TITLE STRUCTURE AND EXPRESSION OF GLUTAMINE SYNTHETASE IN ROOT NODULES OF *PHASEOLUS VULGARIS* L.

AUTHOR Malcolm John Bennett BSc (Hons) Manchester

STRUCTURE AND EXPRESSION OF GLUTAMINE SYNTHETASE IN ROOT NODULES OF *PHASEOLUS VULGARIS* L.

Malcolm John Bennett BSc (Hons) Manchester

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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.

A paper (described below), written in collaboration with Dr J.V.Cullimore and Dr D.A.Lightfoot, has been accepted for publication and includes material presented in Chapters 3 and 5.

M.J.Bennett, D.A.Lighfoot, J.V.Cullimore (1989) cDNA sequence and differential expression of the gene encoding the glutamine synthetase γ polypeptide of *Phaseolus vulgaris* L. Plant Mol. Biol.

SUMMARY

Glutamine synthetase (GS) catalyses the assimilation of ammonia with glutamate, to form glutamine. In higher plants GS is an octameric enzyme of M_r 360,000 located in plastids and the cytosol. In the legume *Phaseolus vulgaris* L., GS is encoded by four nucleur genes (*gln-α*, *gln-β*, *gln-γ* and *gln-δ*) which encode the cytosolic α, β, and γ GS subunits of M_r 39,000, and the plastid δ GS polypeptides of M_r 44,000, accordingly. The aim of the work is to study the regulation of expression and structure of *P. vulgaris* root nodule GS.

This thesis has reported the construction of a *P. vulgaris* nodule cDNA library, and the isolation and characterisation of a full length nodule GS cDNA clone. The polypeptide encoded by this cDNA and two previously identified GS cDNA clones, pR-1 and pR-2 (see Gebhardt *et al*, 1986, EMBO J. 5, 1429-1435) have been produced *in vitro* by transcription/translation and shown to co-migrate on two-dimensional gels with the cytosolic γ, β and α GS polypeptides respectively.

An RNase protection technique has been used to specifically and quantitatively determine the abundance of the *gln-β*, *gln-γ* and leghaemoglobin (*Lhb*) mRNAs. Differences in the relative abundances of *gln-β* and *gln-γ* mRNAs at different stages of nodulation suggest that these two genes are divergently regulated, whereas the *gln-γ* and *Lhb* mRNAs are coordinately expressed during nodule development, detectable at least one day prior to the onset of dinitrogen fixation. Furthermore, the detection of the γ polypeptide, although at a reduced level, in nodules grown in the absence of dinitrogen (under an atmosphere of 80% Argon: 20% oxygen) suggests that a product of dinitrogen fixation does not provide the primary signal for, but may have a role in the level of, *gln-γ* nodule expression. A study of the abundance of the *gln-γ* and *Lhb* mRNAs from a variety of *P. vulgaris* organs has identified that in contrast with the nodule specific expression of *Lhb* mRNA, *gln-γ* mRNA is also detectable, albeit at a lower abundance, in stems, petioles, and green cotyledons.

The structure of *P. vulgaris* nodule and plumule GS has been studied through the resolution of the component GS isoforms, and the determination of their respective GS polypeptide compositions by ion exchange fast protein liquid chromatography and two-dimensional western blotting respectively. These studies have identified that both α + β, and β + γ GS subunits may assemble, perhaps randomly, to produce a heterogenous mixture of GS isoforms. However, a study of the changes in GS isoforms during nodulation has shown that the β_8 isoform is present at higher activity than would be expected, which could reflect the differences in the temporal and/or spatial expression of the γ and β GS subunits within the nodule.

The three cytosolic GS cDNAs have been expressed individually in *Escherichia coli*, synthesising GS subunits of the correct M_r, which appear for α, γ and β to be mainly soluble, intermediate in solubility and insoluble respectively. The soluble α, β and γ GS subunits are able to assemble into kinetically active oligameric isoenzymes, in the absence of any specific plant assembly factors. In addition, the α and γ cDNAs have been shown to complement an *E.coli glnA* mutation, suggesting that the recombinant α and γ higher plant GS enzymes can effectively function in the *E.coli* nitrogen assimilatory pathway.

ABBREVIATIONS

AAT	aspartate aminotransferase
ALN	allantoin
ALT	allantoic acid
$Ar:O_2$	80% argon: 20% oxygen
AS	asparagine synthetase
ASN	asparagine
ASP	aspartic acid
ATP	adenosine triphosphate
AZA	azaserine
bp	base pairs
CAT	chloramphenicol acetyl transferase
CIAP	calf intestinal alkaline phosphatase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
Fd	ferredoxin
FH	tetrahydrofolate
GDH	glutamate dehydrogenase
GLY	glycine
GOGAT	glutamate synthase
GS	glutamine synthetase

Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IEC FPLC	ion exchange fast protein liquid chromatography
Kb	kilo bases
Kbp	kilobase pairs
Kd	kilo daltons
LB	Luria-Bertani medium
Lhb	*Leghaemoglobin*
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
M_r	molecular weight
mRNA	messenger ribonucleic acid
MSO	methionine sulphoximine
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
$N_2:O_2$	80% nitrogen: 20% oxygen
NH_4^+	ammonium
NiR	nitrite reductase
NR	nitrate reductase
OAA	oxaloacetic acid
2-OG	2-oxogluturate
PBM	peribacteroid membrane
PEPC	phosphoenol pyruvate carboxylase
PRPP	phosphoribosyl pyrophosphate
Poly (A)	poly adenylic acid
P. vulgaris	*Phaseolus vulgaris* L.
P. sativum	*Pisum sativum*
RNA	ribonucleic acid

RUBISCO	ribulose bisphosphate carboxylase/oxygenase
SDW	sterile distilled water
SDS	sodium dodecyl sulphate
SER	serine
SSC	standard saline citrate
TE	Tris-EDTA buffer
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
tRNA	transfer ribonucleic acid
XDH	xanthine dehydrogenase

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Plant growth and development involves the integrated interaction of three distinct genetic systems, the chloroplast, mitochondrion and nucleus (see Mellet, 1988; Newton, 1988 for reviews). During symbiotic interactions the development of the nodule organ involves a fourth genome, namely from the endosymbiont (see Rolfe and Gresshoff, 1988 for a review).

Advances in molecular biology and biochemistry has enabled the study of the regulation of expression of the genes involved in the nodule symbiosis and other developmental programmes. This thesis presents results of experiments aimed at understanding the regulation of the plant glutamine synthetase (GS) genes and their products, with particular reference to their roles during nodule development.

The review of the scientific literature discusses aspects of plant biochemistry and molecular biology involved directly or indirectly with the theme of the thesis, with particular reference to data emerging during this period of research. The final section discusses the aims of the work to be presented in this thesis.

1.2 AMMONIA ASSIMILATION

Nitrogen is the single most limiting nutrient to plant growth. Plants primarily obtain nitrogen from the soil as an inorganic form such as nitrate or ammonium (NH_4^+), the former through the action of a permease nitrate transport protein (Jackson *et al*, 1973). NH_4^+ is assimilated directly in the root. In contrast, nitrate is reduced via nitrite to NH_4^+ by the nitrate reductase (NR) and nitrite reductase (NiR) activities, either in the root or, through xylem mediated transport, in the illuminated leaf (see Beevers and Hageman, 1980, for a review) and assimilated in these organs. If this nitrogen supply proves inadequate, certain leguminous and actinorhizal species have the ability to interact symbiotically with dinitrogen fixing soil bacteria, to obtain part, if not all, of their nitrogen from the symbiosis (see section 1.3).

All inorganic nitrogen, whether obtained from the soil (such as nitrate) or from the atmosphere (dinitrogen), is converted into NH_4^+ before incorporation into organic combination is possible.

Prior to 1970, of the number of known reactions involving NH_4^+, glutamate dehydrogenase [Eq.(1)] (GDH) (E.C.1.4.1.2-4) was generally considered to be the probable route of assimilation.

$$\text{2-oxoglutarate} + NH_4^+ + \text{NAD(P)H} \xleftarrow{\text{GDH}} \text{glutamate} + H_2O + \text{NAD(P)} \quad (1)$$

In 1970 however, Tempest *et al* proposed an alternative route in *Klebsiella aerogenes*, involving both glutamine synthetase [Eq.(2)] (GS) (E.C.6.3.1.2.) and a previously undescribed enzyme, pyridine nucleotide linked glutamate synthase [Eq.(3)] (GOGAT) (E.C.1.4.1.13).

$$\text{glutamate} + NH_4^+ + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + \text{Pi} \quad (2)$$

$$\text{2-oxoglutarate} + \text{glutamine} + \text{NAD(P)H} \xleftarrow{\text{NAD(P)H-GOGAT}} 2\ \text{glutamate} + \text{NAD(P)} \quad (3)$$

The proposed route (FIG 1.2) provided a means of initially incorporating the NH_4^+ into glutamine (GLN), using the GS amide synthetase activity, and subsequently transferring the amide-N to the 2-oxoglutarate (2OG) α-C position through the transaminating activity of GOGAT. Thus GS and GOGAT were able to act in conjunction to form what is known as the 'glutamate synthase cycle'. Through such a cycle glutamate (GLU) could act as both substrate and product of NH_4^+ assimilation, in which one molecule of GLU recycles whilst the second can be used in transamination reactions to form other amino acids (see FIG 1.2). In addition, GLN could become the product, featuring in the synthesis of other compounds such as asparagine (ASN), through amide-N group transfer, regenerating GLU. Thus, through this cycle, both GLN and GLU can be synthesised from the assimilation of NH_4^+, and used in subsequent anabolic reactions.

Further work has identified that the glutamate synthase assimilatory route is widely distributed, functioning in other bacteria (see Ginsburg and Stadtman *et al*, 1973), the cyanobacterium *Anabaena sp.*

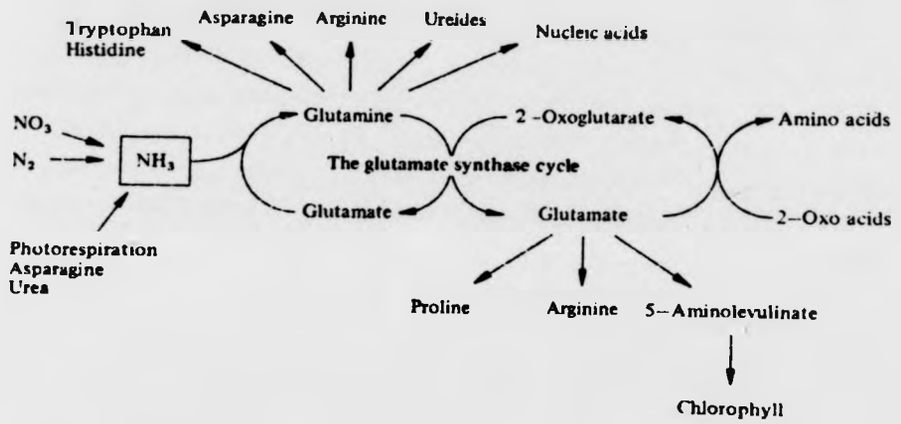


FIG 1.2 Metabolism of ammonia in higher plants (reproduced from Miflin and Lea, 1982)

(Stacey *et al*, 1979), the fungus *Neurospora crassa* (Quinto *et al*, 1977) and higher plants (see Miflin and Lea, 1980). In the latter group, the GOGAT enzyme can occur as both NAD(P)H dependent in non-green tissue (Dougall, 1974) and ferredoxin (Fd) dependent (E.C.1.4.7.1.) in green tissue (Lea and Miflin, 1974).

A considerable body of evidence has been collected which suggests that the glutamate synthase cycle, rather than GDH, provides the major route of NH_4^+ assimilation in higher plants (see Miflin and Lea, 1980; 1982 for reviews). Lines of evidence include the kinetic characteristics of the respective enzymes, in which the K_m for NH_4^+, is apparently considerably lower for GS relative to GDH. Thus GS is able to function at very low NH_4^+ concentrations, below the K_m of GDH, although the K_m value for GDH has been observed to vary in relation to NH_4^+ and NADH levels (Pahlich and Gerlitz, 1980).

The subcellular localization of the three NH_4^+ assimilatory enzymes, in which both GS and GOGAT are present in the leaf chloroplast, whereas NADH-GDH is in the mitochondria (Wallsgrove *et al*, 1979), has enabled the study of the isolated organelles ability to assimilate NH_4^+. Isolated chloroplasts readily assimilated NH_4^+ (Lea and Miflin, 1974), whereas mitochondria failed to do so, unless under non physiological conditions such as 15mM NH_4^+ in an anaerobic atmosphere (Davies and Teixiera, 1975). Although a chloroplastically located NADPH-GDH activity has previously been reported (Lea and Thurman, 1972), the use of inhibitors, such as methionine sulphoximine (MSO) and azaserine (AZA) which specifically inhibit GS and GOGAT (but not GDH), respectively, likewise conclude that isolated chloroplasts assimilate NH_4^+ via the glutamate synthase cycle (Anderson and Done, 1977). Furthermore, ^{15}N and ^{13}N labelling kinetic studies, combined with the use of the above inhibitors, also confirm that NH_4^+ assimilation takes place solely through the glutamate synthase cycle in a variety of plant tissues or whole plants (Meeks *et al*, 1978; Rhodes *et al*, 1980).

Although most genetic studies of the role of the different NH_4^+ assimilatory activities have been performed in bacteria and yeast, higher plant mutants in both Fd-GOGAT (Somerville and Ogren, 1980) and plastidic GS (Wallsgrove *et al*, 1987) have been isolated. These mutants exhibit conditionally lethal phenotypes under photorespiratory

conditions, thus suggesting that all NH_4^+ released during photorespiration, is reassimilated by the glutamate synthase cycle.

1.3 NODULE NITROGEN ASSIMILATION

Certain leguminous and actinorhizal species (see section 1.4A) are capable, through their symbiotic associations with selected nitrogen fixing bacteria, of utilising dinitrogen as a nitrogen source, resulting in the production of NH_4^+ (Kennedy, 1966). Robertson *et al* (1975) noted that the NH_4^+ excreted by the bacteroids is assimilated in the host cytosol, based on the observed repression of the endosymbionts NH_4^+ assimilatory machinery (see section 1.5C) in contrast to enhanced levels of the corresponding host enzymes (see Miflin and Cullimore, 1984).

Although GDH was originally proposed to assimilate dinitrogen fixed NH_4^+ in root nodules (Kennedy, 1966), Meeks *et al* (1978) subsequently identified the direct incorporation of radiolabelled NH_4^+ into the GLN amide group. The ability of MSO to inhibit the incorporation of labelled nitrogen into GLN or any other organic compound substantiated a role for GS in the initial assimilatory reaction. Furthermore, AZA blocked the GLN amide group transfer to 2OG, resulting in labelled GLN accumulation, confirming a role for GOGAT and the glutamate synthase cycle in the assimilation of dinitrogen fixed NH_4^+. Both plant GS and NADH-dependent GOGAT levels are enhanced in nodules relative to uninoculated roots, increasing in activity in parallel with leghaemoglobin synthesis and nitrogenase activity (Lara *et al*, 1983; Chen and Cullimore, 1988; see Cullimore and Bennett, 1988, for a review), whereas no such correlation could be obtained for the GDH activity (Sen and Schulman, 1980). Plant nodule GS and NADH-GOGAT exhibited cytosolic and plastidic localisations (Awonaike *et al*, 1981; Chen and Cullimore, pers. commun.). In addition, a Fd-GOGAT activity has also been detected in soybean nodules (Suzuki *et al*, 1984).

The previously described metabolic reactions are particularly energy demanding processes which, together with the nodule growth and maintenance requirements and the synthesis of organic transport compounds, are estimated to use upto 15-30% of the plants net photosynthate (Rawsthorne *et al*, 1980). Although the primary stages of NH_4^+ assimilation are common to all legumes, later reactions involved in

nitrogen compound synthesis are seen to vary. Legumes are classified as either amide or ureide exporters. Amides, such as ASN or GLN, are usually synthesised in legumes of more temperate origin, such as *Pisum*, *Vicia* and *Lupinus*, whereas ureide exporters are usually tropical and subtropical legumes such as *Glycine*, *Phaseolus* and *Vigna*, synthesising allantoin (ALN) and allantoic acid (ALT). The above compounds make up the major nitrogen solutes of the xylem stream of nitrogen fixing plants, although many other protein and non-protein amino acids are present (see Lea and Miflin, 1980; Schubert *et al*, 1986).

A) SYNTHESIS OF AMIDE TRANSPORT COMPOUNDS

Amide exporting plants assimilate organic nitrogen into both GLN and ASN, the latter being the major xylem sap nitrogenous constituent possibly because of its greater solubility and lower metabolic activity relative to GLN (see Sieciechowitz *et al*, 1988, for a reveiw). GLN is synthesised through the direct amidation of GLU (section 1.2), whereas ASN synthesis requires the GLN dependent transamidation of ASP, catalysed by asparagine synthetase (AS) [Eq.(4)] (Ta *et al*, 1987). ASP is synthesised through the transamination of oxaloacetate (OAA) from GLU by ASP aminotransferase [Eq.(5)] (AAT) (FIG 1.3A).

ASP + GLN + ATP ---> ASN + GLU + ADP + Pi (4)

OAA + GLU ---> ASP + 2OG (5)

In lupin nodules both AS and AAT enzyme activities were found to increase concomitantly with GS activity during the onset of dinitrogen fixation (Scott *et al*, 1976). In soybean (an ureide exporter) both AAT and AS initially increase during early nodulation stages, but with the onset of ureide synthesis both activities subsequently decrease (Werner *et al*, 1980). AAT is present as both cytosolic and plastidic isoforms, termed AAT-P1 and AAT-P2 respectively, in lupin and soybean nodules (Reynolds and Farnden, 1979; Boland *et al*, 1982). AAT-P2 is specifically induced during nodulation, and found in both infected and uninfected soybean nodule cells, whereas the AAT-P1 isoform is present exclusively in the uninfected cell (Hanks *et al*, 1983).

Utilisation of GLN and ASN as transport compounds requires 5 and 4 carbon skeletons which are ultimately derived from leaf photosynthate. It is estimated for lupin that 25-30% of the photosynthate delivered to

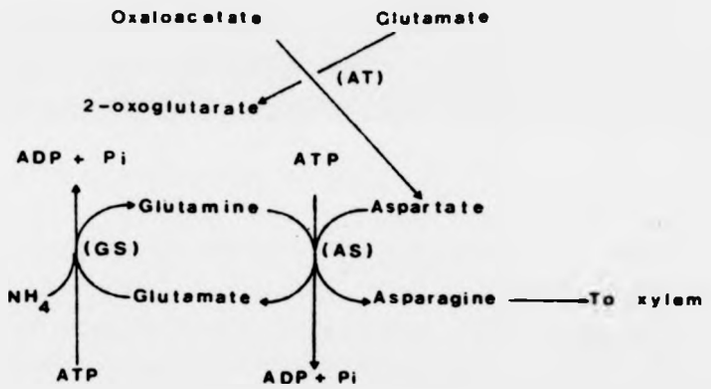


FIG 1.3A Asparagine biosynthesis. Enzymes are indicated in parentheses: AT, aspartate aminotransferase; AS, asparagine synthetase; and GS, glutamine synthetase (reproduced from Dixon and Wheeler, 1986).

the nodule is returned as nitrogenous solutes (Layzell *et al*, 1979). Photosynthate is metabolised via glycolysis and OAA synthesised through the anapleurotic action of phosphoenolpyruvate carboxylase (PEPC) [Eq.(6)], providing an excess of TCA intermediates available for assimilatory processes (Coker and Schubert, 1981). In amide exporters the PEPC enzyme activity is correlated with increases in nitrogen

$$PEP + CO_2 + H_2O \rightarrow OAA + Pi \quad (6)$$

fixation. For alfalfa, PEPC is estimated to provide 25% of the carbon required for the assimilation of the NH_4^+ (Vance *et al*, 1983).

B) UREIDE SYNTHESIS

Ureides have importance in the assimilation and transport of dinitrogen fixed NH_4^+, as exhibited by their selective accumulation in nodulated but not uninoculated plants, and their rapid labelling by ^{15}N (for a review see Reynolds *et al*, 1982). Ureide biosynthesis is relatively more complex than amide synthesis, involving *de novo* purine synthesis and subsequent oxidative catabolism (Matsumoto *et al*, 1977).

