizobia*. 2 That the senescence of the nodules leads to a breakdown of the peribacteroid membrane, recognition of the bacteroids as foreign followed by a hypersensitive response by the plant against the bacteroids.

In situ hybridisations were performed using both mutant and wild type derived nodules in order to ascertain wether the expression of *gln-α* and PAL 1 occured in the same cell type. If the induction was due to general plant senescence expression of the relevant genes would be likely to be seen in all cell types of the senescing nodules. However, if the induction was due to a hypersensitive response a higher or exclusive induction of the genes would be expected in the infected cells of the central zone of the nodule. The results obtained with the *in situ* technique were however disappointing and proved inconclusive. The Enod 2 probe gave the expected expression pattern with a high message abundance in the nodule inner cortex (alternatively termed nodule

parenchyma) as shown for both soybean and pea nodules (van de Wiel *et al* 1990). The expression of the other genes studied in the nodules (*gln-α*, PAL 1 and *gln-γ*) yielded no conclusive results. The levels of hybridisation for all probes in the wild type nodules was higher in the infected tissue than in the uninfected cells. However, this phenomenon was similar to that observed with the sense probes used as a control, thus suggesting that the higher levels of hybridisation observed in the infected cells of the CE3 nodules is due to the higher density of cytoplasm in these cells as opposed to any increased levels of expression. The lack of expression of *gln-γ* in the CE3 nodules is in contrast to the high levels of GUS staining observed by Forde *et al* (1989) in *Lotus corniculatus* plants transformed with a *gln-γ* promoter/ß-glucuronidase construct. The differences in the results obtained in this study and that of Forde *et al* (1989) may be due to an increased sensitivity of the GUS fusion approach or the fact that younger nodules were used for the transgenic work than those used in this study. The main peak of *gln-γ* expression in wild type nodules occurs between days 10 and 16 post-inoculation (Bennett *et al* 1989). It is probable that a combination of these two possible reasons was responsible for the inconclusive result with the *gln-γ* probe. The overall lack of success of this experiment was due to the lack of definition in the central tissue

of the CE119 derived nodules. This lack of definition was due to senescence of the central region to the extent that the cell walls had degraded leaving an amorphous region with no viable cells. This situation obviously precluded any ability to discern any differences of expression between infected and uninfected cells.

Chapter 4

Investigation of the possible coordinate expression of *qln-a* and PAL 1

4.1 Introduction

Following the demonstration that *gln-α* and possibly *gln-ß* are co-induced with PAL 1 in senescent nodule tissue of *P. vulgaris* it was decided to study the expression of the two genes in tissues and under conditions where it is known that PAL 1 is induced in order to study any possible general coordinate expression of *gln-α*, *gln-ß* and PAL 1. The systems used for the study are the elicitation of cell suspension cultures with fungal elicitors, the wound response in hypocotyls and the reaction of leaf tissue to compatible and incompatible pathogens.

The elicitation of cell suspension cultures has become a model system to study the expression of phenylpropanoid pathway genes during induction. Either biotic elicitors, such as those heat released from the fungal pathogen *Colletotrichum lindemuthianum* cell walls (Cramer *et al* 1985) or abiotic elicitors, such as UV light (Chappel and Hahlbrock 1984).

An experiment was designed using wounded hypocotyls which underwent various treatments before total RNA was extracted and used for RNase protection assays. These

treatments were based on the knowledge that wounding of hypocotyls is known to induce the PAL genes of *P. vulgaris* (Liang *et al* 1989b), that reduced glutathione is capable of inducing PAL genes (Wingate *et al* 1988) and that high levels of cinnamate inhibit this response (Bolwell *et al* 1986).

Infection of bean hypocotyls with compatible and incompatible races of *Colletotrichum lindemuthianum* is known to evoke differential responses, the incompatible pathogen induces a hypersensitive response with a rapid induction of plant defense genes, including PAL, while the compatible pathogen gives a disease response with a delayed expression of the defense genes (Cramer *et al* 1985). Experiments were performed to study the behaviour of GS genes during the hypersensitive response. Compatible and incompatible races of the bacterial pathogen *Pseudomonas phaseolicola* were aerosol inoculated onto the lower lamina of bean leaves as described, the leaves were then incubated for varying lengths of time in a high humidity cabinet before harvesting. These leaves were then used in RNA extractions and subsequently RNase protection assays were performed on the extracted total RNA.

4.2 Studies on the expression of PAL 1 and gln-α in suspension cultures

All of the work performed on the suspension cultures, up to and including the point of harvesting, was performed at the Royal Holloway and Bedford New College, Egham on suspension cultures of *P. vulgaris* cv. Immuna. These suspension cultures were maintained as previously described by Dixon and Bendall (1978a). The cell cultures were treated with fungal elicitors from *Colletotrichum lindemuthianum* as described in Dixon *et al* (1981). In the first experiment samples were harvested at one hour intervals from and including time zero. The samples were harvested by vacuum filtration and snap frozen in liquid nitrogen before being taken to Warwick on dry ice and stored at -70°C. RNA was extracted from the tissue and RNase protection assays were performed on the extracted total RNA as described in materials and methods (section 2.7.4).

Several problems were encountered with this experiment. Firstly purification of the total RNA away from polysaccharide, which was present in the samples at a high concentration, proved difficult. When this was achieved the RNA was of a poor quality with many of the RNA samples being partially degraded resulting in many bands being visible in the RNase protection assays. This made it dificult to define accurately the signal relating

to the GS message (figure 4.1). In addition to this problem it was obvious that both the control (water addition) and induced (fungal elicitor addition) treatments had elicited the production of PAL 1 message in the cultures. In addition the cultures used for this experiment were coloured at the start of the experiment possibly indicating a high basal level of phenylpropanoid enzyme activity, this may be due to a high number of sub-cultures the cells had undergone or mechanical damage to the cells during culture. Either of these conditions could lead to an elicitation of the control cells during the set up of the experiment.

Along with the RNase protection assays protein extractions were also performed on the six hour time point sample. The protein extract was loaded on an ion exchange column on a HPLC and the GS activity of the separated GS isoenzymes was determined using the GS transferase assay (see materials and methods section 2.8.3 for details). The results from this separation are shown in figure 4.2. The seed leaves show α8 and ß8 isoenzymes from the tendergreen cultivar, the first peak at around fraction 59 represents the ß8 isoenzyme and the second peak at around fraction 65 represents the α8 isoenzyme. The position and breadth of the peaks obtained with the suspension culture samples suggest that the isoenzyme present in the cell suspension cultures is composed of a mixture of α and ß subunits with no

Figure 4.1: A measure of the abundance of message for the following genes: *gln-α*, PAL 1 and *gln-ß* using the RNase protection assay on samples of total RNA extracted from elicitor treated and control suspension cultures. A represents control water treated cells and B represents elicitor treated cells. Tracks 1-7 and 8-14 represent a time course beginning at 0 hours and finishing at 6 hours with samples taken at 1 hour intervals.

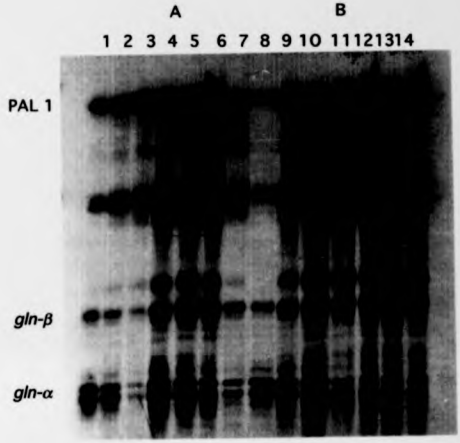
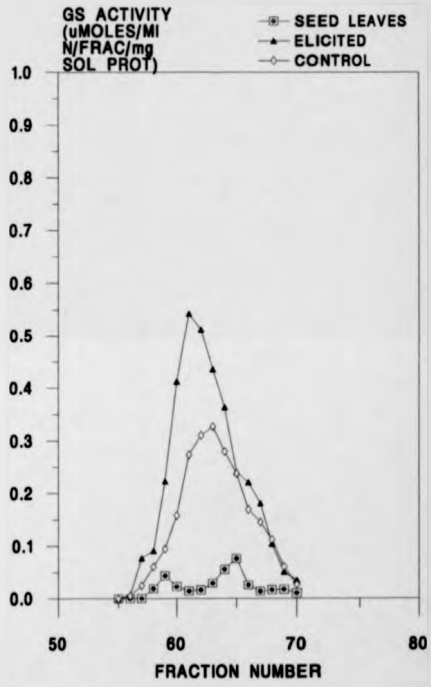

A
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PAL 1
gln-β
gln-α

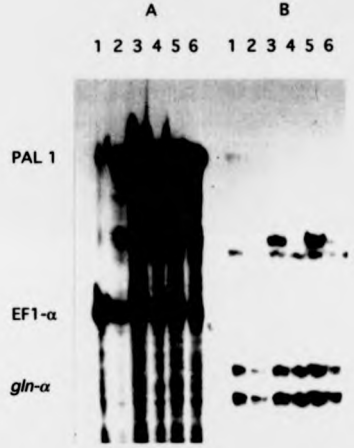
Figure 4.2: Graphical representation of the HPLC ion exchange chromatography of the GS isoenzyme activities present in crude extracts from the 6 hour time points of elicitor treated and control water treated suspension cultures.


GS ACTIVITY
(uMOLES/MI
N/FRAC/mg
SOL PROT)
SEED LEAVES
ELICITED
CONTROL
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
50
60
70
80
FRACTION NUMBER

evidence for the presence of γ or ∂ subunits. However, some caution is necessary in the interpretation of these results as the suspension cultures are derived from a different cultivar (immuna) which has been shown to contain differences in *gln-α* (this study) Differences in the a subunits of the two cultivars may affect the retention time of isoenzymes containing the α subunit on the salt gradient used for the separation of the isoenzymes.