De novo purine biosynthesis proceeds by a series of reactions requiring phosphoribosyl pyrophosphate (PRPP), GLN, ASP, CO_2, GLY, methenyltetrahydrofolate (methenylFH_4) and formylFH_4, resulting in the formation of inosine monophosphate (IMP). GLN and ASP are derived from the nodule GS, GOGAT and AAT activities, whereas GLY and the C1 derivatives are obtained from SER, synthesised by the phosphorylated pathway (see Schubert, 1986). The component enzymes of purine synthesis, such as PRPP amidotransferase, phosphoglycerate dehydrogenase, SER hydroxymethylase and PRPP synthetase, are present at elevated levels in nodules, relative to uninoculated roots, increasing in activity coordinately with GS, GOGAT and AAT as nitrogen fixation and ureide transport begins (Reynolds *et al*, 1982). Studies by Atkins, Boland, Schubert and others (see Schubert, 1986, for a review) identified that the synthesis of IMP was closely associated with the nodule plastid fraction.

The exact route of purine oxidative catabolism was originally highly contentious (see Reynolds *et al*, 1982; Schubert, 1986), and has resulted in the identification of a pathway distinct to that identified in microorganisms (Atkins *et al*, 1985). The component activities,

such as xanthine dehydrogenase (XDH), uricase, allantoinase (Atkins, 1981; Schubert, 1981), 5'nucleosidase and purine nucleotidase (Christensen and Jochimsen, 1983), were identified at elevated levels in nodules of ureide transporting plants, relative to uninoculated roots or nodules of amide exporting legumes, increasing with dinitrogen fixation, NH_4^+ assimilation and ureide export. These activities featured quite distinctive subcellular compartmentation (for a review see Schubert, 1986). IMP dehydrogenase, 5'nucleosidase and purine nucleotidase were associated with the nodule plastid fraction (Boland and Schubert, 1983), thus interacting directly with the purine biosynthetic activities, enabling purines to be shuttled directly into ureide biogenesis. In contrast, XDH, uricase and allantoinase were localised in the cytosol, peroxisome and endoplasmic reticulum (ER) respectively (Hanks *et al*, 1981).

Examination of the nodule cellular organisation by Newcomb and Tandom (1981) observed that infected cell peroxisome development was restricted relative to uninfected cells, which also featured the proliferation of the ER. The implications of such cytological observations on the cellular distribution of ureide metabolism were confirmed by biochemical analysis of fractionated uninfected and infected cell protoplasts (Hanks *et al*, 1983; Shelp *et al*, 1983). Accordingly, NH_4^+ assimilatory, *de novo* purine synthetic and oxidative reactions upto XDH, are predominantly associated with the infected cell. This was further substantiated by Triplett (1985), who identified the exclusive infected cellular localisation of XDH using immunocytological studies. In contrast, later stages of purine oxidation and ureide formation, catalysed by allantoinase and uricase, are predominantly associated with uninfected cells, (Bergmann, 1983) (FIG 1.3B). Such a spatial arrangement is in agreement with the enzymes characteristics and the physiology of the cellular compartments. For example, the purine synthetic enzymes which, together with nitrogenase, display an oxygen lability, are thus protected by the low oxygen tension of the infected cell, whereas uricase, which requires oxygen and has a high K_m for oxygen, is appropriately localised in the uninfected cell (see Schubert, 1986).

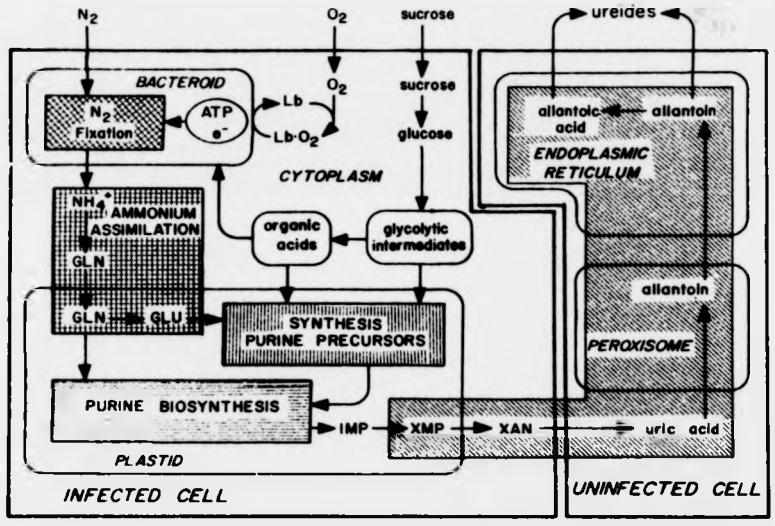


FIG 1.3B Subcellular organisation of ammonia assimilation in the nodule (reproduced from Schubert, 1986).

1.4 BIOLOGICAL NITROGEN FIXATON AND NODULE DEVELOPMENT

A) BIOLOGICAL NITROGEN FIXATION: DISTRIBUTION AND IMPORTANCE

The ability to fix nitrogen, termed diazotrophy, is restricted to the eubacteria and recently discovered members of the archebacteria. The diazotrophic phenotype is present in a wide range of species, although its rather haphazard distribution has lead to speculation on its evolution (Postgate and Eady, 1988). Biological nitrogen fixation can occur in a number of free living organisms including the anaerobic methanogenic archebacteria and *Clostridium*, facultative anaerobes such as *Klebsiella*, aerobes like *Azotobacter* and the microaerobic *Azospirillum*. In addition, certain cyanobacteria such as the unicellular *Gloetheca* and filamentous *Anabaena* have developed ways to temporally or spatially separate the oxygen-generating photosynthetic from the oxygen-sensitive nitrogen fixing reactions. Other bacteria only fix nitrogen, to a greater extent, when interacting symbiotically with certain host plants.

The nitrogen fixing plant-microbe interactions vary in complexity. The most simple involves the selective colonisation of the rhizosphere around certain cereals (corn, sorghum) by *Azospirillum* and featuring the selective exchange of metabolites (see Dobereiner *et al*, 1988 for a review). Interactions between the cyanobacterium *Anabaena* and the waterfern *Azolla* results in leaf dorsal cavity lobe formation, which the cyanobacterium colonises but remains extracellular (Tel-Or *et al*, 1984). By far the most complex association, in which specialised structures are formed on the host plant, involves bacteria such as actinomycetes (*Frankia*), *Rhizobium* and *Bradyrhizobium*. The respective bacteria penetrate the host plant resulting in the formation of the nodule in which the nitrogen is fixed (see section 1.4B). *Rhizobium* and *Bradyrhizobium* mediated nodulation is restricted to the *Leguminoseae*, with the exception of the non-legume *Ulmaceae Parasponia*, whereas actinomycetes form actinorhizal nodules on a variety of non-legume angiosperms, such as *Alnus* (Smith and Gianinazzi-Pearson, 1988).

Such two partner interactions may prove an oversimplification of the complex associations between bacteria and plant occuring in the rhizosphere. A number of recent reports have identified a role for a

third bacterium, such as some pathovars of *Pseudomonas syringae* (Knight and Langston-Unkefer, 1988), in increasing legume forage and grain yields.

All the above symbiotic interactions feature the donation of carbon metabolites by the plant host to the bacteria, in exchange for fixed nitrogen. The increasing complexity of the symbiosis involves the refinement of this interaction, to enable the host to provide the ideal environment for nitrogen fixation. These conditions include a low oxygen tension, due to the irreversible inactivation of nitrogenase by oxygen, and large amounts of energy for dinitrogen reduction, providing reasons for the predominance of the symbiotic and photosynthetic nitrogen fixing systems. It is estimated that grain legumes contribute 20% of total biologically nitrogen fixed, which accounts for over 70% of nitrogen fixation of agricultural importance (Dixon and Wheeler, 1986).

B) NODULE DEVELOPMENT AND MORPHOLOGY

The developmental changes which lead to the formation of a nitrogen fixing nodule have been well studied morphologically (for a review see Robertson and Farnden, 1980; Rolfe and Gresshoff, 1988). Vincent (1980) categorised nodule development into four stages: stage 1, preinfection; stage 2, infection and nodule formation; stage 3, nodule function and maintenance; and stage 4, nodule senescence.

The preinfection stage initially involves a flavanoid mediated signal from the plant root to the *Rhizobium* in the rhizosphere (Peters *et al*, 1986), resulting in rhizobial attachment to an emerging root hair cell (see Rossen *et al*, 1987, for a review). The root hair features a pronounced curling, due to uneven growth, entrapping the bacteria in a pocket. The bacteria penetrate the plant cell wall through its partial dissolution (Turgeon and Beuer, 1985), resulting in changes in root cell wall growth and the formation of the infection thread, initiating stage 2 of nodulation. The infection thread initially invades the root hair cell and then the root cortex where it continues to grow and ramify. However, the actively dividing bacteria within the infection thread remain separate from the plant cell contents. Further signalling between the host and bacteria is evident from the initiation of cell division in undifferentiated root parenchyma cells several hours prior

to root cortex infection thread penetration, resulting in the formation of a mitotically active root nodule meristem (Calvert *et al*, 1984). The infection thread grows towards the meristematic regions. Upon contact, the rhizobia bud off from the tips of the infection thread into the cell cytoplasm, in an endocytotic process, and become enveloped by the host, plasmalemma derived, peribacteroid membrane (PBM) (Robertson *et al*, 1978). The PBM maintains the separation of the host cell cytoplasm and the bacteria, which continue to divide and enlarge, differentiating into the bacteroids, enabling dinitrogen fixation (stage 3). Not all nodule cells become infected, approximately 50% remained uninfected, postulated to serve distinctive roles in the fixation, assimilation and transport of nitrogen (see FIG 1.3B and section 1.3). Nodule senescence (stage 4), normally occurring after plant flowering, is associated with the disruption of the PBM and the lysis of the bacteroid. The breakdown of leghaemoglobin and the haem cofactor results in the characteristic nodule senescent brown and green discolouration (see Vance *et al*, 1986).

Legume nodules are categorised according to their morphology. The first category includes the temperate legumes *Medicago*, *Pisum*, *Vicia* and *Trifolium*, that feature an elongate-cylindrical nodule with a meristem that functions for an indeterminate period, hence indeterminate nodules. Such nodules contain a number of zones of cells at distinctly different stages of development (FIG 1.4A). The nodule meristem is derived from cortical tissue close to the xylem, proliferating in advance of the infection thread, and can maintain mitotic activity for several months. Nodule cells immediately distal with the meristem zone, termed the invasion zone, are invaded by the infection thread, leading to bacterial release and proliferation. In the early symbiotic zone, host cells differentiate into infected and uninfected types, associated with rhizobial differentiation into bacteroids, which feature increased size and exhibit pleomorphism. The late symbiotic zone contains fully differentiated host infected and uninfected cells and bacteroids, performing dinitrogen fixation and assimilatory functions, whereas the senescent zone features host cell and bacteroid degeneration. Tropical legumes, such as *Phaseolus*, *Vigna*, *Arachis* and *Glycine*, develop a spherical nodule with a meristem that functions for a determinate period, hence determinate nodule. Initially the visible nodules are small and white, featuring prolific infection thread formation, cortical cell division and associated bacterial infection. Cortical cell

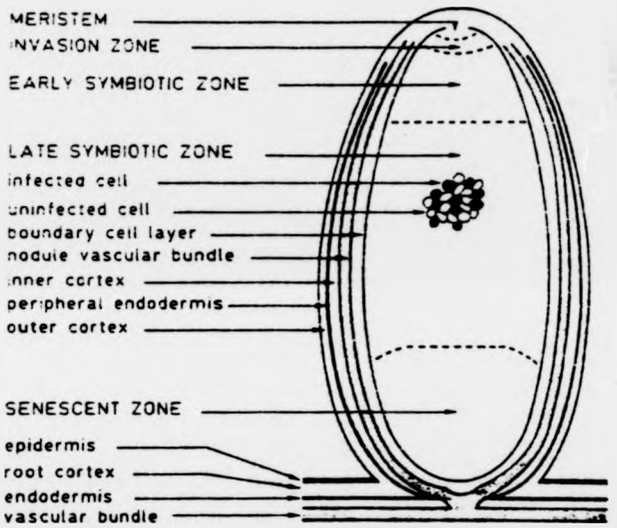


FIG 1.4A Overall organisation of an indeterminate nodule (reproduced from Nap, 1988).

division continues until one week after the onset of nitrogen fixation, resulting in a mature nitrogen fixing nodule exhibiting a red colouration. Any further increase in nodule size is related to cell expansion. Following flowering the nodule begins to senesce, and due to haem breakdown, exhibits a characteristic brown and green discolouration. Therefore, the developmental phases are separated temporally in determinate nodules, instead of spatially as in indeterminate nodules. The non-legume *Parasponia* and other primitive legume nodules differ in that their nodule meristem, is derived from the pericycle, as in lateral root formation, whereas more advanced nodule types rely on *de novo* cortical cell division. These variant nodule morphologies are a result of host dependent developmental programmes, as demonstrated by one rhizobial strains' ability to induce all 3 nodule morphologies on a variety of legumes and *Parasponia* (Trinick and Galbraith, 1980).

In both determinate and indeterminate nodule types the central infected zone is surrounded by the uninfected nodule cortex. The peripheral endodermal cell layer divides the cortex into an inner and outer layer, serving as an oxygen barrier due to the lack of any intercellular spaces and additionally, in determinate nodules, the presence of suberised walls. The vascular supply is located in the inner cortical region, enabling rapid transfer of assimilated nitrogen to the shoot (FIG 1.4A).

C) SYMBIOTIC GENE EXPRESSION

The legume nodule is an unique plant organ, and as such features a developmental programme as complex as other plant organs. This involves numerous genes of both bacterial and host origin and their differential expression distinguishes root nodule development from other organs (see Nap and Bisseling, 1988). The following discusses aspects of bacterial and host gene expression and regulation during root nodule development.

i) RHIZOBIAL SYMBIOTIC GENES

Numerous symbiotic genes have been identified which control the establishment of a nitrogen fixing symbiosis. In the (fast growing) rhizobia such genes are located on a large (150-1000Kb) so called SYM

plasmid, whereas in the (slow growing) bradyrhizobia, the symbiotic genes are chromosomally encoded.

The symbiotic genes required for the initial establishment of nodulation are termed *nod* genes, which on mutation inhibit, reduce or delay root nodulation (Debellé *et al*, 1986). In *R.meliloti*, more than 15 *nod* genes have been identified (Kondorosi *et al*, 1988; see Rossen *et al*, 1987, for a review). They include the *nodDABC* genes, which are found in, and are interchangeable between different rhizobia, hence termed the common *nod* genes (Downie *et al*, 1985). Mutations within the *nodDABC* genes result in a Nod^- phenotype (Kondorosi *et al*, 1984). In contrast, mutations in other *nod* genes display an altered host range, delay or reduce nodulation (see Kondorosi *et al*, 1988, for a review). The *nod* genes are regulated by the constitutively expressed *nodD* gene product. NodD, on binding a specific flavanoid released by the host root, acts as a positive transcriptional activator by binding to a consensus sequence upstream of all *nod* genes. The *nodDABC* have been assigned roles at the earliest stages of nodulation, such as root hair curling (Downie *et al*, 1985), infection thread formation (Djordjevic *et al*, 1985) and the induction of cortical cell division (Dudley *et al*, 1987). The nature of the rhizobial signal has been examined by Bhuvaneswari and Solheim (1985) and Schell *et al* (1988) who have reported the identification of a factor produced in wild type, but not *nodABC* mutated, rhizobia that induces root hair curling and meristematic activity. The factor is characteristically heat stable, non-proteinaceous, with a molecular weight of less than 500 daltons (Schell *et al*, 1988), akin to a plant growth regulator.

Although *nod* gene products are involved in the signalling to the plant host to initiate nodule development, other symbiotic genes are required for further nodule development. For example, a Ti plasmid cured *Agrobacterium tumefaciens* strain containing only the *R.meliloti* *nod* genes, although able to induce a nodule structure, lacked the ability to induce infection thread formation and intracellular bacterial colonisation (Hirsch *et al*, 1984). Mutations in genes involved in regulating the next stage of development display such an 'empty' nodule phenotype, which include mutants involved in extracellular acidic polysaccharide (*exo*) (Finan *et al*, 1985) and cyclan glucan biosynthesis (Dylan *et al*, 1986). The exact role of the *exo* genes in infection is at present unclear, although the polysaccharides are thought to play a role

as an 'avoidance' mechanism against the host defence response (Nap *et al*, 1987).

The symbiotic genes involved in nitrogen fixation are not essential for any developmental stage, since mutations in these genes result in a morphologically normal nodule, but they are required for an effective dinitrogen fixing symbiosis. These genes are categorised either as *nif* or *fix*. Rhizobial *nif* genes are structurally or functionally similar to the *K.pneumoniae nif* genes, whereas *fix* genes are, through their mutated phenotype, deemed essential for nitrogen fixation. *Nif* gene regulation has been best studied in *K.pneumoniae* (see Merrick, 1988, for a recent review). Control is applied at two levels. Firstly, GlnB, GlnD, NtrC and NtrB provides a global nitrogen regulatory mechanism, controlling a variety of nitrogen assimilatory operons including the *nif* regulatory genes *nifLA*. Secondly, a *nif* specific regulation through the action of the transcriptional activator NifA regulates the expression of the other *nif* genes. However, the physiology of symbiotic dinitrogen fixation regulation is very different from *K.pneumoniae*. Firstly, rhizobia do not fix significant amounts of dinitrogen outside the symbiosis, secondly, rhizobia export the majority of their fixed nitrogen, and thirdly the nitrogen rich conditions within the nodule do not suppress the induction of dinitrogen fixation. These differences are reflected in the regulatory mechanisms. Although NifA remained a positive regulator of the *nif* operon and a number of other *fix* operons, *nifA* is not regulated by NtrC (Szeto *et al*, 1987). Instead *nifA* is regulated by the *fixLJ* gene products, which on mutation abolished both symbiotic and microaerobic *nif* and *fix* gene induction (see Kahn *et al*, 1988, for a review). In rhizobia, the global regulatory system (*fixLJ*) is responsive to the oxygen concentration, and not to combined nitrogen, as for the *ntr* system of *K.pneumoniae*, in agreement with nodule physiological conditions.

ii) PLANT SYMBIOTIC GENES

Attempts to identify plant genes involved in nodulation through classical genetic studies have resulted in the isolation of a number of mutant phenotypes from a variety of legumes, but their molecular bases are poorly understood (see Vance *et al*, 1988, for a review). In contrast, modern molecular techniques have identified a variety of genes

(see Nap and Bisseling, 1987) and enzymes (see Robertson and Farnden, 1980) that have roles during nodulation. A number of these proteins are found only in nodules, and not in roots and other organs, which has become the definition of a plant nodulin (van Kammen, 1984). Nodulin genes can be expressed differentially during nodule development, the majority being expressed around the onset of nitrogen fixation and functioning in the establishment and maintenance of a nodule environment conducive to dinitrogen fixation and assimilatory requirements (termed late nodulins, see Govers *et al*, 1985). Only a few nodulins have been detectable at early stages of nodulation, having appropriate roles in the structure and development of the organ (termed early nodulins) (FIG 1.4B). Examples of such nodulins are discussed below.

Gloudemans *et al* (1988) have identified two root hair mRNAs termed RH-42 and RH-44 whose expression are either induced or enhanced respectively within 24 hours of rhizobial inoculation. Their roles are at present unknown, but they could be involved in the initial infection process, such as infection thread formation since *nodABC* mutants fail to achieve such changes of expression. Between two and four early nodulins have been identified whose expression coincides with nodule meristem induction and nodule emergence through the root epidermis. These include the soybean early nodulin Ngm-75, also termed ENOD2 (Franssen *et al*, 1987), which encodes a putative hydroxyproline rich glycoprotein, as previously observed in nodules (Cassab, 1986) and postulated to have structural and microbe interactive roles. In soybean, Ngm-75 is transiently expressed during nodule structural establishment and is proposed to have a structural role, whereas another early nodulin, Ngm-44b, displayed an extended expression pattern more closely reflecting the nodule meristematic activity (Gloudemans *et al*, 1987).