This experiment was repeated at Egham and total RNA was extracted from the tissue samples using an alternative method (see materials and methods section 2.7.1), this extraction method was reasonably effective in purifying the RNA away from polysaccharide contaminants but failed to produce RNA of a quality high enough to produce clean results with the RNase protection assay (figure 4.3). However, this experiment did demonstrate that PAL 1 had been elicited by the fungal elicitor but that there appeared to be a much lower induction of *gln-α* signal of the expected size. There was however evidence of degradation products in the hybridisations in which the *gln-α* was used. There are two possible reasons for this observed degradation: 1. the total RNA is degraded yielding RNA species of a defined size or that 2. there are cultivar differences between the tendergreen cultivar *gln-α* and the *gln-α* present in the immuna cultivar resulting in a mismatch

Figure 4.3: A measure of the abundance of message for the following genes: *gln-α*, PAL 1 and EF1-α using the RNase protection assay on samples of total RNA extracted from elicitor treated cell cultures. A represents samples hybridised with PAL 1 and EF1-α probes. B represents samples hybridised with the *gln-α* probe. Tracks 1-6 represent samples taken at 1.5 hour time points beginning at 0 hours and finishing at 7.5 hours.


A
B
1 2 3 4 5 6 1 2 3 4 5 6
PAL 1
EF1-α
gln-α

between the probe and its recognition sequence resulting in partial digestion of the protected fragments. To test these two hypotheses RNA was extracted from plumule tissue, as it is known that *gln-α* is highly expressed in this tissue and that no other GS gene is expressed at this developmental stage (Cock *et al* 1991), from three cultivars of bean: tendergreen, immuna and canadian wonder. This RNA was used in RNase protection assays using the PAL 1 and *gln-α* probes. The results of these assays are shown in figure 4.4 and demonstrate that there are cultivar differences between tendergreen and the other two cultivars such that the protected band typically seen with tendergreen samples is not present in immuna but is replaced by two smaller fragments. This is probably due to a single mismatch in the probe/target hybridisation which is cleaved during the RNase digestion of the samples.

Protein was extracted from the suspension culture time course samples and the GS isoenzymes were separated by passing the crude extract down an ion exchange HPLC column. The activities of the separated isoenzymes was measured using the transferase assay (see materials and methods, sections 2.8.1, 2.8.2 and 2.8.3). The resulting enzyme activities are shown graphically in figure 4.5. The results presented confirm the earlier observation that in all of the time points apart from time point 1 the same isoenzyme is present in the control and elicited

Figure 4.4: A measure of the abundance of message for the following genes: *gln-α* and PAL 1 using the RNase protection assay on samples of total RNA extracted from seed leaf tissue from various *P. vulgaris* cultivars. Tracks 1 and 2 represent RNA from immuna. Tracks 3 and 4 represent RNA from tendergreen. Tracks 5 and 6 represent RNA from canadian wonder.

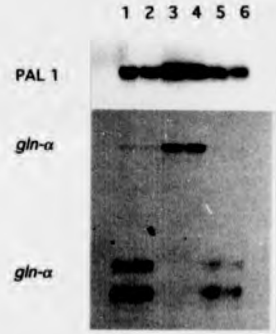
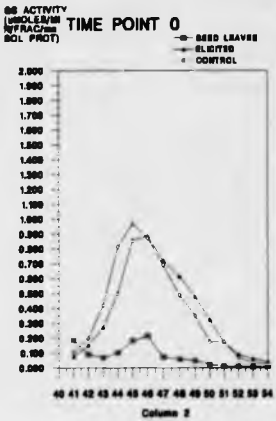
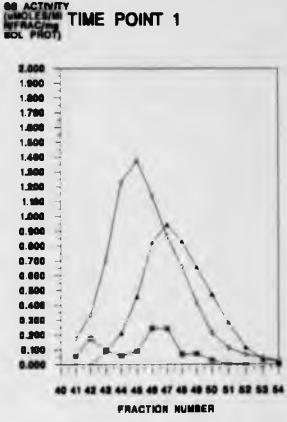
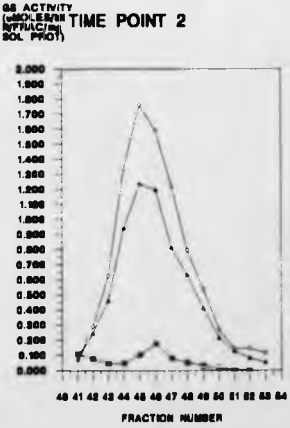
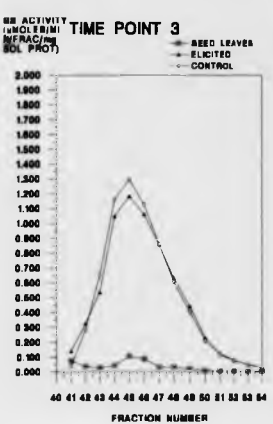

1 2 3 4 5 6
PAL 1
gln-α
gln-α

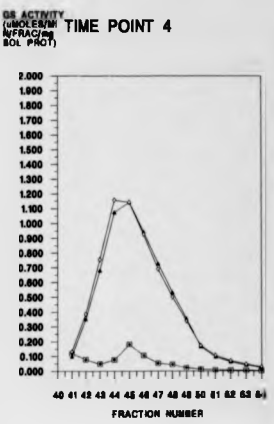
Figure 4.5: Graphical representation of the HPLC ion exchange chromatography of the GS isoenzyme activities present in crude extracts from 0, 1.5, 3, 4.5, 6 and 7.5 hour time points (0-5 respectively) of elicitor and control water treated suspension cultures.

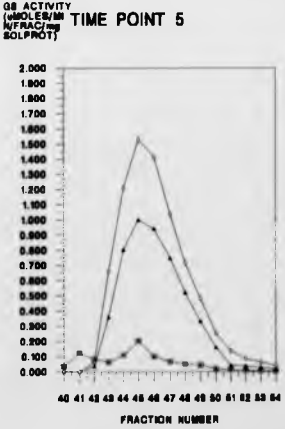

GS ACTIVITY
TIME POINT 0
SEED LEAVES
SLIOTSO
CONTROL
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
Column 2


GS ACTIVITY
TIME POINT 1
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
FRACTION NUMBER


GS ACTIVITY
TIME POINT 2
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
FRACTION NUMBER


GB ACTIVITY (uMOLES/MI N/FRAC/mg SOL PROT)
TIME POINT 3
BEED LEAVES
ELICITED
CONTROL
2.000
1.900
1.800
1.700
1.600
1.500
1.400
1.300
1.200
1.100
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
FRACTION NUMBER


GB ACTIVITY (uMOLES/MI N/FRAC/mg SOL PROT)
TIME POINT 4
2.000
1.900
1.800
1.700
1.600
1.500
1.400
1.300
1.200
1.100
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
FRACTION NUMBER


GB ACTIVITY (uMOLES/MI N/FRAC/mg SOLPROT)
TIME POINT 5
2.000
1.900
1.800
1.700
1.600
1.500
1.400
1.300
1.200
1.100
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
FRACTION NUMBER

cell cultures. The control sample of time point one displays a different activity profile in which the peak of enzyme activity is eluted earlier than in the other samples. This is more likely to be due to a problem with fraction collection than a real difference in this sample. By comparision with the extract from seed leaves of tendergreen it is likely that this isoenzymes making up the peak of GS activity are rich in α and ß subunits, although as previously mentioned care has to be taken in assigning peaks of activity in this case due to the cultivar differences between immuna and tendergreen. There is however little change in the levels of GS activity between the samples with the activity present in the elicited samples not rising above that of the control samples.

Due to the difficulties encountered in extracting RNA of a sufficient quality for RNase protection assays from the suspension cultures some total RNA was obtained from R Dixon (Samual Roberts Nobel foundation, Ardmore, Oklahoma). A time course treated with a fungal elicitor was supplied in addition to a control water treated time course. The total RNA supplied had previously been assayed for changes in glucanase and chitinase message levels (Edington *et al* 1991). This RNA was assayed using the RNase protection technique as described in materials and methods section 2.7.4. The results are displayed both as a photographical representation of the

autoradiographs (figure 4.6) and graphically after measurement of the signal using a scanning densitometer (figure 4.7). The levels of PAL 1 message were strongly induced in the elicitor treated cultures, but signal for PAL 1 was undetectable in the control treatment. This pattern of expression for PAL 1 has been demonstrated previously in elicited cell cultures of bean (Dixon *et al* 1981, Lawton *et al* 1983b, Cramer *et al* 1985a, Edwards *et al* 1985, Lawton and Lamb 1987). *Gln-α* was present in the control samples but was detected at a relatively low and constant level. The *gln-α* message was induced with the elicitor treatment although the induction occured later than the induction of PAL 1 message, with the peak at time point 3. In addition *gln-α* is expressed at a lower level than that observed for PAL 1 in the elicited samples. *Gln-ß* shows a different expression pattern to both PAL 1 and *gln-α* as the message for this gene is not induced with the elicitor treatment above levels seen in the control tissue although there is a slight possibility that the gene is induced to a lesser extent in the elicitor treated cells at time points 2 and 3. *EF1-α* is expressed at a relatively constant level throughout both the control and elicited time courses. This expression pattern is unsurprising as it has been demonstrated that the expression of this gene is linked to the levels of translation (Aguillar *et al* 1991, Ursin *et al* 1991).