The majority of (approximately 20) nodulins are expressed in mature nodules, around the onset of nitrogen fixation. These include leghaemoglobin (*Lhb*), the high affinity oxygen binding protein. *Lhb* controls the free oxygen concentration of the infected cell cytoplasm (Robertson *et al*, 1984), ensuring the integrity of the oxygen sensitive bacteroid nitrogenase enzyme (see Appelby, 1984, for a review). In soybean, Marcker *et al* (1984) has observed that the *Lhb* genes are expressed in an exponential manner, initially at low levels 7-8 days after infection, then followed by a dramatic increase in *Lhb* mRNA concomitant with the majority of other nodulin genes. A number of

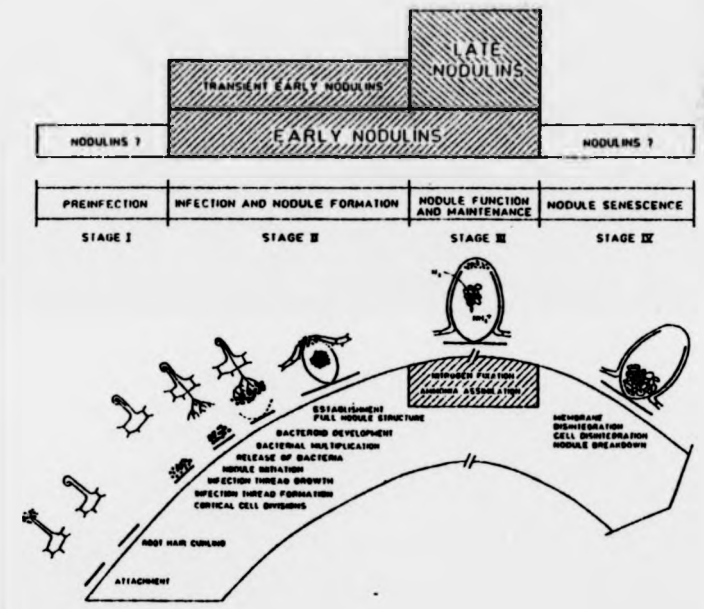


FIG 1.4B Schematic representation of the stages, and sequence of events, in the formation of a nitrogen fixing root nodule, and the involvement of nodulins in these stages (reproduced from Nap, 1988).

nodulins that have roles in nodule metabolism have been identified and characterised. These include the NH_4^+ assimilatory enzymes, GS and GOGAT (section 1.2) whose nodule activities increase coordinately with *Lhb* synthesis and nitrogenase activity (Lara *et al*, 1983; Chen and Cullimore, 1988). Such increases in GS activity were associated with the induction of a nodule specific GS gene in *P. vulgaris* (Cullimore *et al*, 1984) and alfalfa (Dunn *et al*, 1988), whereas *P. sativum* featured the enhanced expression of GS genes expressed in other organs (Tingey *et al*, 1987a). In soybean, contradictory observations have proposed (Sengupta-Gopalen and Pitas, 1986), and denied (Hirel *et al*, 1987) the presence of such a nodule specifically expressed GS gene (see Cullimore and Bennett, 1988, for a review; or section 1.43 for further details). Studies on the nodule NADH-GOGAT enzyme of *P. vulgaris* (Chen and Cullimore, 1988), has identified two isoforms, NADH-GOGAT I and NADH-GOGAT II. NADH-GOGAT II is apparently nodule specific, whereas NADH-GOGAT I is also present at low levels in uninoculated roots.

A number of activities associated with ureide biogenesis, such as purine nucleosidase (Larsen and Jochimsen, 1987), xanthine dehydrogenase (Nguyen *et al*, 1986) and uricase (Nguyen *et al*, 1985), have been proposed to represent nodulins, with the latter enzyme representing the second most abundant nodule protein. cDNAs encoding the nodule uricase subunit have been isolated (Nguyen *et al*, 1985; Sanchez *et al*, 1987), and apparently represent a nodule expressed member of a small multigene family. Other metabolic nodulin cDNAs cloned, but involved in nodule carbon metabolism, include the soybean nodule sucrose synthetase (Thummler and Verma, 1987). Additional nodulin activities includes malate dehydrogenase (Appels and Haaker, 1987), PEPC (Deroche *et al*, 1983) and AAT-P2 (P.H.S.Reynolds, pers.commun.). Further potential nodulin candidates are listed by Robertson and Farnden (1980). All the above activities increase concomitantly with GS, *Lhb* and nitrogenase activity during nodule development.

During nodule development it appears essential, to maintain an effective symbiosis, that the bacteroids are separated from the host cytoplasm by the PBM. The integrity of the soybean PBM appeared associated with a nodule specific choline kinase activity (Mellor *et al*, 1986), with ineffective nodules featuring little such activity. The PBM represents a barrier between the host and endosymbiont, and features in the regulation of nutrient exchange between the cytoplasm and bacteroid.

A number of nodulins associated with the PBM include soybean N-23 and N-26 (Fuller *et al*, 1983; Fortin *et al*, 1985), which have surface and transmembrane associated PBM positions, from primary sequence secondary structure predictions (Mauro *et al*, 1985).

iii) REGULATION OF PLANT GENES DURING NODULATION

Attempts to identify factors involved in the regulation of nodulin genes has centered on the analysis of the proposed *cis* acting sequences at the 5' promoter regions. Sequence alignment studies between a variety of nodulin genes have identified a variety of putative regulatory consensus sequences (Mauro *et al*, 1985; Sandal *et al*, 1987). The importance of nodulin gene 5' regions was demonstrated when a 2Kb soybean *Lhbc3* promoter was fused to a reporter CAT (chloramphenicol acetyl transferase) gene and transformed into *Lotus corniculatus*. The transgenic plants were observed to express the CAT activity in a nodule specific manner (Stougard *et al*, 1986). Jorgensen *et al* (1988) have also demonstrated such a nodule specific expression pattern using a soybean N-23 5' promoter fragment fused to a CAT reporter gene, in transgenic *Lotus* and *Trifolium repens*. Thus all necessary *cis* acting elements required for a nodule specific and developmentally correct expression pattern are present at the 5' region. Stougard *et al* (1986) and Jorgensen *et al* (1988) further delineated these elements through Bal31 deletion studies, and subsequently nodule *trans* acting factor(s) have been observed to bind to two AT rich sequences within these regions (Jensen *et al*, 1988). Jensen *et al* (1988a) have reported similar such sequences in the *Sesbania rostrata Lhb* genes, to which soybean and *S.rostrata* nodule nuclear factors bind with equal affinity. This and other conclusions above, have lead a number of authors to suggest (see Schell *et al*, 1988) that the *cis* and *trans* acting factors controlling the nodule specific expression of nodulin genes are conserved throughout legumes.

Although the ultimate control of the nodule specificity of gene expression is exerted at the gene level, nodule physiological conditions have been repeatedly claimed to play a role in the induction and regulation of nodulin genes. Such conditions include the low nodule oxygen tension. Larsen and Jochimsen (1986) have demonstrated enhanced levels of the nodule uricase activity in soybean callus tissue, by

lowering the oxygen tension. However, studies on pea root expression during microaerobic growth conditions only detected an increase in alcohol dehydrogenase mRNA abundance (Govers *et al*, 1986). Haem has been observed to play a role in the post-translational regulation of the soybean *Lhb* when expressed in yeast (Jensen *et al*, 1986) and also in the regulation of nodule sucrose synthetase activity (Thummler and Verma, 1987), but haem's *in vivo* significance as a regulator of nodulin gene regulation is unclear. In addition, NH_4^+ has been proposed to play a role in the regulation of soybean GS (Hirel *et al*, 1987), although its role as a primary effector of nodulin gene expression is unclear (see sections 1.5 and 5.4). Since nodulin gene expression is not induced by physiological changes within the root, the invading bacteria could provide the signal. Studies using a variety of rhizobial mutants that block nodulation at a number of developmental stages has enabled the study of such a role. Gloudemans *et al* (1988) have examined changes in plant gene expression during the early stages of nodulation using *nodC*, *nodD* and *exo* rhizobial mutants. Only wild type and *exo* rhizobium were able to enhance the abundance of the RH-42 and RH-44 mRNAs, thus the *nodC* and *nodD* appeared to regulate RH-42 and RH-44. *R.meliloti* mutants affecting later stages of nodulation, such as the *exo* genes, during infection thread formation and nodule invasion, are observed to express early nodulins, such as ENOD2, but failed to induce late nodulins, such as *Lhb* (Dickenstein *et al*, 1988; Dunn *et al*, 1988; Norris *et al*, 1988). In contrast, *nif* or *fix* mutants, which on inoculation were unable to actively fix dinitrogen but produced a normal nodule structure with infection threads and intracellular bacteria, expressed all early and late nodulin mRNAs, although at a lower level (Dunn *et al*, 1988; Norris *et al*, 1988). The regulation of late nodulin gene expression has been further examined using the previously noted *Lhb* CAT fusion constructs in transgenic alfalfa plants. The nodule expressed CAT activity provided a quantitative assay for measuring *Lhb* promoter activity in nodules formed with a variety of *R.meliloti* mutants (Schell *et al*, 1988). Such studies have detected reduced CAT activity in *nif* and *fix* inoculated nodules, whereas *exoB* and *exoH* mutants failed to induce any CAT activity at all. Thus *Lhb* gene expression appears to be induced concomitantly with infection thread formation and/or intracellular bacterial release. The authors presented a working model of nodulin gene regulation,

involving successive bacterial gene signalling either through the common *nod* or *exo* gene products, enabling intracellular colonisation and eventual formation of a dinitrogen fixing nodule.

1.5 GLUTAMINE SYNTHETASE

A) THE GS ENZYME, STRUCTURE AND REGULATION

The central role of GS in nitrogen metabolism is reflected in the activity's universal distribution throughout the bacterial (Reitzer and Magasanik, 1987), animal (Meister, 1974) and plant kingdoms (Stewart *et al*, 1980).

GS has been best studied in enteric bacteria, and recently an X-ray crystallographically-solved model has been proposed for the *Salmonella typhimurium* GS enzyme (Almassy *et al*, 1986). This and other studies of the GS enzyme from other bacteria, such as *Escherichia coli*, (Ginsburg and Stadtman, 1973) have concluded that the typical prokaryotic GS molecule consists of 12 identical subunits of M_r 50,000 (giving a native M_r of 600,000), arranged as 2 hexagonal rings lying on top of one another in 622 point group symmetry. The model features a number of unusual subunit interactions, including the identification of the GS active sites located at the interface of two neighbouring subunits within each hexagonal planar ring (Almassy *et al*, 1986).

The regulation of the enteric bacterial GS activity features a number of complex controls (see Reitzer and Magasanik, 1987, for a review). This regulation includes feedback inhibition, in which 8 of the 15 known ligands that bind to GS were GLN derived metabolites, including nucleotides and amino acids. In addition, GS is also regulated by covalent modification (Shapiro *et al*, 1967) through the reversible adenylylation of up to all 12 GS subunits of the enzyme molecule. Such adenylylation occurs under conditions of nitrogen excess, and results in the increased sensitivity of GS to most feedback inhibitors.

Other bacteria, such as the cyanobacterium *Anabaena 7120* (Fisher *et al*, 1981), contain a GS that is not susceptible to covalent

modification. In these organisms GS is regulated by additive feedback inhibition mediated by several amino acids (Orr and Haselkorn, 1982).

The picture is further complicated by the identification of multiple isoenzymic forms of GS, initially in *Bradyrhizobium japonicum* and subsequently throughout the gram-negative *Rhizobiaceae* (Fuchs and Kleister, 1980) and the gram-positive sporoactinomycete, *Frankia* (Edmands *et al*, 1987). The two forms of the GS enzyme, termed GSI and GSII, differ in a number of properties. For example, GSI, but not GSII, is subject to adenylylative control and GSII is relatively unstable to heat. In addition, their respective structures differ; GSI has a 'classical' prokaryotic dodecameric subunit structure, subunit M_r 59,000, whereas GSII is hexameric, subunit M_r 36,000.

Studies on fungal GS in *Candida utilis* (Sims *et al*, 1974) and *Neurospora crassa* (Palacios, 1976) have identified subunits of M_r 50,000, (native M_r 360,000) arranged in a typical eukaryotic octameric structure, as two sets of planar tetramers lying on top of one another. Sims *et al* (1974) found that treatment of *C.utilis* GS *in vitro* with NH_4^+ and GLN could lead to depolymerisation and the formation of inactive GS tetramers.

The algae *Chlorella pyrenoidosa* has been reported to feature a highly atypical hexameric GS possessing 322 point symmetry, made up of subunits of M_r 53,000, native M_r 320,000 (Rasulov *et al*, 1977).

In higher eukaryotes, such as higher plants and vertebrates, GS has an apparent native M_r 350,000-400,000, consisting of eight subunits M_r 39,000-47,000, arranged as two sets of planar tetramers lying on top of one another (Stewart *et al*, 1980). The two planar structure was thus conserved between bacterial and eukaryotic GS. Eisenberg *et al* (1987) suggested, from primary sequence comparisons, that plant GS folded in a similar manner to prokaryotic GS, which could infer that the plant GS active site is at the interface of neighbouring subunits.

Higher plant GS does not appear to be regulated through covalent adenylylation (Kingdon *et al*, 1974), as similarly concluded for vertebrate GS (see Meister *et al*, 1974). However, modification of GS through the action of a GS inactivation system involving a low molecular weight protein that produces redox changes in the GS enzymes thiol groups, has been reported in *Lemna minor* (Rhodes *et al*, 1979).

Regulation of GS by nucleotides has been observed in GS isolated from soybean root nodules (McParland *et al*, 1976), pea leaves (O'Neil and Joy, 1975) and rice (Kanomori and Matsumoto, 1972). Soybean root nodule GS is sensitive to both ADP and 5'AMP, two nucleotides whose levels are indicative of the cellular energy charge (Atkinson *et al*, 1968). In addition, Hirel and Gadal (1980) noted that changes in the chloroplast energy charge, together with Mg^{2+} concentrations and pH, upon illumination were such that conditions moved towards an optimum for the leaf plastid GS activity, enabling the efficient assimilation of photorespiratory or nitrite derived NH_4^+.

Feedback inhibition by amino acids and other ligands exhibited varying effects on enzyme activity. Stewart *et al* (1980) interpreted the variable inhibition by several end products of GLN metabolism and the general activation of GS by 2OG as indicative of a fine control mechanism of GS enzyme activity. However, whether any of these *in vitro* GS enzyme characteristics are of importance *in vivo* is still unclear.

B) GS ISOFORMS AND POLYPEPTIDES

Multiple GS isoforms (that are physically separable, usually by ion exchange chromatography) are common in a number of plant associated bacteria, such as the *Rhizobiaceae* and *Frankia*, and also the lower eukaryote, *N.crassa* (see section 1.51). In higher plant greened tissue, two GS isoforms were originally identified in soybean hypocotyl (Stasiewicz and Dunham, 1979) and barley leaf tissues (Mann *et al*, 1979), termed GS1 and GS2. Both subcellular fractionation (Miflin, 1974; Wallsgrove *et al*, 1979) and immunocytological studies (Hirel *et al*, 1982) identified cytosolic and plastidically located GS activities, which were subsequently correlated with the greened barley leaf GS1 and GS2 isoforms respectively (Mann *et al*, 1980). The presence of GS isoenzymes in different subcellular compartments is common to other enzymes of plant nitrogen and carbon metabolism, and examples include the cytosolic and plastidic forms of aspartate kinase (Davies and Miflin, 1978), pyruvate kinase (Ireland *et al*, 1980) and phosphoenolpyruvate carboxylase (Perrot-Rechenmann *et al*, 1982).

The relative proportions of the GS1 and GS2 isoforms are seen to vary between organs within a plant. Mann *et al* (1980) concluded that

GS2 appeared to be restricted to green tissue, and in studies on for example *P. vulgaris*, GS2 appeared predominantly in etiolated and greened leaves, stems and petioles, whereas GS1 was higher in roots, hypocotyls, pods and seeds (see McNally and Hirel, 1983). The functional significance of the distribution of the GS2 isoform became apparent after McNally *et al* (1983) analysed the leaf GS isoform composition of a large variety of C3, C4 and achlorophylous parasitic plants. The authors found a correlation between the presence and relative level of GS2 activity with the degree of photorespiration occurring in the host plant tissue. Furthermore, differences were noted between the GS1 isoforms from rice leaf and root organs, the latter termed GSR (Hirel and Gadal, 1980). In addition, *P. vulgaris* root nodules were observed to contain two cytosolic GS isoforms, a novel isoform termed GSN1 which was distinct from the second root like isoform, GSN2, in both kinetic, chromatographic (Cullimore *et al*, 1983b) and antigenic behaviour (Cullimore and Miflin, 1984).

The regulation of the GS isoforms is of interest since they appear to be controlled by a number of factors. For example, the *P. vulgaris* root nodule GSN1, relative to the GSN2, isoform is specifically induced by the presence of rhizobia during nodule development, and can account entirely for the increase in root nodule GS specific activity. The induction of GSN1 activity is concomitant with the induction of plant *Lhb* synthesis and bacteroid nitrogenase (Lara *et al*, 1983). Photoinduction of GS activity in etiolated leaves of most C3 plants studied generally achieved, a 4- to 10- fold increase in GS2, but not GS1, levels (see McNally and Hirel, 1983). Similarly, in germinating cucumber cotyledons, GS1 is initially the predominant GS isoform, but is superceded, on cotyledon greening, by the GS2 isoform (Nishimura *et al*, 1982).

On reviewing the GS1 and GS2 isoforms physical and kinetic properties, McNally and Hirel (1983) identified differences such as their pH optima, Km for glutamate and stablity in the absence of thiol reagents. Some differences were proposed to be of physiological importance. For example, Hirel and Gadal (1980) noted that GS2 (but not GS1) *in vitro* activity exhibited a great sensitivity to changes in pH and Mg++ cation concentrations and could result in up to 50% and 80% increases of activity respectively. Such properties are postulated to be of great physiological importance in chloroplasts which feature

stromal pH shifts and cation enrichment on illumination. Studies on rice GS isoforms identified variable susceptibilities to regulation by a number of ligands, such as the nucleotides AMP, CTP and GTP for GS1, and NADH for GS2, whereas GSR appeared unaffected (Hirel and Gadal, 1980). The importance of these differences *in vivo* remains to be elucidated. Recently, kinetic differences between root and nodule GS isoforms, in their sensitivity and insensitivity to ß-tabtoxin, a GS inhibitor produced by some pathovars of *Pseudomonas syringae*, has been related to the enhanced yield of plant biomass, nodule number and nitrogenase activity in nodulated alfalfa plants coinoculated with *P.syringae* (Knight and Langston-Unkefer, 1988). The exact principle behind the greater yield remains speculative, although it could involve increased photosynthate supply to the nodule to assimilate reduced nitrogen, a process presumably inhibited in the root organ.

The basis of GS isoform heterogeneity became apparent when Lara *et al* (1984) examined the GS subunit composition of a number of purified GS isoenzymes from different organs of *P. vulgaris*, using two dimensional SDS-PAGE. The leaf contained up to 6 GS polypeptides, of which 2 subunits M_r 41,000 termed α and ß comprise the GS1 isoform, and between 3 to 4 GS subunits M_r 45,000 termed a,b,c and d, make up the GS2 isoform in etiolated and mature green leaves respectively. Roots contain one GS isoform, termed GSR, composed of α and ß polypeptides. Nodules contain a novel GS polypeptide not identified in roots or leaves, termed ϒ, together with the ß subunit, composing the GSN1 and GSN2 isoforms. GSN1 was observed to consist of mainly ϒ, whereas ß was the major subunit of the GSN2 isoform. Subsequently, Cullimore (1985), using a one dimensional SDS-PAGE technique that separated the ϒ and ß GS subunits, identified that purified GSN2, GSN1 and a nodule third GS activity (eluting on the DEAE ion exchange chromatographic void volume) were composed of the ß, ϒß and ϒ GS polypeptides respectively. Further work by Robert and Wong (1986) studying ϒ and ß GS subunit mixing, using native gel separative techniques, identified two major GS specific bands, refering to these somewhat confusingly as GSN1 and GSN2. GSN2 is also present in uninoculated roots and thus analagous to the GSN2 isoform (Lara *et al*, 1983), whereas GSN1 is nodule specific. GSN1 could be subdivided into 8 subcomponent bands which, together with the GSN2 single band, were postulated to represent all 9 possible mixing forms of the ϒ and ß GS subunits within the octameric nodule GS enzyme. The

relative abundance of each mixing form is observed to vary in relation to the plant cultivar and the nodule developmental stage, reflecting the apparent abundance of the constituent γ and β GS subunits. Recently, the α GS subunit has been detected in nodules, albeit at low levels (Padilla *et al*, 1987), further complicating the above. Thus, in *P. vulgaris*, the distribution of GS isoforms reflects the differential distribution of a number of GS subunits.