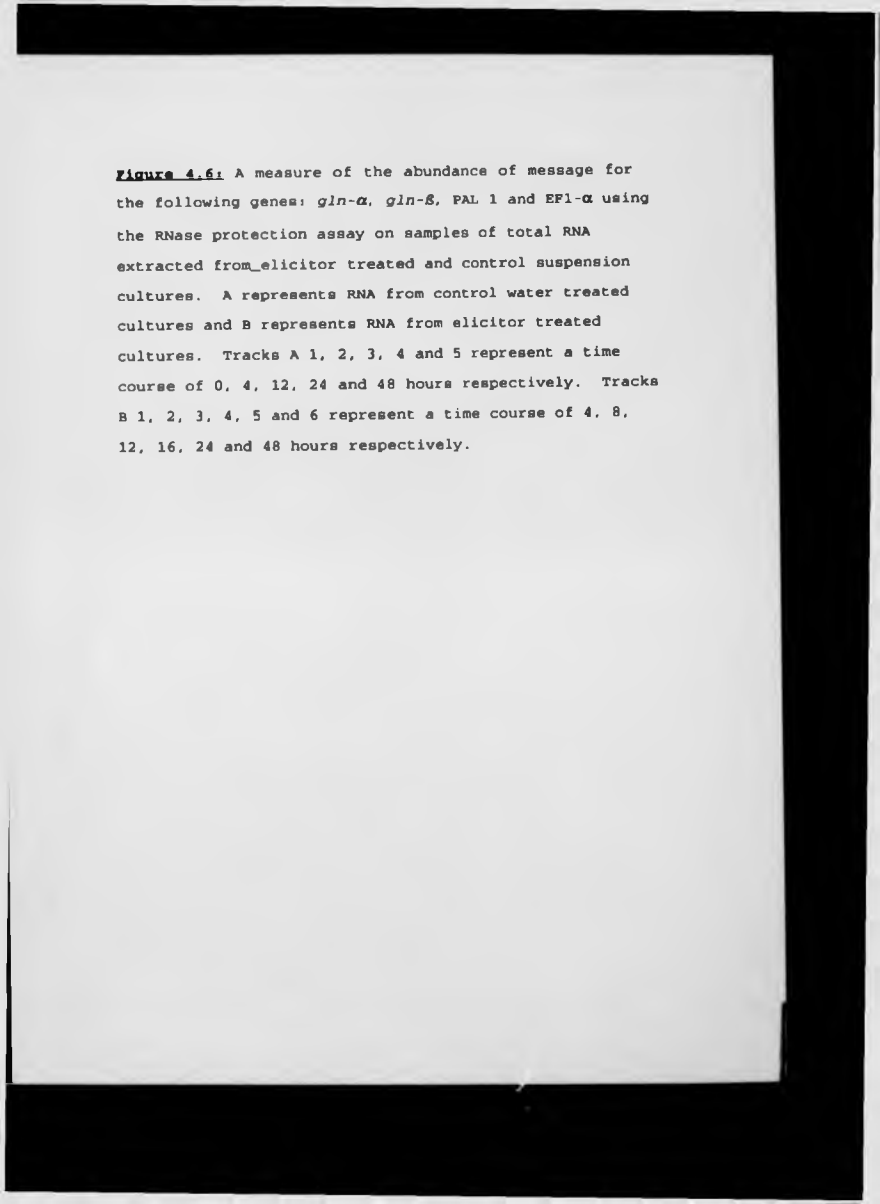


Figure 4.6: A measure of the abundance of message for the following genes: *gln-α*, *gln-ß*, PAL 1 and EF1-α using the RNase protection assay on samples of total RNA extracted from_elicitor treated and control suspension cultures. A represents RNA from control water treated cultures and B represents RNA from elicitor treated cultures. Tracks A 1, 2, 3, 4 and 5 represent a time course of 0, 4, 12, 24 and 48 hours respectively. Tracks B 1, 2, 3, 4, 5 and 6 represent a time course of 4, 8, 12, 16, 24 and 48 hours respectively.

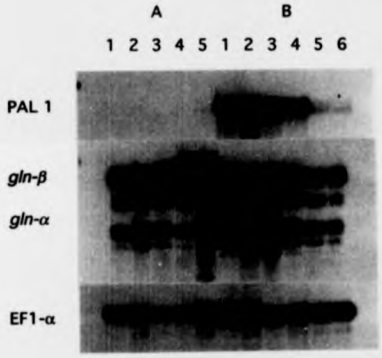
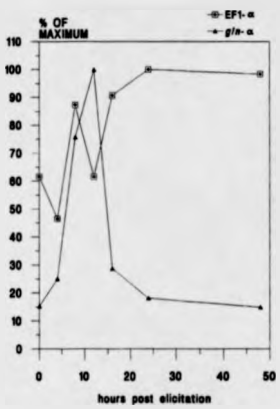
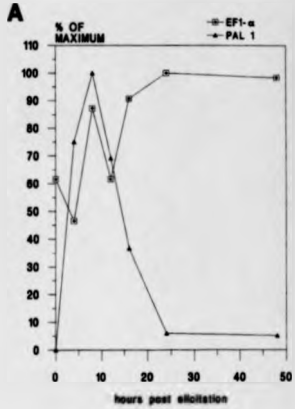
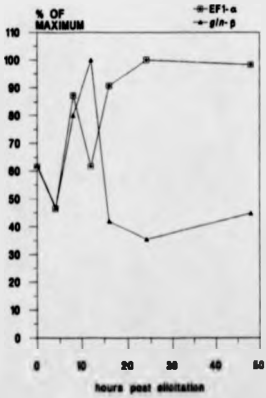

A
B
1 2 3 4 5 1 2 3 4 5 6
PAL 1
gln-β
gln-α
EF1-α

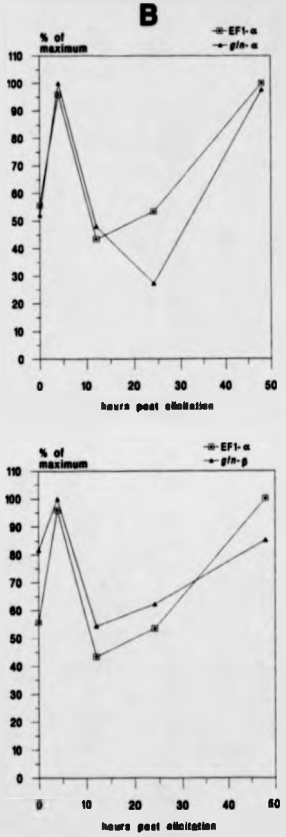
Figure 4.7: Graphical representations of the scanning densitometer analysis of the suspension culture RNase protection assays plotted with EF1-α. A represents the elicitor treated samples and B represents the control water treated samples.


% OF MAXIMUM
EF1-α
gln-α
hours post elicitation

A


% OF MAXIMUM
EF1-α
PAL 1
hours post elicitation


% OF MAXIMUM
EF1-α
gln-β
hours post elicitation


B
% of maximum
EF1-α
gln-α
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post elicitation
% of maximum
EF1-α
gln-β
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post elicitation

4.3 Studies on the expression of PAL 1 and *gln-rich* wounded hypocotyls.

P. vulgaris plants were grown in vermiculite as described in the materials and methods (section 2.1). Plant tissue was used at day fourteen post imbibition. All hypocotyls were removed from the plants and excepting hypocotyls used as controls, were sectioned into petri dishes containing several thicknesses of Whatman 3M paper soaked in the various treatment solutions described below. The control hypocotyls were excised from the plant but were then left intact throughout the treatment until harvesting, when the end 1 cm of hypocotyl was removed. The lids were replaced on the petri dishes which were then incubated under the plant growth conditions. Samples of each treatment were taken at 0, 2, 8, 24 and 48 hours after each treatment began. The treatments were as follows:

control	nutrient solution
wound only	nutrient solution
glutathione	nutrient solution plus 1mM reduced glutathione
cinnamate + glutathione	nutrient solution plus 1mM reduced glutathione plus 1mM cinnamate

Total RNA was then extracted from the samples and RNase protection assays were performed. The results from

the experiment are shown in figure 4.8 as autoradiographs and graphically in figure 4.9 following densitometry of the autoradiographs. PAL 1 message was present in all of the treatments although at widely differing abundances, The expression of the PAL 1 gene showed a definite induction pattern in both the glutathione and glutathione/cinnamate treated hypocotyls where the message peaked in abundance at around four hours after treatment began returning to basal levels by the 24 hour time point. The levels of expression of PAL 1 are lower in the cinnamate treatment compared to the reduced glutathione treatment. In the other two treatments traces of PAL expression were visible early in the time course but persisting longer in the wounded treatment. The signal is to weak in this treatment to tell whether this is an induction of PAL 1 message or background expression of the PAL 1 gene. Levels of the *gln-α* message show similar but not identical expression patterns to those found with PAL 1, there is a low level of *gln-α* expression in the wound only treatment although message is present at a higher abundance in the non-wound control. *Gln-α* message is induced in both the glutathione and glutathione/cinnamate treatments with the peak of induction occurring later than that seen with the PAL 1 peak, at around eight hours. The message is then present over the remainder of the time course at elevated levels compared to the control treatment. *Gln-ß* is

Figure 4.8: A measure of the abundance of message for the following genes: *gln-α*, PAL 1 and EF1-α using the RNase protection assay on samples of total RNA extracted from_hypocotyl tissue. A represents RNA extracted from wounded tissue treated with reduced glutathione, B represents RNA extracted from unwounded tissue, C represents RNA extracted from wounded tissue treated with reduced glutathione and cinnamate and D represents RNA extracted from wounded tissue. Tracks 1-6 represent a time course as follows 0, 2, 4, 8, 24 and 48 hours.

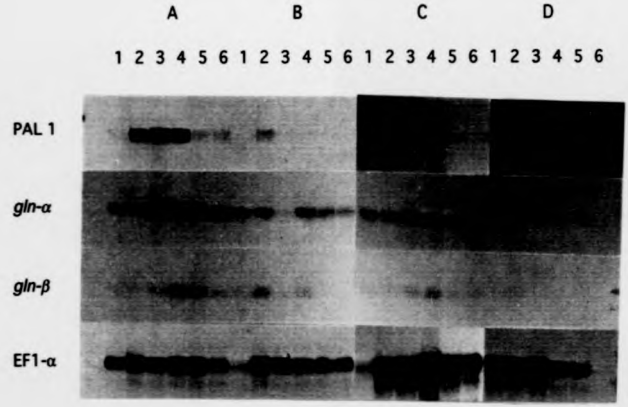
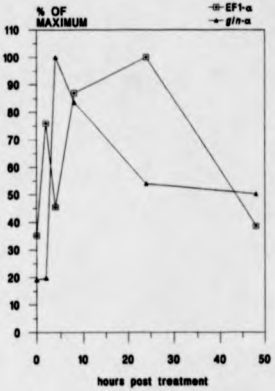
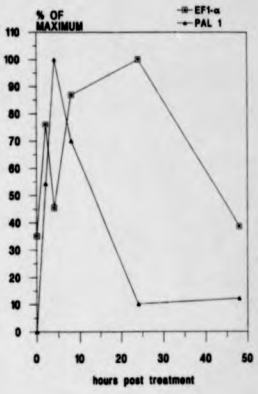
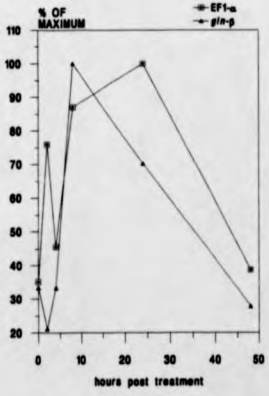

A
B
C
D
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
PAL 1
gln-α
gln-β
EF1-α

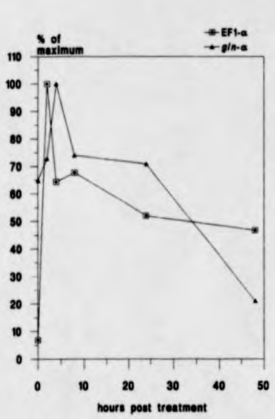
Figure 4.9: Graphical representations of the scanning densitometer analysis of the hypocotyl RNase protection assays plotted with EF1-α. A represents the wounding plus glutathione treatment. B represents the control samples. C represents the wounding plus glutathione and cinnamate treatment. D represents the wounding treatment.