The observed heterogeneity of GS subunits is common to a number of plants. For example, the (tetraploid) *Solanaceae* tobacco chloroplastic GS, after purification and urea isoelectrofocusing, was composed of four Coomassie staining bands (Nato *et al*, 1984), which were proposed to contain upto 5% carbohydrate. In contrast, studies on the closely related (diploid) *Nicotinia plumbaginifolia* (Tingey *et al*, 1987b), using two dimensional separation, only identified one GS subunit charge variant. Furthermore, using a lectin staining procedure to detect carbohydrate,the authors failed to correlate staining with the chloroplastic GS subunit, in agreement with other studies on spinach plastid GS (Ericson, 1985). *N.plumbaginafolia* roots contain two GS subunit charge varients, M_r 38,000 that are not detectable in leaves (Tingey *et al*, 1987b), and such observations are in agreement with GS1 and GS2 isoforms being present in roots and leaves respectively (McNally *et al*, 1983). In *P. sativum*, 5 GS polypeptides were identified, present in all tissues but at varying abundances (Tingey *et al*, 1987a). One GS subunit, M_r 44,000, termed GS2 is present at high levels in the leaf relative to the root, and also at increased levels in the nodule. Another GS polypeptide, M_r 38,000 termed GS1, is present throughout the plant and at a slightly increased level in nodules, and similarly, three GS polypeptides, M_r 37,000 termed GSn, feature a 5- to 10- fold higher expression in nodules relative to roots and leaves.

In other plants, most work has been performed on root nodule GS polypeptides. For example, in alfalfa Lang-Unnasch *et al* (1985) identified three cytosolic GS polypeptides that are present in both root and nodule, whereas two plastid GS subunits are found in nodules but not roots. Sengupta-Gopalen and Pitas (1986) have identified in soybean, using two-dimensional SDS-PAGE, six GS subunits present in both roots

and nodules, and an additional 4 GS polypeptides present only in nodules. Contrary to these observations, Hirel *et al* (1987) noted, through isoelectrofocusing of nodule *in vitro* translation products, 4 major GS polypeptides which were also present in roots and leaves, and thus not nodule specific. Such differences have yet to be resolved.

C) GS GENES AND THEIR REGULATION

A number of GS genes have been isolated from a variety of prokaryotic and eukaryotic sources. The following describes some of their characteristics and modes of regulation.

i) BACTERIAL GS GENES

GS genes have been isolated from a number of bacteria, such as *E.coli* (Colanduani and Villafranca, 1986), *S.typhimurium* (Janson *et al*, 1986), and *Thiobacillus ferrooxidans* (Barros *et al*, 1985). The above bacteria contained a single GS gene copy (*glnA*), arranged within an operon, termed *glnA ntrB ntrC*, encoding GS and two regulatory proteins, NtrB and NtrC respectively.

Expression of the *glnA ntrB ntrC* operon is regulated by three different promoters. The *ntrB* and *glnAp1* promoters maintain low levels of NtrB, NtrC and GS respectively. High level *glnA* expression is only obtained through transcription of the *glnAp2* promoter and requires the binding of NtrC to a specific sequence within this promoter. Under low nitrogen conditions, NtrC is activated (by NtrB and components of the adenylylation system), and acts as a positive transcriptional activator. Such regulation, coupled with adenylylative control of the GS enzyme, provides a high degree of control of GS synthesis and activity strongly correlated to the prevailing nitrogen nutritive conditions.

Other bacteria, such as the cyanobacterium *Anabaena 7120* (Fisher *et al*, 1981), *Bacillus subtilis* (Fisher *et al*, 1984) and *Clostridium acetobutylicum* (Janssen *et al*, 1988) arrange their single GS genes without the apparent *glnA ntrB ntrC* operon structure or NtrC mediated control. *Anabaena* does however retain the use of multiple promoters, termed P1 and P2, homologous with the *Anabaena* RUBISCO and *nifH* promoters respectively, which are selectively used during limiting or

normal nitrogen nutritive conditions respectively (Tumer *et al*, 1983). Under these different physiological conditions, genes such as RUBISCO and *nifH* can be regulated by distinctive promoters, whereas the single GS gene, whose product is required throughout these conditions, possessed both promoter types, utilising one or the other depending on nitrogen supply.

Thus, the bacteria discussed above feature multiple promoters on a single GS gene, which are selectively utilised and regulated by a variety of factors related to the nitrogen supply, providing a simple answer to ensure sufficient GS during variable nitrogen nutritive conditions.

Examination of GS genes from a variety of members of the *Rhizobiaceae*, such as *Bradyrhizobium japonicum* (Carlson *et al*, 1985; Carlson and Chelm, 1986), *Rhizobium leguminosarum b.v. leguminosarum* (Fischer *et al*, 1986), *R.leguminosarum bv phaseoli* (Espin *et al*, 1988), *R.melliloti* and *Agrobacterium tumefaciens* (Rossbach *et al*, 1988a and b), has identified, in contrast with other bacteria, multiple GS gene copies encoding distinctive GS proteins. The GS genes isolated correspond to *glnA*, encoding a typical prokaryotic GS enzyme termed GS1; *glnII*, more closely resembling higher plant GS genes in sequence and structure (Carlson and Chelm, 1986); and a third loci termed *glnT* (Rossbach *et al*, 1988a and b) or *glnIII* (Espin *et al*, 1988), capable of complementing an *E.coli glnA* mutant but appearing cryptic in its respective host.

Both *glnA* and *glnII* feature distinctive modes of regulation. Rhizobial *glnA*, is expressed constitutively without *ntrC* mediated regulation. For example, *B.japonicum glnA* only features a 3- to 10-fold variation in mRNA abundance during various culture and symbiotic conditions (Carlson *et al*, 1985). In contrast, *glnII* is seen to respond to changes in nitrogen availability and the gene sequence contains an NtrC activated promoter (Carlson *et al*, 1987), subject to NtrC mediated regulation (Martin *et al*, 1988). Thus, in the free living *Rhizobiaceae* and apparently *Frankia* (Edmonds *et al*, 1987) the constitutive expression of *glnA* provides adequate GS activity which can be modulated through adenylylative control to respond to transient changes in nitrogen supply. The transcriptional regulation of *glnII* provides a means of responding to larger changes in metabolite levels, such as nitrogen starvation. Such a scheme, involving both transcriptional and

post-transcriptional regulation of GS activity is in common with other bacterial models, yet distinct in its application to different loci and gene products.

The regulation of rhizobial GS would be expected to differ in the symbiotic, with respect to free living state, considering the differences in the physiological conditions (see Miflin and Cullimore, 1984; section 1.4). In *B.japonicum*, growth under microaerobic conditions is sufficient to induce nitrogenase activity, and similarly Martin *et al* (1988) noted that *glnII* could be expressed under such conditions, even in the presence of 10mM NH_4^+. Such puzzling behaviour for a proposed NtrC regulated gene was investigated in an *ntrC* mutant that repressed *glnII* expression irrespective of NH_4^+ levels. Under microaerobic conditions, the *ntrC* mutant featured an induction of *glnII* expression, suggesting that an alternative positive regulator responsive to lowered oxygen levels, such as *nifA*, is controlling *glnII*. Such a regulatory mechanism could be of importance during early symbiotic stages. Ludwig (1980) noted that an *in vitro* re-creation of such conditions through microaerobic growth conditions results initially in GSII activity induction followed by repression prior to nitrogenase activity induction. In contrast, GSI features a greater level of adenylylation after the initiation of nitrogenase activity and is thus responding to NH_4^+ build up. One could postulate that this induction of GSII activity is in response to the biosynthetic requirements during rhizobial colonisation and multiplication prior to nitrogen fixation, and not required in mature nodules. Examination of the *in vivo* importance of either GS activity using single mutations in the *R.meliloti* GS genes (Rossbach *et al*, 1988b) results in a FIX^+ phenotype, suggesting a non-essential role for either GS activity. However, Espin *et al* (1988) have noted that inoculation of *P. vulgaris* with a *R.leguminosarum bv. phaseoli glnA* mutant, reduces nitrogenase acetylene reducing activity by over 35% in relation to wild type rhizobia.

Sequence comparisons of a number of *glnA* genes from a variety of bacteria (Janssen *et al*, 1988) have identified a surprising degree of divergence, only retaining high level homology within regions correlated with the bacterial GS active site. Janssen *et al* (1988) have also noted that the ability to adenylylate a *glnA* gene product is related

to the conservation of the adenylylated tyrosine residue and the surrounding primary sequence.

ii) LOWER EUKARYOTIC GS GENES

Studies in the lower eukaryotic *fungi*, such as *Neurospora crassa* (Quinto *et al*, 1978) and *Saccharamyces cerevisae* (Mitchell and Magasanik, 1983) have concluded that the level of GS is regulated, through the *de novo* synthesis of the enzyme, by the nitrogen source present in the medium. In *N.crassa*, NH_4^+ limitation results in the induction of a GS polypeptide termed α, whereas growth on glutamate results in the induction of a ß GS polypeptide (Davila *et al*, 1980), and genetic studies suggest that both GS subunits are encoded by separate loci (Davila *et al*, 1978). In contrast, *S.cerevisiae* contained a single gene *GLNa* (Mitchell and Magasanik, 1983), that has subsequently been isolated (Gonzalez *et al*, 1985) and Kim and Rhee (1988) have recently presented partial peptide sequences for the *S.cerevisiae* GS polypeptide.

iii) VERTEBRATE GS GENES

In vertebrates, multiple gene and/or cDNA clones have been isolated from a GS gene amplified Chinese Hamster cell line (CHO) (Haywood *et al*, 1986), human liver (Gibbs *et al*, 1987), rat liver (Van de Zande *et al*, 1988) and murine adipocyte cell culture sources (Bhandari *et al*, 1988). Multiple GS clones isolated from a single source appeared identical, suggesting little genetic heterogeneity. In contrast, studies in lower vertebrates, such as elasmobranchs (Smith *et al*, 1987), have identified differences in GS *in vitro* translation product sizes; the liver GS product, which is located within the mitochondrion, is larger than the cytosolically located brain tissue GS product. This has led Smith *et al* (1987) to propose that the liver GS product contains a presequence. Further work will be required to identify if the respective translation products correspond to different gene loci.

iv) HIGHER PLANT GS GENES

GS has been extensively studied at the gene level in a variety of plant species, with particular emphasis on the nodulating *Leguminoseae* (for recent reviews see Coruzzi *et al*, 1988; Cullimore and Bennett, 1988)

The first plant GS cDNA to be cloned was isolated from a *P. vulgaris* root nodule cDNA library prepared to nodule poly(A) RNA, screened using labelled purified GS mRNA obtained by immunoprecipitation of nodule polysomes (Cullimore *et al*, 1984). This partial GS cDNA clone, termed pcPvNGS-01, contained a translated sequence that was 80% homologous to a peptide amino acid sequence prepared from purified alfalfa GS (Donn *et al*, 1984). Subsequently the partial GS cDNA was used to isolate 3 additional, but different, full length GS clones from cDNA libraries prepared to poly(A) RNA from different organs of *P. vulgaris*. pR-1 and pR-2 were isolated from root (Gebhardt *et al*, 1986) and pcGS-δ1 from leaf (Lightfoot *et al*, 1988) cDNA libraries. These GS cDNA clones, which have different sequences, and, on genomic blot analysis, hybridise strongly to different restriction fragments of *P. vulgaris* DNA (Gebhardt *et al*, 1986; Lightfoot *et al*, 1988), were postulated to represent members of a small multigene family containing four, possibly five, GS sequences. A number of *P. vulgaris* genomic clones have been isolated which include pR-1, pcGS-δ1 and pcNGS-01 homologous sequences (B.G.Forde, pers.commun.; Cock and Cullimore, pers.commun.). The latter genomic clone encoded a fifth GS like sequence termed *glnε*, which, since its expression has been undetectable, may represent a pseudo-gene (B.G.Forde, pers. commun).

The pR-1 and pR-2 clones code for polypeptides of 356 amino acids which, together with the partial pcPvNGS-01 clone, are over 80% identical in sequence. The pcGS-δ1 clone however, is only 70% identical to the other GS cDNA clones, encoding N and C terminal extensions to the ORF of 57 and 16 amino acids respectively. The N terminal extension exhibited structural similarities to the cleavable presequence of nuclear encoded plastid stromal proteins (Karlin-Neumann and Tobin, 1986), and when fused to the pR-1 ORF (which encoded a cytosolically located GS polypeptide) enabled uptake into isolated chloroplasts (Lightfoot *et al*, 1988). These observations, together with the higher identity of pcGS-δ1 to a *P. sativum* plastid GS, suggested that the pcGS-δ1 cDNA encoded the *P. vulgaris* plastid located GS.

Gebhardt *et al* (1986) have used an S1 protection technique, which was able to discriminate between the similar GS sequences, to quantify the pR-1, pR-2 and pcPvNGS-01 mRNAs in poly(A) isolated from nodules, roots and leaves. Their results have shown that pcPvNGS-01 homologous mRNA is present only in nodules, pR-1 mRNA is detected in all three

organs, whereas pR-2 mRNA occurs only in roots and leaves. This distribution of individual GS mRNAs corresponded exactly to the organ distribution of one or another of the different GS polypeptides (Lara *et al*, 1984), and hence pcPvNGS-01, pR-1 and pR-2 were postulated to encode the γ, β and α GS subunits respectively (Gebhardt *et al*, 1986). Similar studies by Lightfoot *et al* (1988) identified that pcGS-31 homologous mRNA was expressed at greatest abundance in leaves (and stems), and at much reduced levels in roots and nodules, reflecting the organ distribution of the plastid GS subunits.

Gebhardt *et al* (1986), using this S1 technique, have measured the relative abundance of the pcPvNGS-01 and pR-1 homologous mRNAs at different stages of nodulation. Whereas the relative abundance of pR-1 mRNA to total poly(A) remained constant during nodulation, pcPvNGS-01 mRNA appeared at day 16 (soon after the nodules became visible on the root) becoming the most abundant GS mRNA at day 19. The timing and magnitude of these changes (taking into account the different inoculation procedures) suggests that the production of the γ polypeptide and GSN1 isoenzyme closely follows the appearance of the pcPvNGS-01 mRNA, and hence the induction of the GS γ gene. Similar conclusions have been drawn by Padilla *et al* (1987) who observed, through studying the changes of the nodule GS *in vitro* translation products, that the induction of γ mRNA is correlated to the observed increase in nodule GS activity. In addition, Padilla *et al* (1987) detected α mRNA at a low abundance during early stages of nodulation, prior to the appearance of nitrogenase activity. Ortega *et al* (1986) have similarly noted a transiently high α expression during early stages of root embryo development which was superseded by high levels of β mRNA and polypeptide. Thus all expressed members of the *P. vulgaris* GS multigene family are subject to either a differential organ distribution and/or developmental induction.

GS cDNA and genomic clones have been isolated from other legumes such as pea, alfalfa and soybean. In pea, Tingey *et al* (1987a; 1988) have isolated, sequenced and characterised three full length GS cDNA clones termed pGS185, pGS299 and pGS341 from leaf, root and nodule cDNA libraries respectively.

Through the use of an *in vitro* transcription and translation procedure, the products of the three full length pea GS cDNAs were

correlated to individual native GS polypeptides after two dimensional SDS-PAGE separation (Tingey *et al*, 1988). The pGS185 cDNA encodes a 49 Kd precursor that could be imported into isolated chloroplasts, to give a 44 Kd polypeptide that comigrates with the plastid GS subunit. The pGS299 and pGS341 GS cDNAs encode 38 and 37 Kd polypeptides which comigrate with the GS1 and one of the GSn subunits respectively (Tingey *et al*, 1988). Subsequently, a fourth partial GS cDNA pGS132, apparently differing from pGS341 by just one amino acid within its ORF, hybrid selected a mRNA that encoded an alternative GSn polypeptide (G.Coruzzi and E.Walker, pers. commun.). It is possible that a third closely related GSn encoding cDNA has yet to be isolated.

Genomic blot analysis identified that all three full length pea GS cDNAs hybridised to multiple restriction fragments with differing intensities, suggesting the presence of at least four to five GS genes, in agreement with the number of GS polypeptides identified (Tingey *et al*, 1987a). Subsequently, three pea GS genomic clones have been isolated, corresponding to the pGS185, pGS341 and pGS132 cDNAs (G.Coruzzi and E.Walker, pers. commun.).

The differential tissue distribution and relative levels of expression of each GS cDNAs homologous mRNA closely correlates that of their respective GS polypeptide (see section 1.5B). pGS185 homologous mRNA is abundant in leaves relative to rootsand features, as did pGS299 and pGS341 mRNA, an enhanced expression in nodules, with the latter mRNA achieving a 20- fold higher level of expression (Tingey *et al*, 1987a). More detailed quantitative S1 analysis, which can resolve the closely related pGS132 and pGS341 mRNA, observed that the levels of both homologous mRNAs increase 5- to 7- fold during nodulation relative to uninoculated roots(G.Coruzzi and E.Walker, pers. commun.). Surprisingly, if only the upper nodulated root section is used (removing the root tip), the pGS341 and pGS132 homologous mRNA levels are enhanced 20 and 100 fold respectively. This observation suggests, firstly that the enhancement of these mRNAs upon nodulation is greater than originally anticipated, and secondly that the respective mRNAs feature differences in their spatial distribution within the root.

Studies on soybean GS are less well documented. Sengupta-Gopalan and Pitas (1986) have observed the expression of a nodule specific GS mRNA species homologous to the *P. vulgaris* pcPvNGS-01 cDNA. Hirel *et al*

(1987) have isolated two GS cDNAs from a soybean nodule cDNA library, but failed to identify a nodule specific mRNA, instead detecting an enhanced expression of the two partial GS cDNAs homologous mRNA which are also expressed in roots. The cellular expression of such GS mRNA has been examined by isolating the respective genomic clone, fusing the 5' promoter region to a ß-glucuronidase (GUS) reporter gene and transforming the promoter-GUS fusion into tobacco. Histochemical examination identified the majority of the GUS gene activity within the root, primarily within the root tip and also in the vascular bundles (Verma *et al*, 1988).

In alfalfa a full length GS cDNA and genomic clone have been isolated and fully characterised (Tischer *et al*, 1986) from libraries prepared to alfalfa phosphinothricin resistant cultures which contain amplified GS genes. In addition, a nodule specific alfalfa GS cDNA has been isolated from a root nodule cDNA library (Dunn *et al*, 1988).

In all the legumes studied so far, GS appears to be encoded by a small multigene family, with at least two genes expressed in nodules. Apparently, only *P. vulgaris* and alfalfa contain a GS gene that is expressed in nodules, but not roots or leaves, whereas soybean and pea feature the enhanced expression in nodules of GS genes which are also expressed in other organs.

A GS cDNA, termed pGS1/15, has been isolated from a root cDNA library prepared to the *Solanaceae Nicotiana plumbaginifolia*, which encodes a cytosolic GS polypeptide (Tingey *et al*, 1987b). The homologous GS mRNA is expressed mainly in roots relative to leaves, in agreement with the tissue distribution of the cytosolic GS subunits (see section 1.52). Genomic blotting of *N.plumbaginifolia* DNA has identified several different hybridising fragments, suggesting the presence of a small multigene family. A similar study of the crucifer, *Arabidopsis thaliana* (Peterman *et al*, 1987), identified multiple hybridising GS fragments, characteristic of a small multigene family.

The analysis of monocot GS genes has been far less extensive, with only the recent isolation and characterisation of a *Hordeum vulgare* (barley) GS cDNA (J.Freeman, A.Marquez and B.G.Forde, pers. commun.). The cDNA appears to encode a plastidic GS subunit, containing both N and C terminal extensions relative to the cytosolic GS ORF, characteristic of the dicot plastid GS sequences.

It would thus appear that all the dicot species studied so far exhibit a multiplicity of GS genes, in contrast with most bacteria and the single celled eukaryote, *S.cerevisiae*, which contain single GS genes.

Just as bacteria and fungi GS expression is regulated by the cellular nitrogen supply, there is reason to believe that higher plant GS may also be regulated directly or indirectly through the action of NH_4^+ or one of its products. Evidence for a variety of regulatory mechanisms is discussed below.

Tingey *et al* (1988) have noted that the abundance of etiolated pea leaf plastid GS (pGS185 homologous) mRNA, but not cytosolic GS (pGS299) mRNA, is enhanced by pulses of red light but repressed by far red light, suggesting a classic phytochrome mediated response. However, red light flashed plants fail to reach levels of plastid GS mRNA obtained in light grown controls. Tingey *et al* (1988) have postulated that an additional effector, such as photorespiratory NH_4^+, may be involved in regulating plastid GS expression. Proof for such a suggestion came when pea plants were grown in an air or high CO_2 atmosphere, conditions that permit high or low photorespiratory rates. Plants grown in air contain high levels of plastid GS (pGS185) mRNA compared to plants grown in high CO_2, whereas the level of the cytosolic GS (pGS299) mRNA remain unchanged (G.Coruzzi, pers. commun.).