A

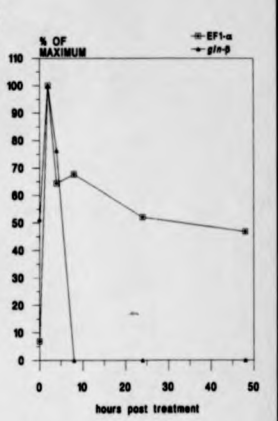

% OF MAXIMUM
EF1-α
gln-α
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post treatment

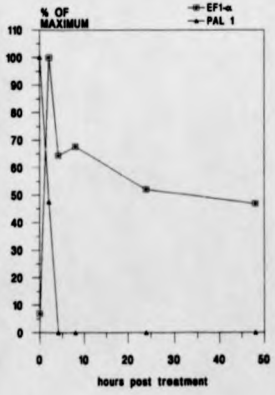

% OF MAXIMUM
EF1-α
PAL 1
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post treatment


% OF MAXIMUM
EF1-α
gln-β
110
100
90
80
70
60
50
40
30
20
0
10
20
30
40
50
hours post treatment

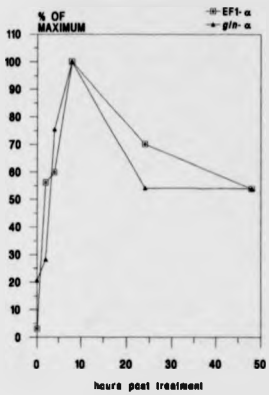

% of maximum
EF1-α
gln-α
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post treatment

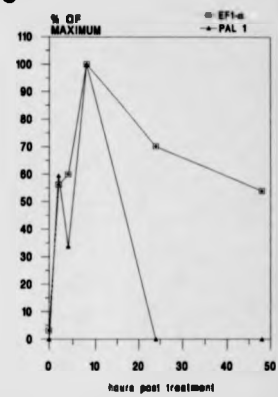
B

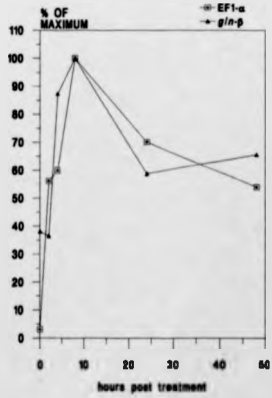

% OF MAXIMUM
EF1-α
gln-β
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post treatment

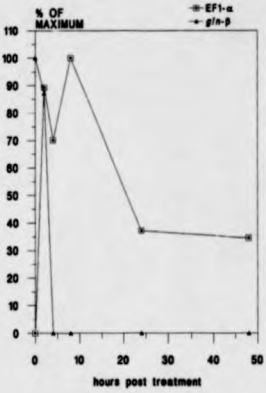

% OF MAXIMUM
EF1-α
PAL 1
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
hours post treatment

C

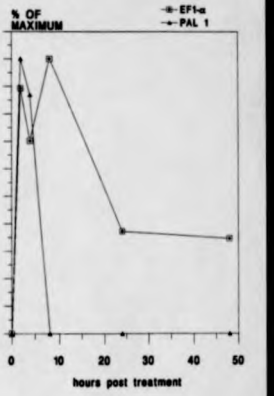

% OF MAXIMUM
EF1- α
gln- α
hours post treatment

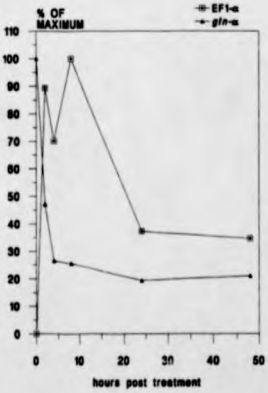

% OF MAXIMUM
EF1-α
PAL 1
hours post treatment


% OF MAXIMUM
EF1-α
gln-β
hours post treatment


% OF MAXIMUM
EF1-α
gln-β
hours post treatment

D


% OF MAXIMUM
EF1-α
PAL 1
hours post treatment


% OF MAXIMUM
EF1-α
gln-α
hours post treatment

present only at low levels in all of the treatments, seen mainly at the start of the time course in the control and wound only treatments, late in the time course peaking at around eight hours with the glutathione treatment and hardly present with the glutathione/cinnamate treatment only detectable in the eight hour time point. the message levels for *EF1-α* remain relatively constant throughout the time course for the various treatments although there may be a slight increase in the message observed in the glutathione/cinnamate treatment. The results obtained with this probe demonstrate that some samples were degraded during the course of the protection assay notably the zero time points of the glutathione and glutathione/cinnamate treatments and the forty eight hour time point of the wound treatment. This problem will also affect the PAL 1 probe as the hybridisations for these two probes were performed in the same assay.

4.4 Studies on the expression of PAL 1 and *gln-α* during leaf infection.

The leaf infection experiment was performed in several sections, all of the work using intact plants was performed at Horticulture Research International, Wellesbourne UK. Samples were then taken to Warwick where the remainder of the work was performed. In the

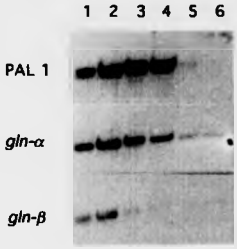
first experiment leaves were harvested at the end of a Wellesbourne study. In this study the leaves of *P. vulgaris* plants had been aerosol inoculated on the lower lamina of the expanding primary leaves with a bacterial suspension of *Pseudomonas phaseolicola* in tap water. Two different races of *P. phaseolicola* were used. One (race three) is incompatible giving a hypersensitive response and the other (race two) is compatible giving a disease response. In addition a control treatment was used where the leaves were spray inoculated with tap water only. The plants were then grown in a high humidity chamber for the duration of the experiment to provide optimum conditions for the bacterial infection. At day fourteen the response of the primary leaves to the pathogens is easily visible with the incompatible pathogen infection resulting in a number of hypersensitive lesions on the leaf lamina, the reaction of the leaves to the compatible pathogen is different with disease symptoms apparent on the leaves as watery lesions beginning to cover the lamina (figure 4.10). The primary leaves were harvested at day fourteen post infection and total RNA was extracted from these leaves and used for RNase protection assays as described in the materials and methods (see section 2.7.4). The results from these RNase protection assays are shown in figure 4.11. There is a high level of PAL 1 message in both the leaves infected with the incompatible pathogen and those infected with the

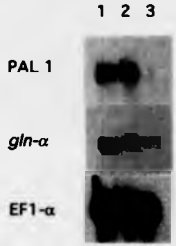
Figure 4.10: Photographs of leaves infected with A *Pseudomonas phaseolicola* race 3 which gives a hypersensitive response and B *Pseudomonas phaseolicola* race 2 which gives a sensitive response. The leaves were photographed at day 14 post inoculation.

A

B

Figure 4.11: A measure of the abundance of message for the following genes: *gln-α*, *gln-ß*, PAL 1 and EF1-α using the RNase protection assay on samples of total RNA extracted from leaves infected with *Pseudomonas phaseolicola*. In the upper region tracks 1 and 2 represent RNA extracted from leaves infected with an incompatable strain (race3). Tracks 3 and 4 represent RNA extracted from leaves infected with a compatible strain (race2). Tracks 5 and 6 represent RNA extracted from control leaves treated with water. In the lower region track 1 represents RNA extracted from the incompatible response, track 2 represents RNA extracted from the compatible response and track 3 represent RNA extracted from the control.


1 2 3 4 5 6
PAL 1
gln-α
gln-β

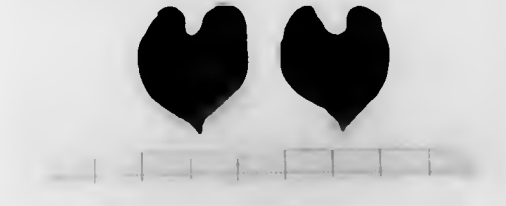

1 2 3
PAL 1
gln-α
EF1-α

compatible pathogen. There is however only an extremely low expression of PAL 1 in the control leaves, this pattern of expression is similar to that observed with the *gln-α* probe although the induction of *gln-α* message levels is lower in the incompatible response and *gln-α* message is present in the control leaves. *Gln-ß* message levels are higher in the race 3 treated leaves than in the race 2 treated leaves whilst expression of *gln-ß* is undetectable in the control treatment. In contrast, the levels of *EF1-α* are similar in all three treatments. The hypersensitive response of bean to fungal pathogens is known to be rapid occuring much faster than the plants response to a compatable strain of the same pathogen (Cramer *et al* 1985b, Lawton and Lamb 1987, Liang *et al* 1989). For this reason a time course study was set up to further study the response of the plants to *P. phaseolicola*. Plants were again inoculated at Wellesbourne by aerosol treatment of the lower lamina as before although a different race of *P. phaseolicola* (race six) was used to give the compatible response. Samples were taken in the form of the primary leaves at three hour intervals beginning at nine hours and finishing at forty eight hours. Leaves at forty eight hours were photographed in order to demonstrate differences in the appearance of the leaves between the different treatments (see figure 4.12). At this time point small brown lesions were easily visible on the leaves infected with

Figure 4.12: Photographs of the upper and lower laminas of leaves infected with A *Pseudomonas phaseolicola* race 3 which gives a hypersensitive response and B *Pseudomonas phaseolicola* race 6 which gives a sensitive response. The leaves were photographed at 48 hours post inoculation.