A role for NH_4^+ in the regulation of GS expression in soybean roots has been proposed by Hirel *et al* (1987). When nitrogen starved roots are treated with NH_4^+, GS mRNA levels are enhanced within 2 hours of application. In contrast, enhanced root GS mRNA levels through the addition of nitrate requires a longer time course. Similar studies in *P. vulgaris* have however, failed to detect any such changes of GS mRNA abundance (R.Mould and J.V.Cullimore, pers. commun.).

Considerable work has been performed on the regulation of GS in root nodules, to determine the factors controlling the induction of GS expression during nodulation (see Cullimore and Bennett, 1988, for a recent review). Two distinct approaches have been used to address this question.

In the first approach, nodules are formed in an atmosphere of 80% argon and 20% oxygen ($Ar:O_2$). Under such conditions, cowpea (Atkins *et al*, 1984a) and soybean nodules (Hirel *et al*, 1987) develop a normal nodule cell and bacteroid structure with nitrogenase activity, but only

hydrogen is produced instead of NH_4^+ due to the absence of dinitrogen. Cowpea nodules, grown in such a manner, contain only 30% of the GS activity exhibited by air grown control nodules, yet samples under both gaseous regimes shared similar initial induction kinetics for nodule GS activity (Atkins *et al*, 1984b). Hirel *et al* (1987), using northern blot analysis to detect GS mRNA, failed to detect any increase of GS mRNA for $Ar:O_2$ grown nodules during nodulation, relative to uninoculated roots, whereas air grown nodule controls featured a several fold increase. In contrast, leghaemoglobin (*Lhb*) mRNA was present, albeit at slightly lower levels, under $Ar:O_2$ relative to air grown gaseous regimes, leading the authors to conclude that transcription of the soybean GS genes was regulated differently to the *Lhb* genes, and stimulated by the availability of NH_4^+ produced through dinitrogen fixation in the nodule.

In a separate approach, nodule GS regulation has been examined using mutated rhizobia which block nodulation at a variety of developmental stages or are unable to fix dinitrogen (see section 1.4). In *P. vulgaris*, Lara *et al* (1983) noted, using two cytologically uncharacterised Tn5 induced Fix^- mutants, that GSN1 (and thus γ gene expression) is present, although at reduced levels. Similarly, Padilla *et al* (1987) noted low levels of γ polypeptide in Fix^- nodules either induced by an Agrobacterium transconjugant containing the *Rhizobium leguminosarum bv phaseoli* sym plasmid or a *Rhizobium* isolated from the tropical legume *Clitoria*. In soybean inoculated with a Fix^- *B.japonicum* mutant, Hirel *et al* (1987) observed a relatively lower abundance of GS mRNA compared to Fix^+ nodules. From the above, it would appear that the induction or enhanced expression of the respective nodule expressed GS genes is possible, albeit at lower levels, even in the absence of dinitrogen or nitrogenase activity. Such a conclusion is further supported by the observed induction of *P. vulgaris* nodule GS γ mRNA prior to the detection of nitrogenase activity (Padilla *et al*, 1987)

In *P. vulgaris*, examination of the expression of the nodule expressed γ GS gene in nodules formed with a variety of cytologically characterised rhizobial mutants, identified reduced levels of γ mRNA in CE108 and F51 nodules (Fix^- with normal cytology); low levels in CE116 nodules (Fix^-, with limited intracellular release of bacteria); and no γ mRNA in nodulated roots inoculated with CE123 or CE106 (Fix^-, with no intracellular infection), even on the addition of NH_4^+ (J.V,Cullimore, pers.commun.). Work by Sengupta-Gopalan and Pitas (1986) using two

B.japonicum mutants, one (*nifD*) mutant forming a normal nodule cell structure, and another which resulted in the early disintegration of the peribacteroid membrane (pbm), identified nodule specific GS mRNA and polypeptides in the former but not the latter. Dunn *et al* (1988) have observed the presence of an alfalfa nodule specific GS mRNA in nodules inoculated with a variety of Fix^- mutants that elicited normal bacteroid and cell structure, albeit at reduced levels, but failed to detect GS mRNA in nodules inoculated with an *exo* mutant that did not penetrate the nodule cell. The induction of nodule GS gene expression appears to be correlated with infection thread formation and bacterial intracellular colonisation.

1.6 AIMS OF THE PROJECT

This thesis aims to study *P. vulgaris* root nodule gene expression, the synthesis of the GS polypeptides and their assembly into GS isoenzymes. Chapter 3 describes the isolation and characterisation of a full length nodule GS cDNA, which encodes the Y GS polypeptide. Chapter 4 considers both nodule and plumule GS isoenzymes and their subunit compositions. Chapter 5 studies both the developmental regulation of GS mRNAs, polypeptides and isoenzymes, and determines the role of dinitrogen. Chapter 6 describes the recombinant synthesis and assembly of the cytosolic GS subunits into their individual GS isoenzymes in *E.coli*, and questions if they are able to efficiently integrate into the bacterial NH_4^+ assimilatory pathway.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

P. vulgaris cv. Tendergreen was used unless otherwise specified, and grown in 6" pots with perlite, under an 18 hour light/6 hour dark cycle (22-24^{o}C) at 100 µmol photons m^{-2} s^{-2}. light intensity, and watered with a nitrogen free nutrient solution. *P. vulgaris* root nodule inoculum *Rhizobium leguminosarum bv. phaseoli* strain R3622 was used unless otherwise stated, and grown as described by Chen and Cullimore (1988). Nodules of cv. Bush Blue Lake used for poly(A) RNA isolation (section 3.2) were grown as described by Cullimore *et al* (1983). Nodules of cv. Tendergreen for the nodule development experiment (section 5.2), were grown as described by Chen and Cullimore (1988). Nodules of cv. Tendergreen used for the argon experiments (section 5.3) were grown under the same growth conditions as described by Chen and Cullimore (1988) as follows. Seeds were soaked overnight in running tap water and germinated in perlite until 5 cm high. Seedlings were transferred to air-tight plastic pots (5 seedlings per 40 cm pot, 12 pots in total), containing sterile leca (a porous pebble growth support), half filled with nitrogen free nutrient solution. On the second day (termed day 0) plants were inoculated with *R.leguminosarum bv. phaseoli* R4962, sealed into the pots (around the stem) with an air-tight (non-toxic) silicone glue, and the root systems supplied with a gas mixture containing 80% nitrogen and 20% oxygen (N_2:O_2), maintained at a flow rate of 200 ml/hr. On day 5, 7 pots were switched from the N_2:O_2 to an 80% argon and 20% oxygen (Ar:O_2) growth regime. At day 15, nodules were harvested from one N_2:O_2 and one Ar:O_2 supplied pot. In addition, two Ar:O_2 pots were switched to a N_2:O_2 atmosphere, two Ar:O_2 pots were supplied with 10mM NH_4^+ applied to the upper nodulated roots of the plants, and two N_2:O_2 pots were switched to an Ar:O_2 regime. Nodules, from each treatment, were harvested at days 16 and 19.

For the experiment described in section 3.4, germinating (white) cotyledons and the embryonic axes were dissected from seeds soaked overnight in running tap water; primary leaves, stems and roots were harvested from plants grown for 16 days in perlite with nitrate as a nitrogen source; green cotyledons and petioles were harvested from plants grown for 7 and 14 days respectively in compost; and nodules were harvested from plants grown for 14 days after inoculation with

R.leguminosarum bv. phaseoli R3622. Imbibed plumule tissue used in section 4.4 was dissected from seeds soaked overnight in running tap water, and germinated plumule tissue was dissected from seeds grown for two days in perlite moistened with tap water. All plant tissue collected was weighed, wrapped in aluminium foil and immediately frozen and stored in liquid nitrogen.

2.2 BACTERIAL STRAINS, GROWTH AND MAINTENANCE

The following *Escherichia coli* strains (with their genotypes) were used for recombinant DNA manipulation and expression studies.

ET8894 *rbs, lacZ::IS1, gyrA, hutC, Δ(rha-ntrC), 1703::Mucts62.*

N5151 *thi1,* $\lambda c1857^{ts}$

MM294 F', *endA1, thi1, hsdR17, supE44,* λ^-.

RR1 F', *hsd S20(*rB^-*,* mB^-*), ara14, LeuB6, proA2, thi1, lacY1, galK2, rpsL20, xyl5, mtl1, supE44,* λ^-.

TG2 *(lac pro), thi, stmA,* $hsdR^-$*,* $hsdM^-$*,* $recA^-$*, scIC 300:Tn10(tet)/F' traD36, proAB, lacIq, lacZ M15.*

LB and minimal M9 media were prepared as described by Maniatis *et al* (1982). YM media contained 0.5 g di-potassium hydrogen phosphate, 0.2 g magnesium sulphate heptahydrate, 0.1 g NaCl, 10.0 g mannitol and 1.0 g yeast extract per litre, adjusted to pH 6.8, and autoclaved until sterile. All antibiotics supplemented to growth media were prepared as described by Maniatis *et al* (1982) (with the exception of ampicillin that was used at double the recommended concentration), and sterile filtered through a 0.2 µm filter. GLN growth supplement was prepared as an aqueous 2.5 mg/ml stock solution, sterile filtered, stored at -20 C, and diluted one hundred fold on use, to give a 25 µg/ml working concentration.

E.coli strains RR1, MM294 and N5151 were grown on LB media. TG2 and ET8894 were grown on M9 minimal media, the latter strain (a *glnA* auxotroph) requiring a GLN supplement, and *Rhizobium leguminosarum bv. phaseoli* were grown on YM agar slopes. For strain maintenance, all bacteria were kept at 4°C on solid media, or stored at -70°C, as described by Maniatis *et al* (1982).

2.3 CHEMICALS

All common organic and inorganic chemicals used were of the highest analytical grade available, and obtained from BDH Chemicals Ltd, Sigma Chemical Co Ltd, Fisons PLC and FSA lab systems. Sephadex and Sephacryl products were purchased from Pharmacia Fine Chemicals Co. Radiolabelled nucleotides and methionine, and the Biotin Streptavidin Peroxidase colour labelling products were obtained from Amersham PLC.

2.4 ENZYMES

All DNA restriction and modifying enzymes were obtained from Amersham PLC, unless specified below:- AMV reverse transcriptase, Life Sciences Ltd; restriction endonuclease *AsuII*, Promega Biotec; calf intestinal alkaline phosphatase, Boeringer-Mannheim; DNase I, RNase A and RNase T1, Sigma; restriction endonuclease *Nde I*, BRL.

All restriction endonucleases were incubated in the compatible Amersham E system buffers. DNA modification enzyme buffers used were as recommended by Maniatis *et al* (1982) unless specified in the text.

2.5 DNA MANIPULATIONS

2.51 DNA RESTRICTION ANALYSIS

DNA was digested with a 4-5 fold excess of restriction enzyme units, under conditions recommended by the supplier. When DNA was cleaved with a combination of enzymes, digestions were performed sequentially, adjusting reaction conditions appropriately for the second enzyme. When partial restriction enzyme digestion was required, DNA was digested at a ratio of 2 units of restriction enzyme activity per µg of DNA for twenty minutes at 37^{o}C. The reaction was stopped by placing on ice and restricted DNA products were loaded directly for agarose electrophoresis. Low resolution restriction maps (accurate to 50-100bp) were generated by pairwise (six cutter) restriction enzyme digestion of the DNA and electrophoretic analysis of the fragment sizes produced,

using 1% agarose gels (120 mm by 120 mm), essentially as described by Maniatis *et al* (1982).

2.52 DNA MODIFICATION

All DNA modification reactions were performed as described by Maniatis *et al* (1982) unless specified below.

2.52A DNA phosphatasing

The method, using calf intestinal phosphatase (CIAP) (D.Hodgson, pers.commun.) removed linear DNA terminal 5' phosphate groups. The reaction contained up to 1 µg of linearised DNA, 0.1 volumes of 10 X CIAP salts (10 mM $MgCl_2$, 1 mM $ZnCl_2$), 0.1 volume of 10 X CIAP buffer (0.5 M NaOH, 20% glycerol pH 9.4) and six units of CIAP enzyme. The reaction was incubated at 37°C for 30 minutes, and stopped by phenol-chloroform extraction and ethanol precipitation of the DNA.

2.52B DNA ligation

The method for high efficiency ligation of *sticky* or *blunt* DNA ends was modified from Rusche and Howard-Flanders (1985). Ten units of T4 DNA ligase was mixed with linearised DNA, 1 µl of 10 X super ligase buffer (250 mM Tris acetate pH 7.5, 50 mM $MgCl_2$, 10 mM hexamine cobaltic chloride and 1 mM ATP), 1 µl of 5 X dithiothreitol (DTT) (final concentration 0.5 mM) and made up to 10 µl with SDW (sterile distilled water). The reaction was incubated overnight at 15°C and the ligated DNA used directly for transformation.

2.53 DNA TRANSFORMATION OF *E.coli*

All *E.coli* DNA transformations were performed using the modified salts procedure (Maniatis *et al*, 1982) unless otherwise stated below.

2.54 PLASMID DNA ISOLATION

Small and large preparations of plasmid DNA were isolated essentially as described by Maniatis *et al* (1982).

2.55 DNA LABELLING

The nick translation method was used as described by Rigby *et al* (1977), with the single modification of using a doubled concentration of the 10 X nick translation buffer in the reaction. The labelled probe was isolated from unincorporated nucleotides using a 1 ml Sephadex G 50 column (as described by Maniatis *et al*, 1982), Cerenkov counted (using a

LKB Wallac liquid scintillation counter), and used directly for hybridisations.

2.56 COLONY HYBRIDISATION

The method allowing *in situ* hybridisation of bacterial colony plasmid DNA with a radioactive nucleic acid probe was taken from Maniatis *et al* (1982).

2.57 DNA HYBRIDISATION

All colony nitrocellulose filters were hybridised as follows. Filters (with bound DNA) were initially incubated in 10 ml of prehybridising solution (containing 0.5 ml of a 1 mg per ml sheared salmon sperm DNA, boiled for 10 minutes, and added to 9.5 ml of 5 X SSPE [900 mM NaCl, 50 mM $NaPO_4$ pH 7.7, 5 mM sodium ethylene diamine tetra-acetic acid (EDTA)], 5 X Denhardts solution [0.1% bovine serum albumin (BSA), 0.1% FICOLL, 0.1% polyvinylpyrrollidone (PVP)], 0.2% sodium dodecylsulphate (SDS), 20 µg/ml poly A, U or C, and heated to 65°C), for three hours at 65°C, with constant agitation. The filters were then reincubated in fresh prehybridisation buffer containing the denatured radiolabelled DNA probe, and hybridised overnight with shaking at 65°C. Filters were washed, using an SSC [20 X SSC consists of 3.0 M NaCl and 0.3 M sodium trisodium citrate] (plus 0.1% SDS) washing buffer at 65°C. Low and high stringency washes were performed with 2 X or 0.1 X SSC (and 0.1% SDS) at 65°C respectively.

2.58 AUTORADIOGRAPHY

^{32}P-labelled nucleic acids were exposed to X-ray film (Fuji), using an intensifying screen, at -70 C. ^{35}S-methionine labelled proteins, fixed in polyacrylamide gels, were initially fluorographed in 50 ml of Amplify (Amersham) for 20 minutes, rinsed in Coomassie destain solution (40% methanol and 7% acetic acid), vacuum dried onto Whatman 3MM paper and exposed to X-ray film (as above) using an intensifying screen. ^{35}S-methionine labelled polypeptides, Western blotted onto nitrocellulose (section 3.3), were exposed directly to X-ray film without an intensifying screen, at room temperature. All X-ray film was processed in a manual minilab X-ray processor (X-ograph Ltd) under safe light conditions, using Kodak LX-24 X-ray developer for 1-5 minutes, and fixed in Kodak LX-40 solution for 5 minutes.

2.59 CLONING OF RECOMBINANT GS EXPRESSION CONSTRUCTS

The *P. vulgaris* GS pcGS-α1, pcGS-ß1 and pcGS-Y1 cDNAs were cloned into the pEV3 expression plasmid, to give *E.coli* expression vectors pcGS-Eα, pcGS-Eß and pcGS-EY, as follows (see FIG 6.2B). pcGS-α1 was initially restricted with *BglII* and then partially digested with *HindIII*. Among a number of fragments released, a 1300 bp DNA fragment was isolated by preparative gel elution. The pcGS-α1 fragment was cloned into the *BamHI* linearised (and phosphatased) pEV3 via a three way ligation involving an additional *BamHI-HindIII* (pUC19 derived) adaptor DNA fragment. pcGS-ß1 was initially restricted with *BglII* and then partially digested with *BamHI*. Among a number of fragments released, a 1400 bp DNA fragment was isolated by preparative gel elution, and the pcGS-ß1 fragment was cloned into the *BamHI* linearised (and phosphatased) pEV3. An M13 mp18 derived pcGS-Y1 construct was digested with *HincII*, releasing a 1400 bp blunt ended fragment that was gel isolated and ligated into the T4 polymerase *filled in ClaI* site of pEV3. All manipulations should result in the *in frame* fusion of expression vector and plant GS cDNA derived coding sequences (see FIG 6.2B).

2.510 DNA SEQUENCING AND ANALYSIS

The DNA insert of the full length nodule GS cDNA pcGS-Y1 (also known as F14) was sequenced using both the methods of Sanger *et al* (1977) and Maxam and Gilbert (1980). About 80% of the cDNA was sequenced on both strands. Most of the sequence analyses were performed using the Beckman Microgenie Software on an IBM PC computer. Secondary structure predictions used the programmes PREDICT (comprising a suite of ten individual secondary structure prediction programmes compiled at the Department of Biophysics, University of Leeds) and PLOTSTRUCTURE (which presents a graphic representation of the Chou-Fasman prediction and was obtained from Dr B. Foertsch, Max Planck Institute of Munich).

2.6 RNA MANIPULATIONS

2.61 ISOLATION OF TISSUE RNA

Small scale preparations of plant total RNA, for use in RNase mapping experiments (see sections 3.4 and 5.2), were isolated as described by Logemann *et al* (1987) with the single modification that the

RNA (previously extracted with guanidium chloride, ethanol precipitated and resuspended in 500 µl of SDW) was further purified by LiCl precipitation by adding 117.5 µl of 10 M LiCl to the sample, to achieve a final 2M LiCl concentration. Samples were incubated at 4°C overnight and centrifuged in a microfuge for 10 minutes at 4°C. The pellet was further washed with 3M NaAc pH 5.2, as described by Logemann *et al* (1987). Large scale total *P. vulgaris* cv. Bush Blue Lake 274 nodule RNA isolation and subsequent preparation of poly (A) enriched RNA, for use in the construction of the nodule cDNA library (see section 3.2), was performed as described by Lightfoot *et al* (1988). Total RNA was extracted from 15 g of nodules isolated from *P. vulgaris* cv. Bush Blue Lake 274, using the SDS phenol method (Burrell *et al*, 1985). Poly (A) enriched RNA was purified by two cycles of oligo (dT) cellulose chromatography.