A

B

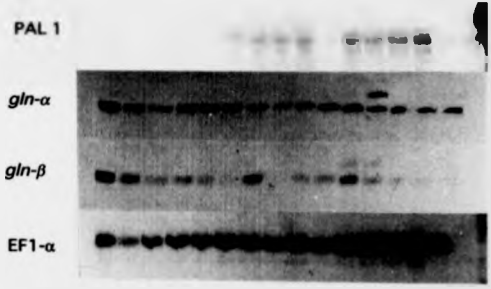


the race 3 strain whilst only small watery lesions were visible on the race 6 treated leaves. Samples from this time course were extracted for total RNA, as previously described, and this RNA was used for RNase protection assays. The results, as autoradiographs, are shown in figure 4.13. These autoradiographs were scanned using a densitometer and the results are presented graphically in figure 4.14. PAL 1 shows an induction in the race three treatment between fifteen and thirty three hours peaking at twenty four hours, the PAL 1 message in the race six treatment could only be observed after a five day exposure of the RNase protection assay, there appears to be an increase in the message towards the end of the time course although the message never rises to the level observed in the race three treatment during the span of the time course. *Gln-α* also shows an induction in the race three time course peaking at between 35 and 42 hours but not returning to basal levels by the conclusion of the time course. The *gln-α* assay was exposed for three days in order to observe the signal present at the intensity seen in figure 4.13. The *gln-α* message levels remain relatively constant during the course of the race six time course. The signal for the *gln-α* message in the race 6 samples was also low as the assay was exposed for five days for the signal to be detectable at the levels shown in figure 4.13. The displayed assays for *gln-ß* message have received the same exposure times as those

Figure 4.13: A measure of the abundance of message for the following genes: *gln-α*, *gln-ß*, PAL 1, EF1-α and CAB using the RNase protection assay on samples of total RNA extracted from leaves infected with *Pseudomonas phaseolicola*. A represents RNA extracted from leaves infected with a compatible strain (race 6) and B represents RNA extracted from leaves infected with an incompatable strain (race 3). Track C represents a water inoculated control, tracks 1-14 represent a time course running from 9 to 48 hours with samples every 3 hours.

A

C 1 2 3 4 5 6 7 8 9 10 11 12 13 14



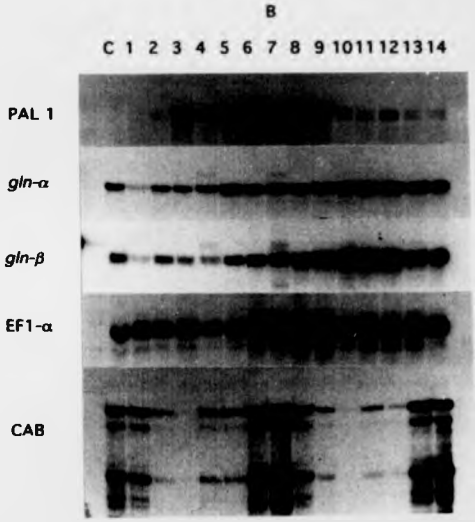
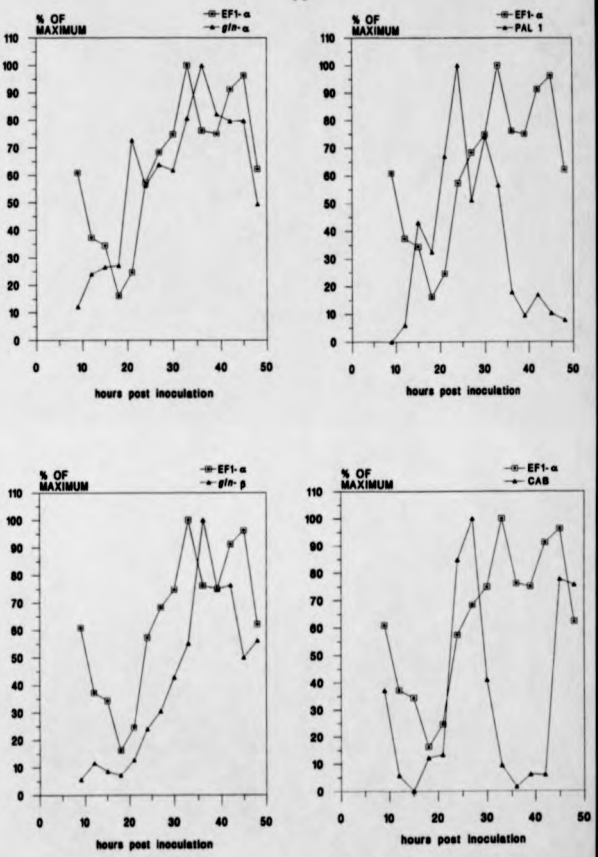
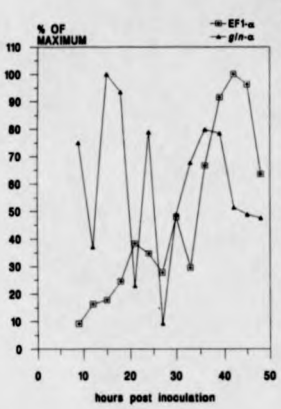

B
C 1 2 3 4 5 6 7 8 9 10 11 12 13 14
PAL 1
gln-α
gln-β
EF1-α
CAB

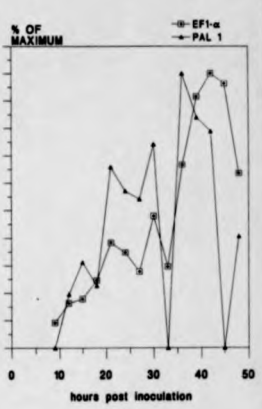
Figure 4.14: Graphical representations of the scanning densitometer analysis of the leaf infection RNase protection assays plotted with EF1-α. A represents the race three infected samples and B represents the race 6 infected samples.

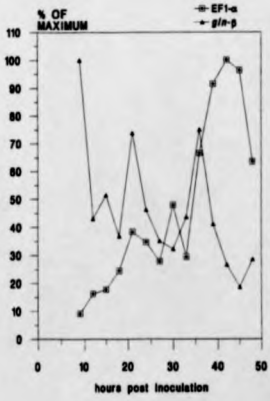
A




% OF MAXIMUM
EF1-α
gln-α
hours post inoculation

B


% OF MAXIMUM
EF1-α
PAL 1
hours post inoculation


% OF MAXIMUM
EF1-α
gln-β
hours post inoculation

shown for *gln-α*, the expression of the *gln-ß* message shows a similar pattern as that for *gln-α* although the expression is higher in the race three time course and lower in the race six time course. Two different probes were used to display a control pattern of expression different to that of the test probes. The first of these, *EF1-α*, however showed a pattern of expression increasing over the course of the two time courses, this expression pattern is similar to that displayed by the two GS messages tested. To ascertain as to whether the expression patterns seen in these treatments are a general response to infection RNase protection assays were performed, on the race 3 infected time course, using another probe. A chlorophyll ab binding protein (CAB) probe was used which showed a diurnal pattern unlike that shown for the other probes used in this study. This expression pattern for CAB has previously observed for this gene (Piechulla *et al* 1987, Tavladoraki *et al* 1989, Adamska *et al* 1991) and demonstrates that the expression patterns observed for the other genes studied are not part of a general response to infection.

4.5 Discussion

There has been a large amount of work performed on the increase in PAL activity and the induction of PAL 1 gene expression in suspension cultures of *P. vulgaris* cv. immuna using several different methods of elicitation. The majority of the earlier work used autoclaved RNase A as the elicitor (Dixon and Bendall 1978b, Dixon *et al* 1980). A different elicitor, heat released from the cell walls of the pathogen *Colletotrichum lindemuthuanum* (the causative organism of anthracnose on *P. vulgaris*), was later used more commonly with cell cultures, demonstrating that PAL enzyme activity rises up to twelve hours (Dixon *et al* 1981, Lawton *et al* 1983a, Lawton *et al* 1983b, Cramer *et al* 1985b, Edwards *et al* 1985, Bolwell *et al* 1985b, Robbins *et al* 1985, Cramer *et al* 1985b, Bolwell *et al* 1986, Lawton and Lamb 1987). This peak of activity is later than the peak of enzyme production which occurs at *circa* four to five hours demonstrating that a post-translational modification is necessary for enzyme activity (Lawton *et al* 1983, Cramer *et al* 1985b, Edwards *et al* 1985), this modification is the addition of a dehydroalanine residue at the active site (Hanson *et al* 1981b). The induction of PAL 1 message is extremely rapid, measurable after only a few minutes post-elicitation (Edwards *et al* 1985) peaking at *circa* four

hours (Lawton *et al* 1983b, Cramer *et al* 1985a, Cramer *et al* 1985b, Edwards *et al* 1985, Lawton and Lamb 1987) just before the peak of enzyme production suggesting that the induction of PAL enzyme is controlled at the level of transcription. This observation was verified by Lawton and Lamb (1987) who performed nuclear run off experiments demonstrating that the level of PAL activity generated in the cultures is dependant upon the level of transcription.

The results obtained from the RNase protection studies on the RNA samples, supplied by R Dixon, with the PAL 1 probe are similar but not identical to those in the literature, there is a large induction of the PAL 1 message although this appears to occur slower in this experiment peaking later (at 8 hours) in the RNA samples used for the experiment (figures 4.6 and 4.7) than had previously been reported peaking at four hours (Lawton *et al* 1983b, Cramer *et al* 1985a, Edwards *et al* 1985, Lawton and Lamb 1987). This could be due to a number of reasons including the age of the suspension cultures when elicited and the batch of fungal elicitor used to treat the cells.

The behaviour of the *gln-α* message has both similarities and differences to the expression pattern shown by PAL 1. The message levels are induced by the fungal elicitor peaking at the same time point (eight hours) however the background levels of the *gln-α* message

are much higher than those observed for the PAL 1 message. Indeed the message levels of *gln-α* are easily detectable in the control treatment while PAL 1 message abundance appears to be below the level of detection of the assay. Expression of *gln-α* in the control tissue samples is not surprising as it is known that this gene is highly expressed in tissues such as seed leaves and complete seeds (Bennett and Cullimore 1988, Swarup *et al* 1990, Cock *et al* 1991), early stages of root development (Ortega *et al* 1986) as well as early in nodule development (Cock *et al* 1990, chapter 3). All of these examples are early developmental stages and are possibly analogous to suspension cultures in mid log phase. The expression of PAL 1 in these tissues is variable, either showing an expression pattern similar to that of *gln-α*, seen in the case of nodule development (chapter 3), or showing no measurable expression, as in this case which clearly demonstrates that the co-induction observed between *gln-α* and PAL 1 is not universal. *Gln-α* showing expression patterns additional to those detectable for PAL 1.

The expression of the *gln-ß* gene may show a slight induction in the elicited treatment although this increase in the level of expression may not represent a significant increase in abundance of the message. This pattern of expression for *gln-ß* has been observed previously in nodule development (Bennett *et al* 1989,

Cock *et al* 1990), leaf development (Cock *et al* 1991) and root development (Ortega *et al* 1986). This gene is not however constituatively expressed as differences in expression between cell types has been observed in nodules and roots (Forde *et al* 1989).

The expression pattern observed for *EF1-α* showed little variation in the expression of this gene indicating that there was little variation in the levels of transcription in this time course (Aguilar *et al* 1991, Ursin *et al* 1991).

The HPLC profiles obtained for the samples generated at the Royal Holloway and Bedford new college (figures 4.2 and 4.5) suggest that the GS isoenzyme present in cell cultures contains α and ß subunits although caution is necessary in the interpretation of this data due to the observed cultivar differences between tendergreen and immuna which may affect the position of elution of any isoenzyme containing a subunits. To confirm the presence of α subunits in this isoenzyme two dimensional gel electrophoresis would have to be performed on the purified isoenzyme. The was no induction of the GS isoenzymes in the time course collected (figure 4.5).

It has been known for sometime that PAL is induced in response to wounding of plant tissues such as hypocotyls (Lawton and Lamb 1987, Liang *et al* 1989b, Jorrin *et al* 1990), and tubers (Ishizuka *et al* 1991). In addition, it has been reported that PAL 1 is induced by

reduced glutathione (GSH) in suspension cultures of *P. vulgaris* (Wingate *et al* 1988) and also that the induction of PAL activity is repressed in the presence of 1mM cinnamate (Bolwell *et al* 1986, Jorrin *et al* 1990). The wounding experiments performed were designed to study the effect of these conditions in wounded hypocotyls of French bean the results of this study are shown in figures 4.8 and 4.9. The wound only treatment produced no marked induction of either PAL 1 or any GS genes studied, this is surprising and may be due to the wound response being very localised although this seems unlikely as the degree of wounding was similar to that used in other studies (Lawton and Lamb 1987, Liang *et al* 1989b, Jorrin *et al* 1990). A second explanation is that the PAL 1 gene is not induced in response to the wounding of hypocotyls, the PAL response being due to expression of other members of the PAL multigene family with which there is no coinduction with any GS gene studied (*gln-α* or *gln-ß*). This is also unlikely as it has been shown previously that PAL 1 is induced in response to a wounding stimulus (Liang *et al* 1989b). The third and most likely cause of the lack of induction of the PAL 1 message is that something in the nutrient solution inhibited the induction of PAL. The control unwounded hypocotyls may provide some evidence for the result observed with the wound treatment. Unsurprisingly there was no PAL induction in this treatment although a faint

PAL signal is discernible in the time zero sample. A similar situation is seen with *gln-ß* where a low message abundance was observed at the two hour time point of the control unwounded hypocotyls, the *gln-α* message is however expressed at a higher abundance showing a low but definite expression in the control hypocotyls. The lack of *gln-α* message in the wound treatment can therefore be due either to a down regulation of *gln-α* triggered by wounding or due to an inhibition of its expression by a factor present in the nutrient solution used in the treatments to which the harvested portions of the control hypocotyls were not exposed. The reduced glutathione (GSH) treatment induced a rapid and large induction of PAL 1 message abundance, similar to the response reported by Wingate *et al* (1988) in cell suspension cultures, with the message levels peaking at 4 hours. The two GS genes behaved differently in this treatment, both were induced but the message abundance for *gln-α* is much higher than that observed for *gln-ß*, the induction peak for *gln-α* appears to occur at the same time point as for PAL 1 but four hours before the observed peak of *gln-ß* message abundance. The *gln-α* message abundance also persisted at an elevated level until the end of the time course whilst the levels of PAL 1 message fell to basal levels by 24 hours. Cinnamate treatment (1mM) of suspension cultures completely inhibits the induction of PAL 1 message (Dron pers comm). However, in the hypocotyl sections only a

partial inhibition was observed with the cinnamate treatment. This treatment was originally included to allow an insight as to whether the expression of *gln-α* is under the same controls as PAL 1 or whether the expression of *gln-α* is induced by a compound produced due to PAL activity. There are two possible reasons for the lack of inhibition observed in the cinnamate treatment 1. It is possibile that cinnamate does not inhibit the induction of PAL 1 in wounded hypocotyls or 2. The cinnamate was not effectively taken up by the hypocotyl sections and was therefore not present in a sufficiently high concentration to inhibit the induction of PAL 1 message. Of these two reasons the second is more plausible as it is unlikely that cinnamate would invoke a different response in hypocotyls than in suspension cultures. The expression of *gln-α* was similar or identical to that observed with the GSH treatment indicating that the levels of *gln-α* expression were unaffected by the partial inhibition of PAL 1. This suggests that *gln-α* expression is unaffected by elevated levels of cinnamate in contrast to the effect these levels have on PAL 1 expression. The levels of the *gln-ß* message in the cinnamate treatment were reduced when compared against that measured for the GSH treatment although the difference between the two treatments is insignificant.

The majority of the work done on the expression of PAL genes during the hypersensitive response of *P. vulgaris* to incompatible pathogens has used strains of the fungus *Colletotrichum lindemuthuanum*, the causative organism of anthracnose, infecting hypocotyls of the *P. vulgaris* plants (Cramer *et al* 1985b, Lawton and Lamb 1987, Liang *et al* 1989b). These studies suggest that the main difference between the induction of PAL 1 and 3 during infection with compatible or incompatible pathogens is not one of whether or not PAL 1 or 3 is induced but more a case of when and where the induction occurs. The response of the hypocotyl is much more rapid and localised to the incompatible pathogen allowing a local lesion to form, preventing the pathogen from establishing an infection. The compatible response leads to a delayed more widespread induction of the PAL genes allowing the disease symptoms to progress eventually resulting in the death of the infected tissue. This situation is not restricted to *P. vulgaris* but occurs in the race specific response of any plant to incompatible and compatible strains of fungal pathogens. Other examples include the response of soybean roots to infection with compatible and incompatible strains of *Phytophthora megasperma* sp. glycinea (Borner and Grisebach 1982, Habereder *et al* 1989), the localised response of potato leaves to infection with *Phytophthora infestans* (Cuypers *et al* 1988), the rapid and localised

response of parsley to infection with *Phytophthora megasperma* sp glycinea and the induction of PAL in tomato cell cultures inoculated with *Verticillium albo-altrum*. In addition, examples of this type of response are not restricted to fungal plant pathogens but are also observed in the plants response to challenge by viruses such as TMV (Fritig *et al* 1973).

This work studies the response of *P. vulgaris* plants to infection with a bacterial pathogen *Pseudomonas phaseolicola*, the causative agent of halo blight. The response of the leaves to the incompatible bacterial strain was similar to that previously demonstrated with fungal infections of *P. vulgaris* hypocotyls (Cramer *et al* 1985b, Lawton and Lamb 1987, Liang *et al* 1989b). However, the response with the bacterial incompatable pathogen occured later than the response observed in the infected hypocotyls (Cramer *et al* 1985b, Lawton and Lamb 1987, Liang *et al* 1989b) were the response occurs after a similar time span as that seen elicited cell cultures (Lawton *et al* 1983b, Cramer *et al* 1985a, Edwards *et al* 1985, Lawton and Lamb 1987). In the experiment performed as part of this study the induction of PAL 1 message occurred later peaking at *circa* 24 hours (figures 4.13 and 4.14). This could be due to a number of reasons such as differences in the response of plants to fungal and bacterial pathogens, differences in the inoculation treatment between studies or differences in the time

taken for bacterial pathogens to infect the leaf after inoculation. The response of the leaves to infection by the compatible pathogen is as expected from reports of fungal pathogens in the literature (figures 4.13 and 4.14). There is an induction of the PAL 1 message but this occurs much later than in the incompatible reaction peaking at *circa* 45 hours post infection. The expression of the PAL 1 gene is weaker than it appears in figure 4.13 as the exposure necessary to visualise the signal from the PAL 1 probe was five days compared to the overnight exposure showed for the PAL 1 probe hybridised to total RNA from the race three infected leaves.

The two GS genes (*gln-α* and *gln-ß*) studied in this experiment showed similar induction patterns. Both were expressed in the samples prior to the appearance of the PAL 1 induction and both were induced above this basal level although after the peak of PAL 1 abundance. The *gln-ß* message was more abundant than that of *gln-α* although both were much weaker than the signal from PAL 1 as they had to be exposed for three days as opposed to the standard overnight exposure shown for the other RNase protection data. The behaviour of the message for the two GS genes with the race six compatible response is completely different to that observed with the race three treatment. Both message levels are much lower than those detected in the incompatible response. Therefore five day exposures have been used to construct figure 4.13.

The *gln-α* message levels remain constant over the time course showing, if anything, a slight tail off towards the end of the experiment when the PAL 1 message was being induced. The *gln-ß* message level is measurable at the beginning of the time course falling off rapidly until the signal becomes undetetectable after the first few time points. This pattern of expression for *gln-ß* is similar to that observed for the gene in previous experiments where the message abundance dropped of when the leaves started to green (Cock *et al* 1991). This study does however show different expression patterns for *gln-ß* with the race three treatment and for *gln-α* in both treatments. This suggests that the expression patterns for the two genes in these samples are due to the infection with *P. phaseolicola* and not due to the normal development of the leaves.

Interestingly, the pattern of expression shown by the two GS genes was similar to that shown by *EF1-α*, which was originally included as a control as it was expected to show a different expression pattern to the other genes studied. This could be caused by a number of factors including 1. The possibility that this is a true response of *EF1-α* to infection with a pathogen: 2. That during the course of the infection the total number of transcripts produced falls resulting in an effective enrichment of those transcripts which are maintained at the levels previous to the infection or 3. That a normal

uninfected leaf would show the same induction of *EF1-α* message which is known to increase when an increase in protein synthesis occurs (Aguilar *et al* 1991, Ursin *et al* 1991). In order to answer that question a further control probe was used in RNase protection assays, this probe (for cab) shows a circadian diurnal pattern observed for this gene in other species (Piechulla *et al* 1987, Tavladoraki *et al* 1989, Adamska *et al* 1991) thus proving that the observed induction of the *EF1-a* message was not due to a general increase in gene activation.