2.62 SYNTHESIS AND CLONING OF NODULE cDNA

The following method used to synthesise nodule cDNA was adapted from methodology by Gubler and Hoffman (1983). The first cDNA strand synthesis reaction consisted of 9 µl of nodule poly(A) RNA (3 µg); 3 µl of 10 X first strand buffer (500 mM Tris pH 8.3, 100 mM $MgCl_2$, 100 mM DTT, and 400 mM KCl); 6 µl of 5 X first strand dNTP's (11.6 mM dCTP, dATP and dTTP, with 2.5 mM dGTP); 1 µl of ^{32}P-labelled dGTP; 3 µl of oligo $(dT)_{12-18}$ (1 mg per ml); 2 µl of RNasin (30 units per µl); 2 µl of Na pyrophosphate; and 4 µl of AMV reverse transcriptase (15 units per µl), mixed and incubated at 42°C for 40 minutes. 1 µl was Cerenkov counted and 1 µl 400 mM EDTA added. The sample was extracted in an equal volume of (1:1) phenol:chloroform, microfuged (at 13,000 RPM) for 5 minutes, and the aqueous phase re-extracted twice with an equal volume of chloroform. All organic phases were re-extracted with 30 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and the two aqueous samples pooled. Unincorporated dGTP label was separated from the cDNA first strand species using a 1 ml Sephadex G 50 column, then ethanol precipitated (with 0.1 volume of NH_4 Ac and 2.5 volumes of cold ethanol) on dry ice for 30 minutes. The first strand cDNA sample was pelleted by microfugation for 30 minutes at 4°C, washed with ice cold 75% ethanol, repelleted, vacuum desiccated, and resuspended to a final concentration of 1 µg per 10 µl. The complementary second cDNA strand synthesis reaction mixture consisted of 10 µl of the first strand cDNA sample template; 10 µl of 10 X second strand buffer (200 mM Tris-HCl pH7.5,

50 mM MgCl , 100 mM NH_4 SO_4, 1000 mM KCl and 500 µg per ml BSA); 2 µl of 50 X second strand dNTP's (2 mM dATP, dCTP, dGTP and dTTP); 2 µl of ^{32}P-dGTP; 1 µl of RNase H (30 units per ml); 5.6 µl of DNA polymerase (5 units per µl); and 69 µl of SDW. The reaction was incubated at 12^{o}C for 60 minutes, transferred to 20 C for a further 60 minutes. An 1 µl sample was removed and Cerenkov counted and 2 µl 400 mM EDTA added. The sample was phenol:chloroform, and then chloroform, extracted as previously described, then unincorporated ^{32}P-dGTP removed using a Sephadex G 50 column and the collected double stranded cDNA sample ethanol precipitated. First and second strands were calculated to be synthesised at 30% and 100% efficiencies respectively. Furthermore, analysis of first and second strand products by alkaline agarose electrophoresis and autoradiography (data not shown), identified that both strands had a relatively similar mobility, suggesting that second strand synthesis was efficiently primed by the action of RNase H and not through hairpin loop formation.

Nodule double stranded cDNA was *end filled* with T4 polymerase, methylated with *EcoRI methylase*, ligated to ^{32}P labelled *EcoRI* linkers and digested with *EcoRI* (as described by Maniatis *et al*, 1982). *EcoRI* linkered nodule cDNA was separated from unligated *EcoRI* linkers on a 10 mm X 150 mm Sephacryl S300 column, fractions collected and samples analysed by alkaline agarose electrophoresis and autoradiography (data not shown). Fractions containing cDNA of between 0.5 and 2.0 Kbp in size were used to construct the nodule cDNA library.

Approximately 2 ng of nodule cDNA was ligated with 5 ng of *EcoRI* linearised pUC19, ligated, and transformed into *E.coli* strain TG2, using the high efficiency method of Hanahan (1983). A total of 1600 recombinant clones containing nodule cDNA inserts were identified by lacZ^{-} selection. Clones containing GS homologous sequences were identified by screening the nodule cDNA library with a ^{32}P-labelled pcPvNGS-01 *Pst*1 fragment by colony hybridisation . For further details, see section 3.2.

2.63 RNA BLOTTING AND HYBRIDISATION

Poly (A) RNA samples were separated on a 1.5% formaldehyde agarose gel, which was subsequently soaked for 20 minutes in 20 X SSC and RNA transferred to a PAL Biodyne nylon membrane (Du Pont) as described by Maniatis *et al* (1982) for Southern transfer. Filters were initially

incubated in 10 mls of RNA hybridising solution (50% formamide, 5 X SSPE, 5 X Denhardts, 0.2% SDS and 20 µg poly U), at 42°C for three hours, then reincubated in fresh hybridising buffer containing the single stranded RNA probe, shaking overnight at 42°C. The filter was washed at high stringency (0.1 X SSC and 0.1% SDS) at 65°C, with constant shaking and changes of buffer for thirty minutes, covered in plastic film, and autoradiographed.

2.64 *In vitro* SYNTHESIS OF RNA

The transcription vectors used for the *in vitro* synthesis of the α and γ GS polypeptides (termed pcGS-α1T and pcGS-γ1T) are as described in Bennett *et al* (1989), and for the β GS polypeptide (termed pcGS-β1T) as described by Lightfoot *et al* (1988). The three *in vitro* transcription plasmids containing the three cDNAs were then linearised in the multicloning site at the 3' end of the cDNA and used to produce (m)RNA by *in vitro* transcription (Krieg and Melton, 1987). The amount of (m)RNA produced was quantified by the incorporation of trace amounts of ^{32}P-UTP. The transcripts were then translated in a wheat germ extract system using ^{35}S-methionine (Lightfoot *et al*, 1988).

The transcription vectors used in the synthesis of single-stranded ^{32}P-labelled anti-sense RNA for RNase mapping of total plant RNA were constructed by Dr J.V.Cullimore as described in Bennett *et al* (1989), to synthesise the following probes. The pcGS-γ1P plasmid was either digested with *EcoRI* or *HincII* to synthesise probe-γA (of approximately 450 bases containing 80 bases derived from the vector) or probe-γB (a 239 bases RNA probe containing 25 bases derived from the vector). The pcGS-β1P plasmid was digested with *HaeIII* and used to produce probe-β (of 166 bases, 4 bases of which are derived from the vector). The pLhb-1P plasmid was digested with *EcoRI* and used to synthesise a probe of about 350 bases, 45 bases of which are derived from the vector.

2.65 RNase MAPPING PROCEDURE

Hybridisations, RNase digestions, and analysis of the probe fragment protected from RNase digestion by its complementary mRNA, were performed as described by Krieg and Melton (1987). Note that more than one probe may be used in the same hybridisation. To quantify the amount of the GS mRNA present in the total RNA samples, varying amounts of a known concentration of *in vitro* synthesised (m)RNAs (see section 2.64)

were used in the same way as the plant RNA samples, to construct a calibration curve.

2.66 *In vitro* TRANSLATION AND IMMUNOPRECIPITATION

Nodule poly (A) RNA was translated in a rabbit reticulocyte lysate (New England Nuclear), as described by Pelham and Jackson (1976). Immunoprecipitations were performed as described by Cullimore and Miflin (1984).

2.67 RNA ELECTROPHORESIS

RNA was electrophoretically separated either on alkaline (1.5%) agarose gels (for Northern blotting) or by using 6% denaturing polyacrylamide gels (for RNase mapping), as described by Lightfoot *et al*, (1988).

2.7 PROTEIN MANIPULATIONS

2.71 PREPARATION OF CRUDE PLANT PROTEIN EXTRACTS

Approximately 0.5 g of plant tissue was extracted in a mortar and pestle with 2.5 ml of GS extraction buffer [10 mM Tris-Cl buffer pH 7.5 containing 5 mM glutamic acid (GLU), 10 mM Mg-acetate (MgAc), 10% glycerol, 1 mM DTT, 0.1% ß-mercaptoethanol (ßME), 0.05% TritonX-100 and 0.02% phenylmethylsulphonylfluoride (PMSF)], at 0°C. The homogenate was centrifuged in microfuge tubes for 3 minutes and the supernatant desalted on a 2.5 ml Sephadex G 50 column. The sample was filtered through a 0.2 µm filter and made up to a 2.5 ml total volume with extraction buffer. 0.4 ml of this crude extract was mixed with 0.8 ml ice cold acetone (stored at -20°C, serving as a stable protein sample suspension). 0.1 ml of the extract was retained for total soluble protein determination (as described by Bradford, 1981) and GS transferase activity estimations (see section 2.73), and the remaining 2.0 ml analysed by FPLC (see section 2.74).

2.72 PREPARATION OF CRUDE *E.coli* PROTEIN EXTRACTS

E.coli cI857 containing strains (N5151 and ET8894, see sections 6.3 and 6.4), which expressed the pcGS-Eα, pcGS-Eß and pcGS-Eγ expression plasmids at temperatures of 37°C and above, were inoculated with an overnight culture and grown (in 500 ml of either LB [N5151] or M9

minimal [ET8894] media, with anti-biotic selection, in a 2 l flask, at 250 RPM) initially at 30°C, until achieving an OD_{600} of 0.6. λP_L promoter expression was achieved by transferring the exponential phase culture to a 42°C incubation temperature (shaking at 250 RPM) for normally a two hour period. Bacterial cells were harvested by centrifugation at 3,000 RPM for 10 minutes, and the cell pellets were rapidly frozen in liquid nitrogen and stored at -20°C. The pellet (from approximately 25 ml of the original culture) was resuspended in 2.5 ml of GS extraction buffer (section 2.71), and the bacterial cells lysed using three cycles of French pressing (10,000 lbs inch^{-2}). Total protein samples were mixed with an equal volume of SDS sample loading buffer (section 2.76), and either boiled and used immediately, or frozen at -20°C for storage. Soluble and insoluble fractions were obtained by centrifuging the cell homogenate in microfuge tubes for 10 minutes. The insoluble sample pellet was washed and repelleted twice, with GS extraction buffer, to remove any traces of the soluble supernatant, and subsequently boiled in 100 µl of SDS sample loading buffer (section 2.76) and used immediately, or frozen at -20°C for storage. The soluble sample was further prepared, for FPLC analysis, as described in section 2.71.

2.73 FPLC CHROMATOGRAPHY

A Gilson (model 302) FPLC system was employed, controlled by an Apple computer using the Gilson HPLC gradient manager V1.2 package. A TSK DEAE-5PW analytical ion exchange column (7.5 X 75 mm, Beckman and LKB) and a TSK G4000 SW analytical gel filtration column (7.5 X 600mm, LKB) were used throughout. Chromatography was performed essentially as described by Chen and Cullimore (1988), except running buffers consisted of the previously described GS extraction solution. All buffers had been passed through a 0.45 µm filter and degassed, prior to use. Molecular weight (M_r) marker proteins, thyroglobulin (670,000), ß-galactosidase (540,000), ferritin (443,000), catalase (232,000), alcohol dehydrogenase (160,000) and haemoglobin (60,000), used in the calibration of the FPLC gel filtration column, were all assayed essentially as described by Cullimore (1980).

2.74 GLUTAMINE SYNTHETASE ACTIVITY MEASUREMENTS

Sample extracts were assayed for GS transferase (GSt) and semi-biosynthetic (GSs) activities as described by Cullimore and Sims (1980) and Cullimore (1980) respectively.

2.75 PROTEIN ELECTROPHORESIS AND STAINING PROCEDURES

Proteins were separated on one dimensional and two dimensional (isoelectrofocusing in the first dimension) 12.5% SDS polyacrylamide gels essentially as described by Laemmli (1970) and Roscoe and Ellis (1982) respectively. Proteins were fixed and stained within the polyacrylamide matrix using Coomassie stain buffer (50% methanol, 7% acetic acid and 0.025% Coomassie blue) for 60 minutes with constant shaking, and destained with multiple changes of destain buffer (40% methanol and 7% acetic acid). Stainable SDS-PAGE M_r markers (BIORAD) contained myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). Gels were dried by vacuum desiccation at 80°C onto Whatman 3MM paper.

2.76 WESTERN BLOTTING

The protein electroblotting method, termed Western blotting, was modified from Burnette (1981) for the transfer of proteins separated by SDS polyacrylamide gels to a nitrocellulose filter. The gel was sectioned for Coomassie blue staining of molecular weight markers and Western blotting of the remainder of the gel. The latter was equilibrated in Western transfer buffer (25 mM Trizma Base, 192 mM glycine, 20% methanol) for 2 minutes, then overlaid with a prewetted nitrocellulose filter and placed in a Biorad electroblotting apparatus. The proteins were transferred for 3 hours at 50 volts (approximately 0.3 amps) under constant voltage conditions. The apparatus was dismantled and the nitrocellulose filter containing the transferred proteins was placed in PBS (10 mM $NaPO_4$ buffer pH 7.5, 130 mM NaCl).

2.77 COLONY IMMUNODETECTION

E.coli expression vector clones were screened for recombinant GS Y polypeptide expression (see section 6.2) essentially as described by Crowl et al (1985), and using the staining procedure described below.

2.78 IMMUNOSTAINING PROCEDURE

The nitrocellulose filter with attached antigen had non-specific protein binding sites blocked with 2% Marvel .. in PBS for 30 minutes. The blocked filter was sealed in a plastic bag with 10 ml PBS containing the anti-GSN1 antibody (Cullimore and Miflin, 1984) using 90 µl of 0.2 mg lyophilised serum per ml PBS buffer. The filter was shaken overnight at 200 RPM at 30 C, then removed from the plastic bag and washed with frequent changes of PBS containing 0.1% TWEEN 20, with constant shaking for 30 minutes. The filter was resealed in a new plastic bag containing 5 mls of PBS and 16.5 µl of biotinylated protein A, shaken for 60 minutes at 200 RPM, 30 C and removed and washed as previously described. The filter was reincubated as previously described with 5 mls PBS and 16.5 µl streptavidin peroxidase, but for only 30 minutes. The filter was washed with several changes of PBS + 0.1% TWEEN and then with PBS alone over a 30 minute period. The filter was developed by incubating in an immunostaining solution (3% NaCl, 20 mM Tris pH 7.5 and 0.2% H_2O_2 in 50 mls SDW mixed with 30 mg 4-chloro-1-naphthol dissolved in 10 mls of methanol). Once the desired band intensity was achieved, the reaction was stopped by washing in tap water repeatedly over a 30 minute period. The blot was dried overnight on Whatman 3MM paper, storing in darkness, before photographing.

2.79 NITROGEN GAS DETERMINATION

Contaminating nitrogen gas (see section 5.3) was determined by gas chromatography using a Pye series 104 oven fitted with a Kathometer detector. 0.5 ml of gaseous sample was injected into a 6 ft column packed wth molecular sieve (60-80 mesh) heated to 50 C with a Helium carrier gas eluting at a rate of 30 ml/min.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF A FULL LENGTH NODULE GS cDNA

3.1 INTRODUCTION

GS activity in higher plants has been separated using ion exchange chromotography (IEC), into a number of isoforms, that differ in their subcellular localisations and organ distributions (see section 1.5 for a full description). Studies on leaf GS (Mann *et al* 1980) have identified a cytosolic isoform, termed GS1, and a plastid located isoform, GS2, whereas roots were found to contain a single cytosolic GS1 like isoform, termed GSR. Interestingly, two cytosolic isoforms have been identified in root nodules of *P. vulgaris* (Cullimore *et al*, 1983), a novel nodule isoform termed GSN1 and a root like isoform, GSN2.

Both polypeptide analysis and recombinant cloning techniques were used to establish the molecular basis for the observed GS isoform heterogeneity. Two dimensional gel electrophoretic examination of GS subunits purified from a variety of *P. vulgaris* organs identified three cytosolic polypeptides α, ß and ϒ of M_r 41,000 and four plastidic subunits termed a,b,c and d, of M_r 45,000, which were expressed in plant organs in a differential manner (Lara *et al*, 1984). α was expressed in roots and leaves, ß was present in nodules, roots and leaves, whereas ϒ appeared only in the nodule, and plastidic (a,b,c and d) subunits were present in leaf. In parallel to the above study, GS encoding DNA sequences were characterised. Initially, a partial GS cDNA termed pcPvNGS-01, was isolated from a nodule cDNA library (Cullimore *et al*, 1984). Subsequently three different full length GS cDNA clones, termed pR-1 and pR-2 (Gebhardt *et al*, 1986) and pcGS-δ1 (Lightfoot *et al*, 1988) were isolated from root and leaf cDNA libraries respectively. In addition, two genomic clones homologous to the pcPvNGS-01 and pR-1 cDNAs have been isolated (B.G.Forde *et al*, submitted), the former appearing to contain a fifth linked GS gene, termed *glnε* (B.G.Forde, pers. commun.). The isolation and characterisation of distinctive GS clones, together with genomic blotting studies (Gebhardt *et al*, 1986; Lightfoot *et al*, 1988), have identified a five member GS multigene family in *P. vulgaris*.

A number of studies have been performed to define the relationship between the GS polypeptides and their encoding gene/cDNAs. Lightfoot *et al* (1988) have identified that the pcGS-δ1 cDNA encoded the plastidic subunits, termed δ (see section 1.5 for a description). Gebhardt *et al* (1986) have identified, using an S1 mapping procedure, the differential

tissue distribution of GS mRNAs homologous to the three cytosolic GS cDNAs pR-2, pR-1 and pcPvNGS-01. The pR-2 homologous mRNA was detected in roots and leaves, and pR-1 homologous mRNA was found to be expressed in roots, leaves and nodules, whereas pcPvNGS-01 mRNA was expressed only in nodules. Gebhardt *et al* (1986) have postulated by correlating the differential expression of the three GS mRNAs with the selective tissue distribution of the three cytosolic GS polypeptides (as observed by Lara *et al*, 1984), that the pR-2, pR-1 and pcPvNGS-01 GS cDNAs encoded the α, ß and ϒ subunits respectively. This suggestion however, remains to be confirmed directly.

The following chapter describes the isolation of a full length (pcPvNGS-01 homologous) nodule GS cDNA, the identification of its own and other cytosolic GS cDNAs encoded subunit, the comparison of its sequence with other *P. vulgaris*, plant, animal and bacterial GS sequences and the characterisation of its homologous mRNAs novel differential organ expression pattern.

3.2 ISOLATION AND CHARACTERISATION OF GS cDNA CLONES FROM A NODULE cDNA LIBRARY

Total RNA was extracted from four-week old root nodules of *P. vulgaris* cv. Bush Blue Lake, and poly(A) RNA then isolated by oligo(dT) cellulose chromatography. The nodule poly(A) RNA was translated in a rabbit reticulocyte lysate system. A sample of the translation products was immunoprecipitated with an anti-GS antiserum (Cullimore and Miflin, 1984). Both total and immunoprecipitated nodule radiolabelled products were separated by one dimensional sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS PAGE) (FIG 3.2A). Examination of the total translation products identified a major 30Kd translation product, as previously observed by Campos *et al* (1987). Two polypetides immunoprecipitated with the anti-GS antiserum and these corresponded in size to the ϒ and ß GS subunits (Lara *et al*, 1984), the latter being at a greater relative abundance. Therefore, the nodule poly(A) RNA appeared to contain translatable full length mRNA encoding both the ϒ and ß GS subunits and thus provided a suitable template for cDNA synthesis and cloning of these two GS mRNAs.

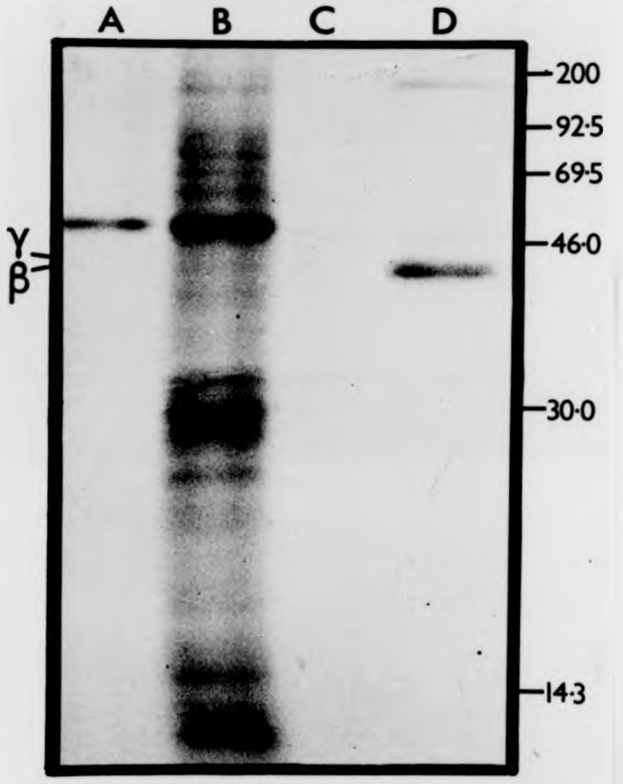


FIG 3.2A Total nodule and immunoprecipitated nodule GS translation products separated by one-dimensional SDS-PAGE. Rabbit reticulocyte lysate *in vitro* translations, containing ^{35}S-methionine, were performed in the absence (sample A) or presence (samples B-D) of 0.5 µg of nodule poly (A) RNA. Samples C and D were immunoprecipitated either using pre-immune or anti-GS antisera respectively (as described by Cullimore and Miflin, 1984). Molecular weight markers are ^{14}C labelled phosphorylase b (M_r 92,500), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), lysozyme (M_r 14,300).