Chapter five

Attempts to clone *glnA*

5.1 Introduction

The GS enzyme in *P. vulgaris* is composed of a wide variety of isoenzymes consisting of a mixtures of four subunits α, ß, γ and ∂ (Lara *et al* 1984). Three of these subunits (α, ß, and γ) are cytosolic and can randomly assemble to form the active octameric holoenzyme, the proportion of each subunit depending on the levels of expression of each subunit in a particular cell at a particular time (Bennett and Cullimore 1989, Cai and Wong 1989). The ∂ subunit is plastidic forming an isoenzyme made up exclusively of ∂ subunits.

Full length cDNA clones have been isolated for each of the four subunits (Gebhardt *et al* 1986, Lightfoot *et al* 1988, Bennett *et al* 1989). Each of these cDNA clones show a differential expression pattern with the expression of the subunits is controlled by both developmental and environmental stimuli at the level of transcription (Gebhardt *et al* 1986, Bennett *et al* 1989, Cock *et al* 1990, Cock *et al* 1991). Each of the four cDNA clones is derived from a separate gene termed *gln-α*, *gln-ß*, *gln-γ* and *gln-∂* respectively. Genomic clones had been isolated for all of the genes at the start of this study except for *gln-α*. In addition, a fifth putative silent gene *gln-ε* has been isolated, making up a multigene

family of five members (Turton *et al* 1986, Forde *et al* 1989, Cock *et al* 1992).

The following chapter describes the attempts made to obtain a genomic clone for *gln-α*, both by screening of a representative lambda library and a search for a suitably sized restriction fragment, to construct an partially enriched genomic library. This work was undertaken to obtain the 5' flanking regions of *gln-α* in order to study both the expression of the gene in transgenic plants and to compare these sequences with those from the PAL 1 gene.

5.2 Preparation of *gln-α* cDNA for production of probe for screening.

The plasmid pGS-αM1 was used to prepare both of the probes used in the screening protocols. This plasmid consists of the entire *gln-α* cDNA cloned into the *Pst*I site of the pGEM-4Z polylinker. Probe production was as described in the materials and methods (section 2.5.2) using the pGS-αM1 plasmid linearised with *Bgl*II as the template for a transcription probe and a gel isolated *Hind*III fragment of *circa* 600bp for labelling by random priming. The specificity of the two probes was expected to be different with the transcription probe being specific to *gln-α* (as the template contains the 5' untranslated region) while the multi-primer probe was

expected to recognise other members of the GS gene family (as the fragment was derived from the coding sequence). This was checked by colony hybridisation to the *E. coli* host to various cDNA's representing the members of the GS gene family. The results of this colony hybridisation are shown in figure 5.1 where it can be observed that RNA probe was specific for the *gln-α* cDNA whilst the random primed probe also weakly hybridised weakly to cDNA derived from *gln-ß* and *gln-γ* but not to that derived from *gln-∂*.

5.3 Screening of a partial digest *Phaseolus vulgaris* genomic library.

A *Sau*3A partial digest library prepared from *P. vulgaris* cv. Tendergreen root genomic DNA, utilising lambda DASH™ (Stratagene) as the vector (Cock *et al* 1992) was used for the screening. The titre of the library was checked prior to the screening of the library when 2,000,000 plaques were propagated up on four large plates (at least 11 haploid genome equivalents Bennett and Smith [1976]). Duplicate filters were taken hybridised with either the transcription or the random primed probe. The filters were then washed at low stringency before exposure to autoradiography. No

Figure 5.1i Colony blots of the four GS cDNAs hybridised with A: a 5' *gln-α* transcription probe and B: a multi primer probeproduced from a *Hind* III fragment from the coding region of the *gln-α* cDNA.

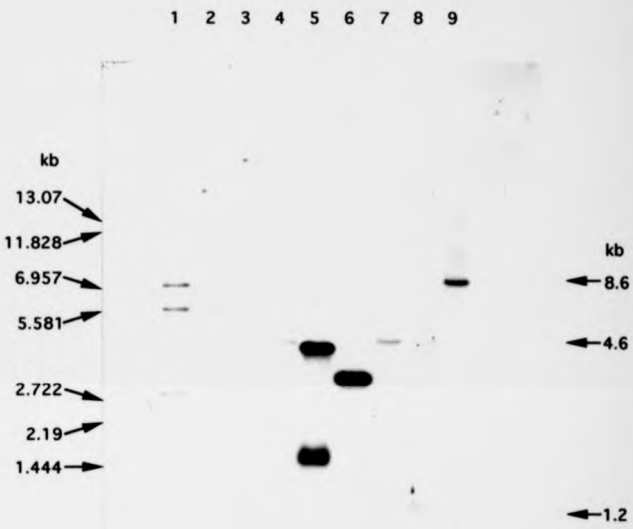

A
gln- α
gln-β
gln-δ
gln-γ
B
gln- α
gln-β
gln-δ
gln-γ

positives were obtained using this approach and an alternative approach was sought.

5.4 Southern hybridisations to locate a suitable complete digest.

Genomic DNA was isolated from roots of *P. vulgaris* seedlings grown for eight days post imbibition, this DNA was digested to completion with a range of 6 base-cutting restriction enzymes (*Eco*RI, *Bam*HI and *Bgl*II) and the digests were then run on a 0.6% agarose gel. In addition positive and negative controls were included on the gel in the form of *gln-α* and *gln-ß* cDNA plasmids, Radiolabelled markers were also run, to allow accurate sizing of the fragments. The gel was denatured and Southern blotted onto a nylon membrane (Hybond N Amersham). The membrane was then hybridised against a transcription probe prepared from the template described in section 5.2, washed at low and high stringencies and exposed for autoradiography. The probe hybridised to the control *gln-α* cDNA at a high level whilst the hybridisation to the *gln-ß* cDNA was much weaker possibly due to a low degree of hybridisation to the polylinker. At both low and high stringency washes one band lit up with each digest as seen in figure 5.2, the sizes of the

Figure 5.2: Southern blot hybridised with a 5' *gln-α* transcription probe and washed at high stringency. Tracks represent: 1 and 2 radiolabelled lambda DNA restricted with *Acc* I and run as markers. 3 pUC19 vector restricted with *Bam* HI. 4 pGSR1 (*gln-ß* cDNA clone) restricted with *Pst* I. 5 pGSR2 (*gln-α* cDNA clone) restricted with *Pst* I. 6 pGSR2 (*gln-α* cDNA clone) restricted with *Pst* I and Bgl II. 7 *P. vulgaris* genomic DNA restricted with *Eco* RI (9.5ug). 8 *P. vulgaris* genomic DNA restricted with Bgl II (9.5ug). 9 *P. vulgaris* genomic DNA restricted with Bam HI (9.5ug).


1 2 3 4 5 6 7 8 9
kb
13.07
11.828
6.957
5.581
2.722
2.19
1.444
kb
8.6
4.6
1.2

respective bands are shown below:

Eco RI	4.6kb
Bam HI	8.5kb
Bgl II	1kb

Evidently the *Bam* HI digest gives the highest chance of success of containing the 5' flanking sequences as it is the longest. More information was sought as to the nature of this sequence by performing double digests with *Nde*I a restriction enzyme which cuts towards the 5' end of the *gln-α* cDNA. The digests were run on an agarose gel, blotted and probed as above. At both low and high stringency the following bands were observed on the autoradiograph (see figure 5.3):

Bam HI	8.6kb
Nde I	2.75kb and 4.5kb
double digest	2.75kb

5.5 Discussion

The apparent absence of any *gln-α* sequences in the library used for the initial screening experiments could be for two reasons. Either the library was inherently faulty in some respect or that the library contained no clones containing recognition sites for the *gln-α* probes used in the screening experiments. The first explanation is unlikely as the library had previously been used to

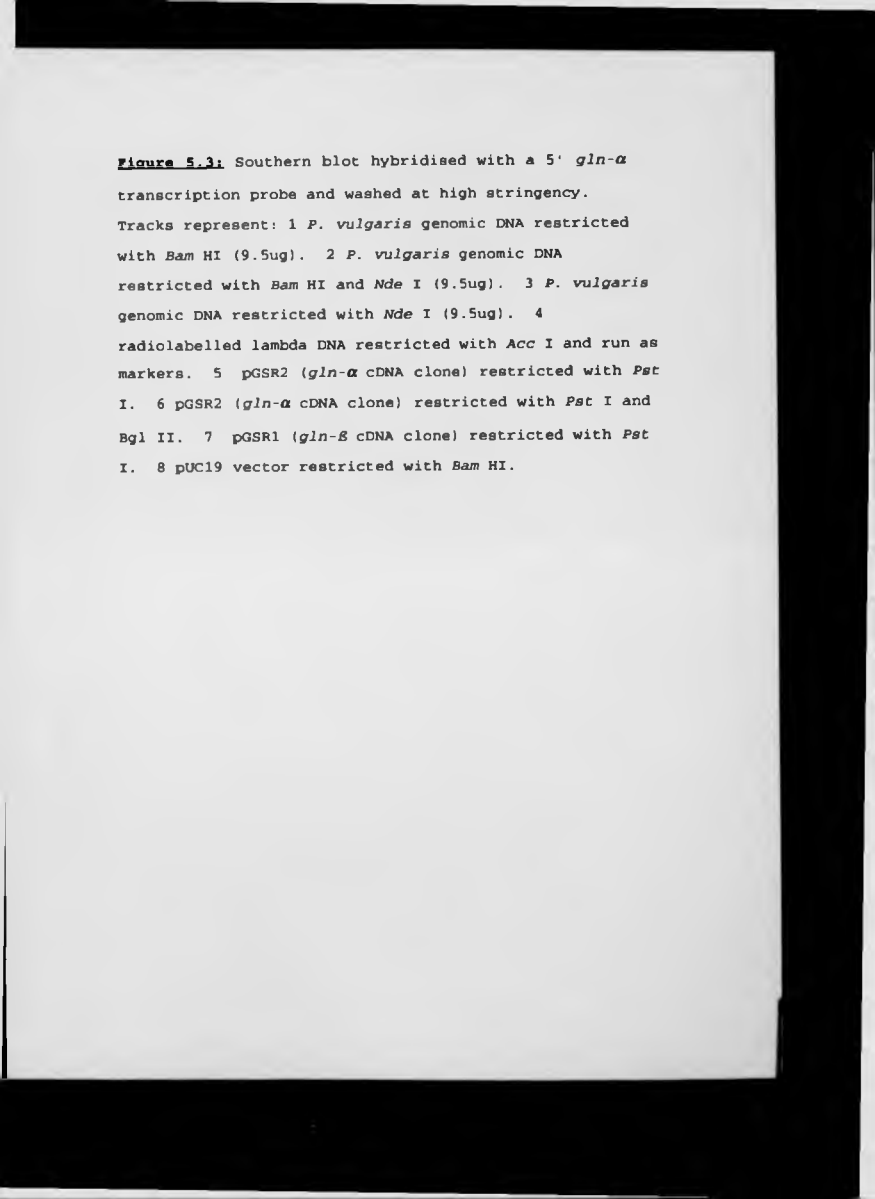
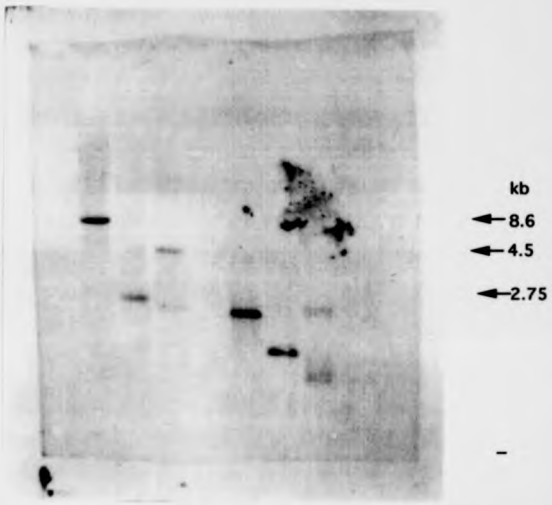


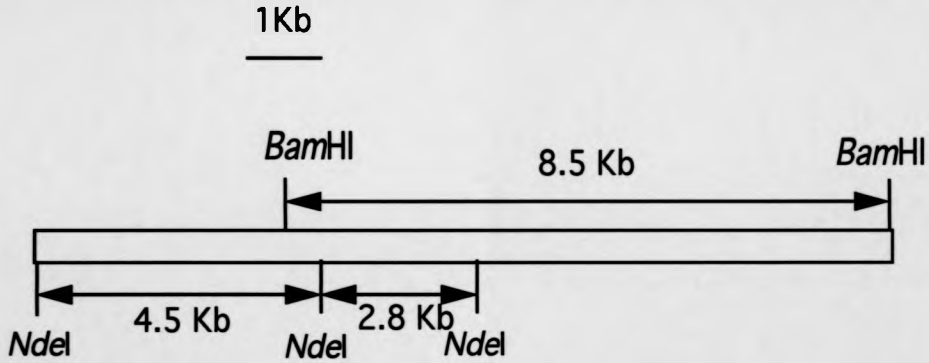
Figure 5.3: Southern blot hybridised with a 5' *gln-α* transcription probe and washed at high stringency. Tracks represent: 1 *P. vulgaris* genomic DNA restricted with *Bam* HI (9.5ug). 2 *P. vulgaris* genomic DNA restricted with *Bam* HI and *Nde* I (9.5ug). 3 *P. vulgaris* genomic DNA restricted with *Nde* I (9.5ug). 4 radiolabelled lambda DNA restricted with *Acc* I and run as markers. 5 pGSR2 (*gln-α* cDNA clone) restricted with *Pst* I. 6 pGSR2 (*gln-α* cDNA clone) restricted with *Pst* I and Bgl II. 7 pGSR1 (*gln-ß* cDNA clone) restricted with *Pst* I. 8 pUC19 vector restricted with *Bam* HI.


1 2 3 4 5 6 7 8
kb
8.6
4.5
2.75

obtain eight clones for the *gln-∂* gene (Cock *et al* 1992). The other reason therefore is the most likely to explain the lack of success with the cloning. The reasons for the under-representation or absence of *gln-α* clones in the library can possibly be explained in several ways 1. It is known that the methylation pattern of plant genomic DNA can cause the DNA to be recognised as foreign by the *E. coli* host and be inactivated and degraded, therefore resulting in an under-representation of that sequence in the library, this will occur in strains such as LE392 (used in this study) which are wild type for either the McrA or McrB loci (Raleigh *et al* 1988, Woodcock *et al* 1989, Graham *et al* 1990). As it is likely that the *gln-α* sequences present in the *P. vulgaris* genome would be methylated in the mature tissue used for the library construction, as the gene is not expressed in mature roots, this could be the reason why a clone was not obtained for *gln-α* from the library used. Another possible reason for the under-represenation of *gln-α* clones in the library is that the clones contain inverted repeats in the sequences of the clones. These repeats have been shown to cause recombination in the bacterial host which obviously results in an underrepresentation of the clone in the resulting library, problem can be overcome if an *E. coli recA* mutant is used as the host (Wyman *et al* 1986, Wertman *et al* 1986, Chalker *et al* 1988).

The sizes of the genomic fragments containing probe recognition sequences observed in the southern blot studies were difficult to explain. A possible explanation at this time is that there could be a large intron in the genomic fragment relating to the 5' untranslated region of the cDNA thus suggesting a restriction map as shown in figure 5.4. The occurrence of a relatively large intron at the 5' end of a GS gene has been observed in *P. vulgaris* with the *gln-∂* gene (Cock *et al* 1992). Unfortunately the presence of this putative large intron would make it extremely unlikely that the *Bam*HI fragment would contain any of the promoter sequences, therefore there was little point in continuing with the 8.5kb *Bam* HI fragment by constructing a sized complete digest libary for screening as planned. In addition it became unlikely that any further attempt to clone the *gln-α* gene would prove fruitful in time to allow any in depth study on the gene after cloning and sequencing had been completed. Therefore it was decided to terminate the attempt to clone the *gln-α* and to concentrate on the other aspects of the study which have been described earlier (see chapters three and four).

Figure 5.4: Restriction map suggested by figure 5.3.


1Kb
BamHI
8.5 Kb
BamHI
4.5 Kb
2.8 Kb
NdeI
NdeI
NdeI

Chapter 6

General discussion and conclusions

The initial observation that *gln-α* may be coinduced with PAL 1 was made in nodules senescing prematurely due to growth under an argon/oxygen atmosphere (Cock and Cullimore unpublished results). This discovery lead to a series of experiments studying the behaviour of *gln-α* both in nodules infected with *Rhizobial* mutants and in other systems where PAL 1 expression was known to occur. Expression of PAL 1 and *gln-α* was induced in the other systems although short time courses suggested that the expression may be staggered with the PAL 1 induction occurring a few hours in advance of the *gln-α* message induction. This observation suggests that the induction of *gln-α* may be in response to a product of PAL metabolism rather than to the same signals that trigger the induction of PAL 1 message. The factor which induces the induction of *gln-α* is unlikely to be ammonium as previous work has demonstrated that GS genes are not induced by exogenous ammonium in roots (Cock *et al* 1990). This is reinforced by the result seen with the cinnamate treatment of the wounding experiment where PAL 1 induction was partially inhibited while levels of *gln-α* expression remained unaffected. Another possibility is that *gln-α* is being induced in response to general stress stimuli and its induction is not directly connected to that of PAL 1 at all. This is however unlikely as induction of the two genes appears to be coordinate in

tissues where there is no stress stimuli such as seed leaves and early in wild type nodule development.

The expression patterns observed for *gln-ß* are more difficult to interpret than those observed for *gln-α* as this gene shows coordinate expression with *gln-α* in some systems, such as *Rhizobium leguminosarum bv phaseoli* strain CE119 derived nodules and *Pseudomonas phaseolicola* race three infected leaves. However, *gln-ß* expression is not induced in response to other stimuli which induce the expression of *gln-α* and PAL 1 such as elicitation of cell cultures and the wounding response in the presence or absence of exogenous glutathione and cinnamate. Expression of *gln-ß* is not high in the young tissues where *gln-α* and PAL 1 expression was found to be high such as plumules and developing nodules. Thus from the experiments performed as part of this study it would appear that *gln-ß* expression occurs under stress stimuli such as senescence and infection. Interestingly *gln-ß* is not induced with fungal elicitation suggesting that the induction is in response to a different set of stimuli to those which elicit the induction of PAL 1. The expression pattern of *gln-ß* appears to suggest, from the available data, that this gene is induced in response to general stress stimuli rather than specific conditions connected with PAL activity.

The correlation between the expression of a PAL gene and induction of GS genes has not previously been

observed although the induction of cytosolic GS message in response to senescence has recently been reported in rice (Kawakami and Watanabe 1988). The possibility of a link between the expression of a GS gene and that of a PAL gene is particually interesting as it would represent an example of either coordinate expression of genes of primary and secondary metabolism or a situation where the expression of a gene of primary metabolism was induced in response to an upregulation of secondary metabolism. Other examples of such phenomena include the induction of S-adenosyl-L-methionine synthetase and S-adenosyl-L-homocysteine hydrolase after the elicitation of parsley cell cultures (Kawalleck *et al* 1992). In addition enzymes of the shikimic acid pathway have been shown to be induced in response to light and wounding (Dyer *et al* 1989, McCue and Conn 1990). This second example is particularly relevant as the shikimic acid pathway is responsible for the production of phenylalanine which is obviously necessary for the phenylpropanoid pathway to be active. The enzymes induced in the response of the shikimic pathway show a coinduction of message patterns with PAL and the authors suggest a possible coregulation of the genes. While it is possible that the induction of *gln-α* is due to a product of PAL activity rather than to a direct coregulation of the two genes it is interesting that both genes for the production of phenylalanine and a GS gene (the product of which would be responsible for

removing ammonium, a product of PAL activity) show similar patterns of expression to that of PAL genes.

chapter 7

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