Double stranded cDNA was synthesised according to the method of Gubler and Hoffman (1983), blunt end filled in and ligated to EcoR1 linkers. Double stranded cDNA of 0.5-2.0 Kbp (Kilobase pairs) in size, was cloned into the EcoR1 site of pUC19 and transformed into the *E.coli* strain TG2. 1600 colonies were screened by colony hybridisation using the nick translated cDNA insert of pcPvNGS-01. The filters were initially washed at a low stringency, to identify all GS cDNA clones present. Subsequently, using a higher stringency washing procedure, cDNA clones more homologous to pcPvNGS-01 were identified (FIG 3.2B) (for further details see Materials and methods). Three putative GS positives termed F23, F27 and F14 were identified which hybridised at both stringencies. These clones were further characterised by restriction mapping (FIG 3.2C). Restriction digestion of the F14, F23 and F27 clones with *PvuII* (which cleaves 0.15 and 0.25 Kbp either side of the pUC19 vectors polylinker insertion sequence) released single inserts of 1.9, 1.1 and 0.8 Kbp (see FIG 3.2C), and thus represented cDNA inserts of approximately 1.5, 0.7 and 0.4 Kbp respectively. Further restriction mapping using *PvuII* (to release the cDNA inserts) and *NdeI* or *AsuII*, identified that F14 contained an *NdeI* and *AsuII* restriction site, F27 appeared to contain neither restriction site, and F23 possessed only an *AsuII* restriction site. A comparison of the three nodule cDNAs restriction maps with the four previously isolated GS cDNAs (see FIG 3.2D), concluded that the F14 and F27 cDNAs were most similar to the partial nodule GS cDNA pcPvNGS-01 (Cullimore *et al*,,1984), since all three shared an unique *AsuII* restriction site, and F14 and pcPvNGS-01 both contained a similarly positioned *NdeI* site. In contrast, the lack of *AsuII* and *NdeI* sites in the F23 cDNA was most similar to the restrictionless 3' pR-1 sequence (Gebhardt *et al*, 1986). Further restriction analysis of the 1.5 Kbp F14 cDNA insert (FIG 3.2C), resulted in an approximate restriction map for the above sites and also *HincII*, *BamHI* and *EcoRI* (FIG 3.2D), which was later confirmed by DNA sequence analysis (see FIG 3.5C). On the basis of these observations, it was decided to work exclusively on the 1.5 Kbp F14 cDNA clone.

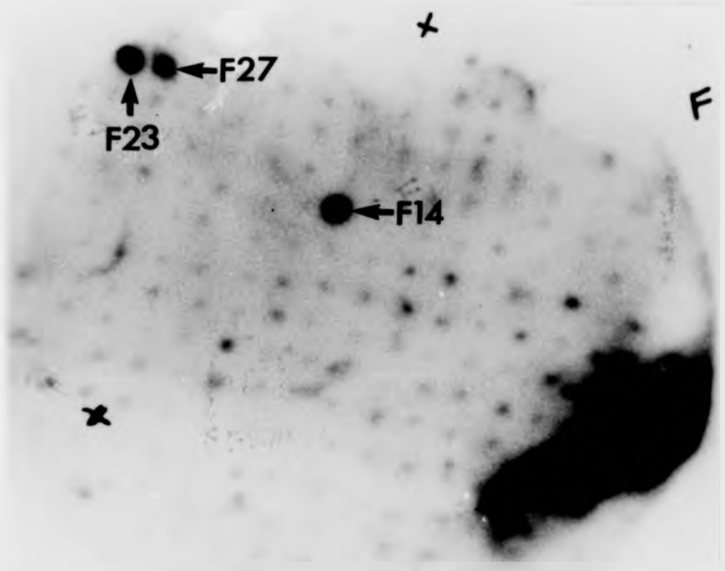


FIG 3.2B GS probe colony hybridisation with nodule cDNA library filter F. Colony filters A to F were hybridised with the radiolabelled pcPvNGS-01 probe, washed at high stringency (0.1 X SSC and 0.1% SDS, 65^{o}C), autoradiographed, and three positive hybridising colonies identified from colony filter F (F14, F23, F27).

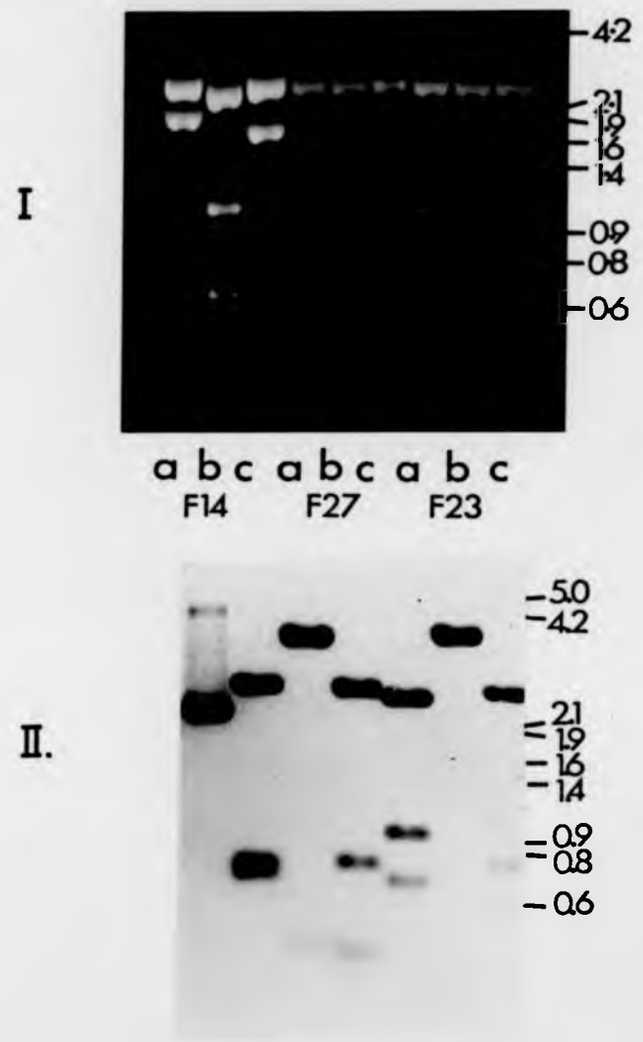


FIG 3.2C Restriction map analysis of plasmid DNA from the putative nodule GS clones F14, F23 and F27. I. F14, F23 and F27 DNA was restricted with *PvuII* (a), *PvuII* and *NdeI* (b) and *PvuII* and *AsuII* (c). II. F14 plasmid DNA was either uncut (a) or restricted with *EcoRI* (b), *BamHI* (c), *EcoRI* and *BamHI* (d), *NdeI* (e), *HincII* (f), *HincII* and *EcoRI* (g). Restricted fragments were separated on a 1% agarose gel and visualised with ethidium bromide staining (FIG's I and II represent positive and negative photographic reproductions). Molecular weight markers are *EcoRI* and *HindIII* restricted λ DNA.

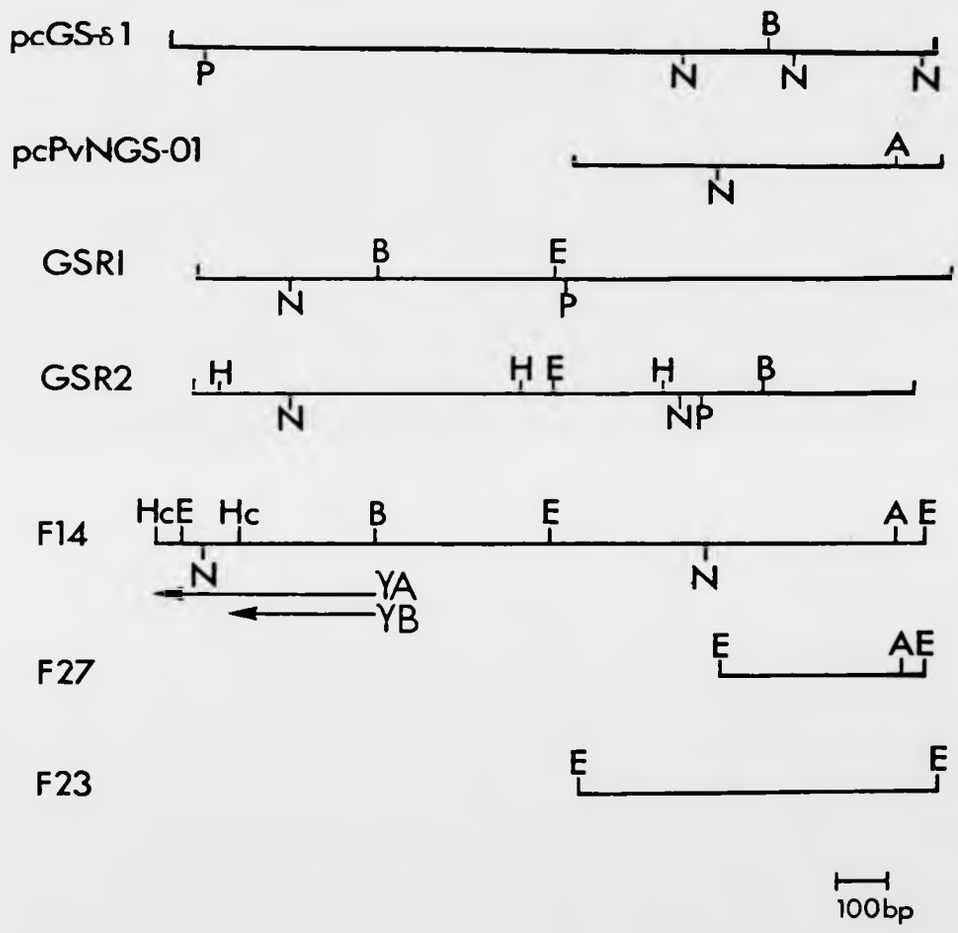


FIG 3.2D Restriction maps of putative nodule GS clones F14, F23 and F27, with respect to the *P. vulgaris* GS cDNA's pcGS-δ1 (Lightfoot *et al*, 1988), pcPvNGS-01 (Cullimore *et al*, 1984), GSR1 and GSR2 (Gebhardt *et al*, 1986). E *EcoRI*; A, *AsuII*; H, *HindIII*; B, *BamHI*; P, *PstI*; N, *NdeI*; and Hc, *HincII*. Arrows marked beneath the F14 cDNA represent the DNA regions used to synthesise the anti-sense RNA probes YA and YB (see section 3.4 for further details).

3.3 IDENTIFICATION OF THE SUBUNITS ENCODED BY THREE *P. vulgaris* GS cDNAs

Three GS cDNAs, pR-1, pR-2 (Gebhardt *et al*, 1986) and the F14 nodule clone were inserted into the pGEM-3Z transcription vector (performed by Dr J.V.Cullimore, Dr D.A.Lightfoot and Dr M.P.Robbins; see materials and methods, section 2.64 for a description). Each of the constructs were transcribed and the RNA product translated in a wheat germ extract system to produce ^{35}S-methionine labelled polypeptide products (performed by Dr D.A.Lightfoot).

α plus ß, and ϒ plus ß GS subunits were extracted from plumule and nodule tissues respectively and mixed together to provide a source of all three native cytosolic GS subunits.

The extracts containing the native GS subunits were mixed with each of the three radiolabelled *in vitro* synthesised GS polypeptides and then separated by two dimensional electrophoresis. The gels were western blotted onto nitrocellulose, and the native GS subunits visualised with the anti-GS antiserum using Biotin-streptavidin peroxidase colour labelling. The radiolabelled *in vitro* synthesised polypeptides were then identified by autoradiography. The result (FIG 3.3) identified that the *in vitro* transcribed and translated GS cDNA synthesised a single polypeptide of approximately M_r 40,000, and that the pR-2 polypeptide comigrated exactly with the α subunit, the pR-1 polypeptide with the ß subunit and the F14 polypeptide with the ϒ subunit. Therefore, firstly, each cDNA must encode a full length GS cDNA containing a complete open reading frame (ORF), and secondly, the pR-1, pR-2 and F14 GS cDNA clones encode the α, ß and ϒ subunits respectively. The three cDNAs, pR-2, pR-1 and F14 were thus renamed appropriately pcGS-α1, pcGS-ß1 and pcGS-ϒ1 and their encoding genes termed *gln-α*, *gln-ß* and *gln-ϒ*, and referred to as such throughout the remaining text.

3.4 THE EXPRESSION OF THE *gln-ϒ* GENE IN *P. vulgaris* NODULES AND OTHER ORGANS

To determine the organ expression and transcript size of the pcGS-ϒ1 homologous mRNA, a pcGS-ϒ1 ^{32}P labelled single stranded anti-sense RNA probe, termed probe-ϒA (see FIG 3.2D for a description),

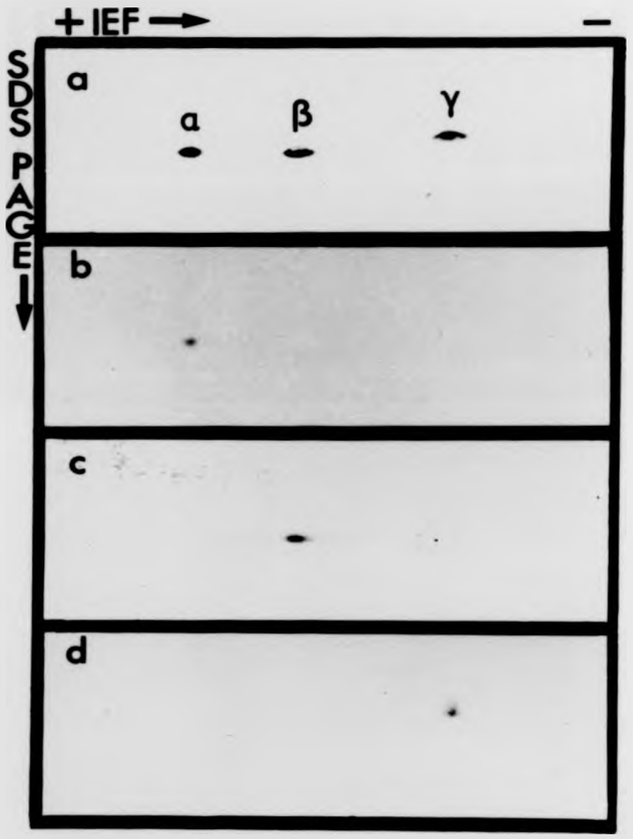


FIG 3.3 Analysis of the polypeptides encoded by the cytosolic GS cDNA clones F14, pR-1 and pR-2. a) Authentic cytosolic GS polypeptides of *P. vulgaris*, α, ß and ϒ, from crude plant extracts were separated by two dimensional gel electrophoresis and detected by western blotting using peroxidase linked anti-GS antiserum. Labelled GS polypeptides synthesised *in vitro* from the cDNA clones (b) pR-2 (renamed pcGS-α1), (c) pR-1 (renamed pcGS-ß1), and (d) F14 (renamed pcGS-ϒ1) were run on similar gels and detected on the blots by fluorography.

was hybridised to poly(A) RNA from leaves, roots and nodules of *P. vulgaris* by northern analysis (FIG3.4A). Probe-γA hybridised to a mRNA species corresponding to 1,500 bases in size, as originally observed by Cullimore *et al* (1984) with their closely related pcPvNGS-01 cDNA. However, the probe-γA hybridised preferentially to root rather than nodule poly(A) RNA, whereas pcPvNGS-01 had previously hybridised to nodule RNA. The northern blot was reprobed with a *P. vulgaris* leghaemoglobin (*Lhb*) RNA probe, whose encoding cDNA was isolated from the same nodule cDNA library screened for pcGS-γ1, but identified by its hybridisation to a *P. sativum Lhb* probe (J.V.Cullimore in Bennett *et al*, submitted). This probe hybridised exclusively with nodule mRNA, thus confirming that the pcGS-γ1 probe-γA was hybridising most strongly with root and not nodule mRNA (FIG 3.4A). Explanations for the apparent difference in behaviour between the closely related pcGS-γ1 (FIG 3.4A) and pcPvNGS-01 (Cullimore *et al*, 1984) cDNAs could include that they encode distinct transcripts yet retain similar restriction patterns. Alternatively, it could represent differences in the choice of the radiolabelled probe, such as the above RNA probe-γA encoding a 5' coding region highly conserved between other GS gene members (and hence able to crosshybridise with these mRNAs), whereas the nick translated pcPvNGS-01 DNA probe used by Cullimore *et al* (1984) encoded a divergent 3' untranslated segment.

To resolve this apparent discrepancy, the RNase mapping technique was employed to provide a means of assaying the abundance of a specific mRNA species as this technique is both quantitative and can discriminate from other closely related mRNA sequences. The technique involved the synthesis of an anti-sense RNA probe in an *in vitro* transcription system. When hybridised to an RNA sample, any unprotected or mismatched RNA probe (resolving single bp mismatches) is digested with RNase A and RNase T1. The probe-γA has been shown to be specific for γ mRNA and not to hybridise to *in vitro* synthesised α, β and δ mRNAs in this assay (J.V.Cullimore in Bennett *et al*, submitted).

Initially the pcGS-γ1 probe-γA was used to screen nodule, root, leaf and stem poly(A) RNA for *gln*-γ gene expression. Probe-γA was approximately 450 bases long (see FIG 3.4B, track P), unprotected by tRNA, root or leaf poly(A) RNA (tracks t, R and L), and gave a 300 bases protected fragment with nodule poly(A) RNA (track N). Surprisingly, a protected fragment was also detectable with stem poly(A) mRNA. To

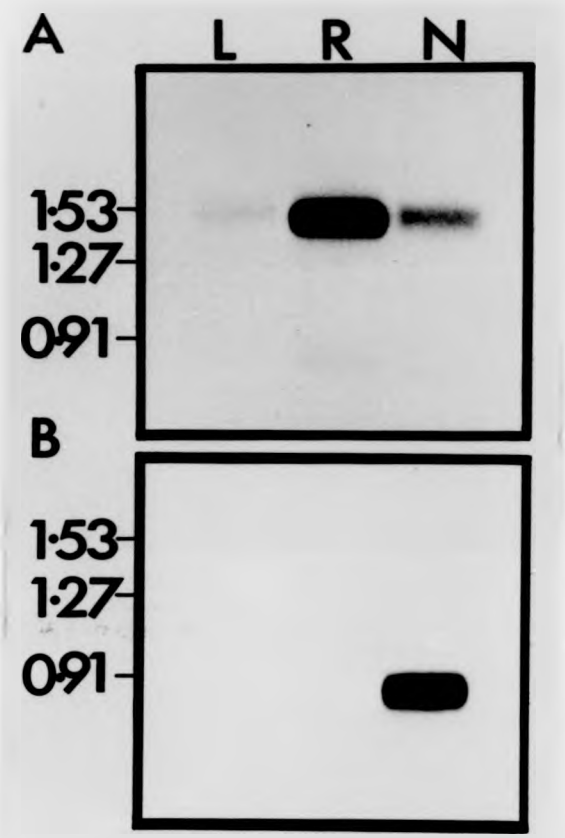


FIG 3.4A Northern blot hybridisation of leaf (L), root (R) and nodule (N) poly (A) RNA with single stranded RNA (A) pcGS-Y1 probe-YA and (B) leghaemoglobin probe-*Lhb*. The filter was washed at high stringency (0.1 X SSC and 0.1% SDS, 65^{o}C) and autoradiographed. Radiolabelled single stranded RNA markers were prepared from a pcGS-δ1 template, as described by Lightfoot *et al* (1988), giving single stranded RNA molecules of the marked size (Kb).

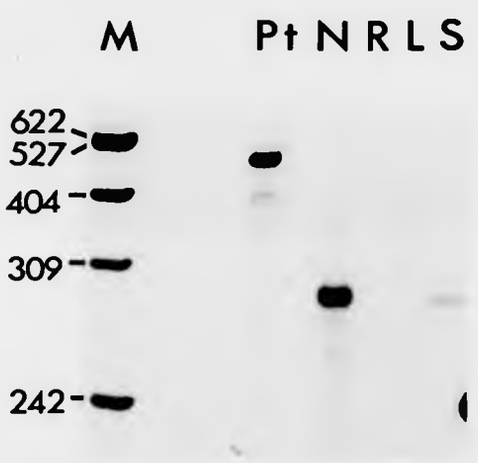


FIG 3.4B Detection of pcGS-Y1 related mRNAs in different organs of *P. vulgaris*, assayed by an RNase protection method. Track (P) shows some of the undigested probe-YA (450 bases). 10 µg of yeast tRNA (t) and 1 µg samples of poly (A) RNA isolated from nodules (N), roots (R), leaves (L) and stems (S) were assayed with the probe-YA by RNase protection. Probe protected by the homologous *gln-Y* mRNA was then detected by gel electrophoresis and autoradiography (approximately 300 bases). Molecular weight markers (M) are end labelled HpaII cleaved pBR322.

pursue this novel observation further, RNA from a number of *P. vulgaris* organs were screened by RNase mapping. In view of the large difference between probe and protected probe fragment sizes obtained for probe-γA (for reasons discussed in section 3.5), a shorter ^{32}P labelled anti-sense RNA probe was used, termed probe-γB (see FIG 3.2D for a description) to study further the *gln*-γ organ expression. As a comparison, a *P. vulgaris Lhb* RNA probe was used to detect a *Lhb* mRNA, in a similar manner. Both probes were used in the same hybridisation for most of the samples, except in nodules due to the strong signal achieved with the *Lhb* probe. It was observed (FIG 3.4C) that *gln*-γ mRNA was detected at high levels in nodules, at about a 10- fold lower level in green cotyledons, and a 50- fold lower level in stems and petioles. No *gln*-γ mRNA could be detected in roots, leaves, embryonic axes or white cotyledons. In contrast, *Lhb* mRNA was only detectable in nodules, estimated to be at an approximately 100- fold higher levels than *gln*-γ mRNA. The production of a triplet of protected fragments by the *Lhb* probe could be interpreted either as the partial digestion of the probe-mRNA hybrid or hybridisation to mRNA from 3 closely related *Lhb* genes.

To investigate whether the observed *gln*-γ mRNA was translated in the identified organs, plant tissue extracts from nodule, stem, petiole, green cotyledon, and root were separated by one dimensional SDS-PAGE and western blotted. Two-dimensional analysis of the cytosolic GS polypeptides (FIG 3.3) has previously shown that the γ polypeptide has a slower mobility than the α and β polypeptides on the second (SDS-PAGE) dimension. Immunodetection using peroxidase linked anti-GS antibodies identified a GS polypeptide of slower mobility in nodule, stem, petiole, green cotyledon but not root extracts, probably representing the γ polypeptide (FIG 3.4D). In stems and petioles the α or β GS subunits were the major cytosolic GS polypeptides and the higher M_r δ plastid GS polypeptide was also present. In contrast γ was a major cytosolic GS subunit in both green cotyledons and nodules. Thus the relative *gln*-γ mRNA levels were reflected in the abundance of the γ polypeptide in the respective plant organs.

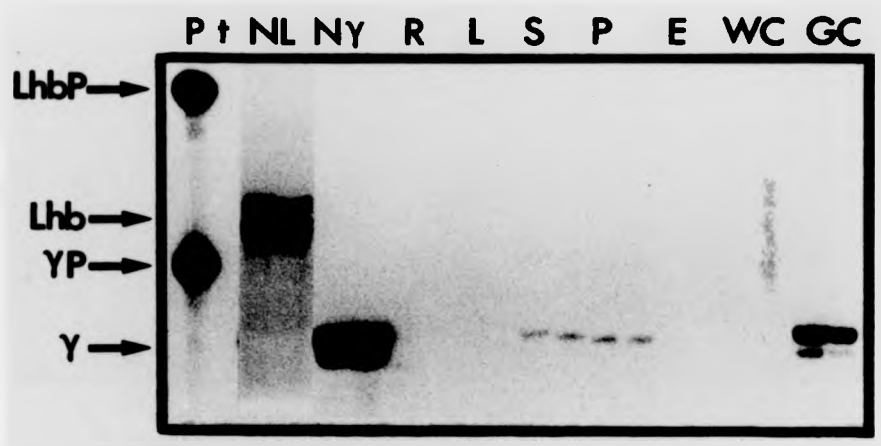


FIG 3.4C Abundance of pcGS-Y1 and pLhb-1 related mRNAs in different organs of *P. vulgaris*, assayed by an RNase protection method. Track (P) shows some of the undigested probes. 10 µg of yeast tRNA (t) and 10 µg duplicate samples of total RNA isolated from roots (R), leaves (L), stems (S), petioles (P), embryonic axes (E), white cotyledons (WC) and green cotyledons (GC) were hybridised using both probes in the same hybridisation. 1 µg and 4 µg samples of nodule RNA were hybridised using the probe-Lhb (NL) and pcGS-Y1 probe-YB (NY) respectively in separate hybridisations. Probes protected by their homologous mRNA were then detected following electrophoresis and autoradiography. Note that the NL tracks were autoradiographed for about 50-fold shorter time compared to the other tracks. The positions of the undigested probes (LhbP and YP) and the expected positions of the probes protected by their homologous mRNAs (*Lhb* and Y) are marked.

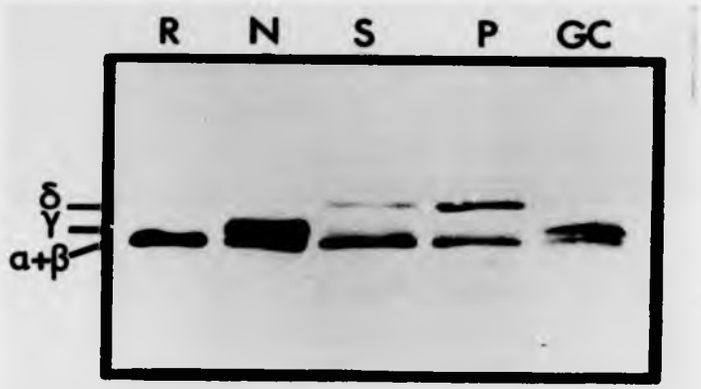


FIG 3.4D Western blot of proteins extracted from roots (R), nodules (N), stems (S), petioles (P) and green cotyledons (GC), using peroxidase linked anti-GS antiserum. The position of the individual GS polypeptides are marked.

3.5 SEQUENCE ANALYSIS OF THE pcGS-Y1 cDNA

The *P. vulgaris* pcGS-Y1 GS cDNA clone was DNA sequenced by Sequencing Systems Ltd (Cambridge) using Maxam and Gilbert methodology (as described by Barker *et al*, 1983).

The complete pcGS-Y1 cDNA insert contained a total of 1521 bp and appeared totally homologous at its 3' end to the previously isolated partial GS cDNA pcPvNGS-01 (Cullimore *et al*, 1984). In addition, sequence homology was identified between the pcGS-Y1 and *gln*-Y genomic clone 5' untranslated and N terminal ORF regions (FIG 3.5A). However, the sequences did not retain any further homology upstream of the nucleotide -68 position (relative to the ATG codon), which represented the major transcription start site of the *gln*-Y gene (B.G.Forde, pers. commun.), and thus pcGS-Y1 contained a 73bp non-homologous 5' extension (termed D73; see FIG 3.5B). D73 appeared unrelated to the GS mRNA as it showed little nucleotide sequence homology to any upstream region of the *gln*-Y gene within 1,000 bp of the divergence position (B.G.Forde, pers. commun.). To determine whether D73 was contiguous to native Y mRNA, an RNase mapping experiment, involving a probe sequence that was anti-sense to the coding sequence of pcGS-Y1 (termed probe-YA, of approximately 450 bases, 80 bases derived from the vector, in addition to the D73 region), was hybridised to nodule (as well as root, leaf and stem) poly(A) RNA. The probe across this divergent upstream region was not protected by nodule poly(A) RNA resulting in a 300 bases, instead of a 370 bases, protected fragment (FIG 3.4B). The pcGS-Y1 GS cDNA is therefore considered to start after D73, and has been numbered accordingly (FIG 3.5C).

The following structural features were identified from the *gln*-Y mRNA derived pcGS-Y1 sequence (FIG 3.5C). The first ATG, which is immediately followed by a single large ORF, is preceded by the sequence GAAG, thus providing a purine at the -3 position but not pyrimidines at the -2 and -1 positions, disagreeing with the model for translation initiation sequences proposed by Kozak (1984). Interestingly, the purine chosen was an adenine, as favoured in all plant GS sequences available (FIG 3.5D). Comparisons of the pcGS-Y1 and other higher plant GS gene translation initiation environments to proposed consensus sequences (FIG 3.5D) gave a good correlation to a motif identified by

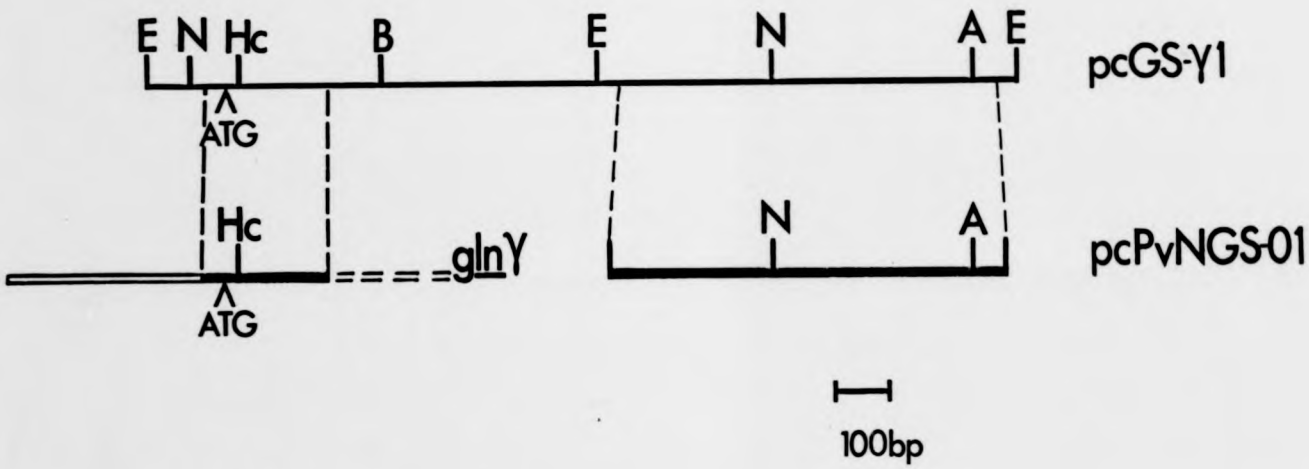


FIG 3.5A Graphic representation of pcGS-γ1 homology to the partial nodule GS cDNA pcPvNGS-01 and the genomic *gln-γ* sequences. ATG represents the initiating methionine residue; open and shaded lines in pcPvNGS-01 and *gln-γ* represent non-homologous and homologous DNA sequences respectively to pcGS-γ1. The dashed line represents non-coding *gln-γ* intron sequence. E represents an *EcoRI* restriction site; N, *NdeI*; Hc, *HincII*; B, BamHI; and A, *AsuII*.

```
                              120-                100-
pcGS-γ1 ATATAAAAAAAATATTTTCATATTTTATATAGAGATGTAAAAAAACATATGGAGGTGCAAG
gln-γ   GCAGTATTTGTTTTGGAAACTGAGAGATAGCATAAACACTATAAAACCCACTGCAACAAC
        80-                 60-                 40-
pcGS-γ1 AATAAACTCTCATATTGAAAGGAAGAGAAAGAGAAAATTTCTCTGTGAAGAGTCTCTGCT
gln-γ   CTTGTATCAACGC----------------------------------------------

        20-                 1+
pcGS-γ1 GAAAGTTTTGGTTTCTTGAAGATGTCATCAATCTCCGATCTTGTTAACCTTAACCTCTCT
gln-γ   ------------------------------------------------------------
```

FIG 3.5B Nucleotide sequence divergence between the 5' pcGS-γ1 and *gln*-γ DNA sequences (B.G.Forde, pers. commun.). Numbering is relative to the A start codon nucleotide of the GS γ polypeptide open reading frame. ---- represents identical residues.

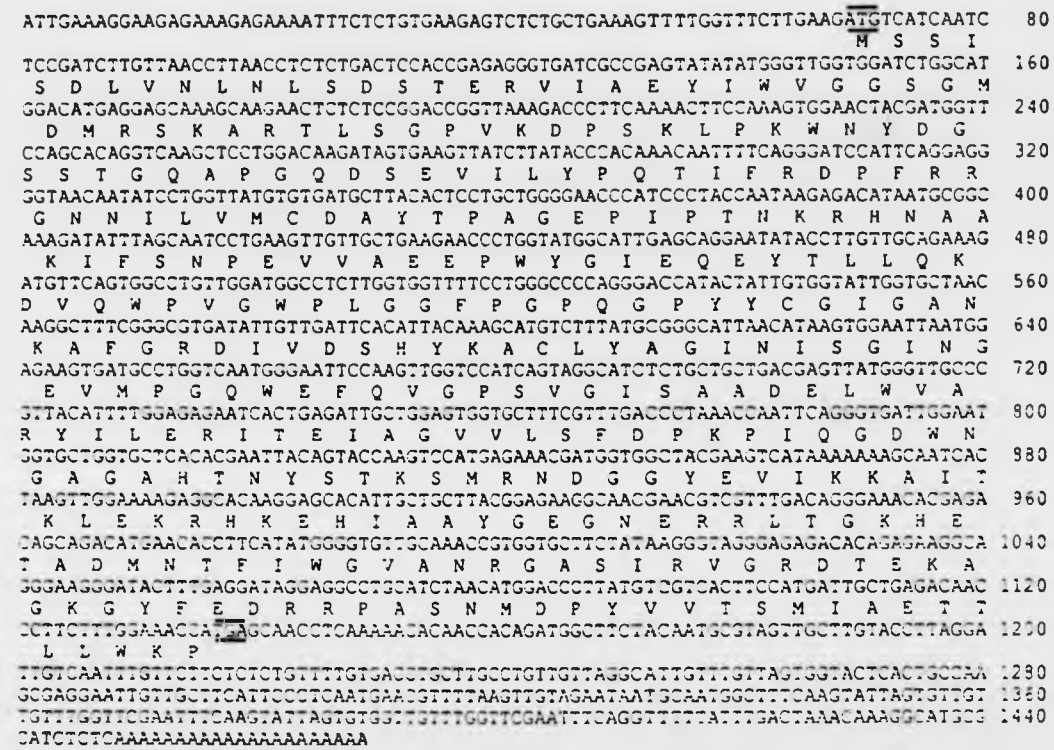


FIG 3.5C DNA sequence and deduced amino acid sequence of the nodule GS cDNA clone, pcGS-Y1. (NB. The divergent 5' D73 sequence [see FIG 3.5B] has not been included)

FIG 3.5D Sequence analysis of the initiation codon environment and termination codon of the pcGS-γ1 and other plant GS sequences, in relation to eukaryotic (Kozak, 1984) or plant consensus sequences (Joshi, 1987; Heidecker and Messing, 1986). GS sequences include pcGS-γ1 (FIG 3.5C), pcGS-α1, pcGS-β1 (Gebhardt *et al*, 1986) and pcGS-δ1 (Lightfoot *et al*, 1988) from *P. vulgaris*; pGS185 (Tingey *et al*, 1988), pGS341 (Tingey *et al*, 1987) and pGS299 (Tingey *et al*, 1988) from *P. sativum*; pGSA19 from *Medicago sativa* (Tischer *et al*, 1986); pGS1/15 from *Nicotiana plumbaginifolia (Tingey et al*, 1987a); and pBarlGS2 from *Hordeum vulgare* (J.Freeman, A.Marquez and B.G.Forde, pers.commun.).

pcGS-γ1	G A A G A T G T C A	-----354-----	T G A
pcGS-β1	C A C C A T G T C G	-----354-----	T G A
pcGS-α1	T A C C A T G T C T	-----354-----	T G A
pcGS-δ1	G A A C A T G G C A	-----427-----	T A A
pGS185	G A A C A T G G C G	-----427-----	T A A
pGS299	A A C C A T G T C T	-----354-----	T A A
pGS341	C A T T A T G T C T	-----354-----	T G A
pGS1/15	T A T T A T G T C T	-----354-----	T G A
pGSA19	A A A C A T G T C T	-----354-----	T A A
BarlGS2	----------T G G C G	-----428-----	T G A

INITIATION CONSENSUS SEQUENCES

KOZAK	A A C C A T G
JOSHI	A A C A A T G G C C T
H + M	N A N N A T G G G C T

Heidecker and Messing (1986), but proved different to other reported eukaryotic (Kozak, 1984) and plant (Joshi, 1987) consensus sequences.

The pcGS-γ1 ORF of 356 codons translated to give a polypeptide of M_r 39.3 Kd, which compares to M_rs of the pcGS-α1 and pcGS-β1 encoded polypeptides of 39.2 Kd and 39.1 Kd respectively (Gebhardt *et al*, 1986). pcGS-γ1 terminated with a TGA stop codon, as generally favoured by plant cytosolic GS sequences (FIG 3.5D).

When the pcGS-γ1 5' and 3' untranslated regions (of 68 and 309 bp respectively) were compared to the corresponding sequences of a number of plant GS cDNAs, other than exhibiting a general AT rich character, as observed for a large number of plant genes by Joshi (1987), there was little identity retained (approximately less than 50%) between the closely related GS cDNAs (data not shown). In contrast, the pcGS-γ1 and pcPvNGS-01 3' sequences were identical, and therefore both clones were probably derived from the same mRNA species. However, in comparison to pcPvNGS-01, pcGS-γ1 contained an additional 23 bp prior to the poly(A) tail, and therefore there are probably different sites of polyadenylation of this mRNA, as shown for other plant mRNAs (Dean *et al*, 1986).

3.6 COMPARISONS OF GS SEQUENCES

A number of GS sequences, initially taken from higher plants and later animal and bacterial sources, were compared using a variety of parameters as discussed below.

The pcGS-γ1 coding region and similar segments of all four *P. vulgaris* GS cDNAs were examined for codon usage (TABLE 3.6A). The cytosolic GS sequences (pcGS-α1, pcGS-β1 and pcGS-γ1) appeared more similar to each other, than to the pcGS-δ1 plastid GS. Although the latter GS cDNA exhibited preferential use of certain cysteine and serine codons, there was little evidence for strong differential codon usage between the four sequences. In addition, there was no evidence for G or C enrichment of the third codon position between plastid or cytosolic *P. vulgaris* GS sequences, or for other dicotyledon GS sequences, in contrast with the marked bias observed with the monocotyledon barley plastid GS sequence (TABLE 3.6B).

Table 3.6A Codon usage of the three cytosolic (α, β and γ) and the chloroplastic* (δ) GS polypeptides of P. vulgaris.

	γ	β	α	δ		γ	β	α	δ		γ	β	α	δ		γ	β	α	δ
Phe-TTT	4	3	4	5	Ser-TCT	5	2	7	6	Tyr-TAT	6	6	10	8	Cys-TGT	3	3	3	1
Phe-TTC	5	6	3	4	Ser-TCC	6	4	3	0	Tyr-TAC	10	10	9	7	Cys-TGC	0	0	0	4
Leu-TTA	2	0	1	4	Ser-TCA	5	5	5	7	ter-TAA	0	0	0	0	ter-TGA	0	0	0	0
Leu-TTG	5	7	5	6	Ser-TCG	1	1	0	0	ter-TAG	0	0	0	0	Trp-TGG	10	10	10	10
Leu-CTT	8	2	8	6	Pro-CCT	12	12	10	15	His-CAT	2	2	2	3	Arg-CGT	5	4	3	4
Leu-CTC	2	4	6	2	Pro-CCC	3	5	3	2	His-CAC	4	4	4	5	Arg-CGC	0	1	1	3
Leu-CTA	0	2	1	4	Pro-CCA	7	7	11	7	Gln-CAA	5	4	5	4	Arg-CGA	0	1	0	1
Leu-CTG	1	4	0	1	Pro-CCG	1	0	1	0	Gln-CAG	5	5	3	4	Arg-CGG	0	0	0	0
Ile-ATT	11	10	16	15	Thr-ACT	5	6	3	3	Asn-AAT	7	6	6	10	Ser-AGT	3	2	4	10
Ile-ATC	8	13	7	7	Thr-ACC	6	8	7	4	Asn-AAC	11	13	9	9	Ser-AGC	3	4	3	2
Ile-ATA	6	4	1	6	Thr-ACA	6	4	5	11	Lys-AAA	10	6	13	6	Arg-AGA	5	4	9	5
Met-ATG	9	5	6	3	Thr-ACG	1	0	0	1	Lys-AAG	10	14	7	10	Arg-AGG	9	8	5	6
Val-GTT	13	14	13	8	Ala-GCT	15	17	12	13	Asp-GAT	11	8	16	13	Gly-GGT	16	17	12	17
Val-GTC	3	3	2	3	Ala-GCC	2	5	4	1	Asp-GAC	8	12	7	4	Gly-GGC	6	3	4	4
Val-GTA	2	1	3	3	Ala-GCA	8	7	12	9	Glu-GAA	12	12	8	11	Gly-GGA	10	14	17	13
Val-GTG	4	5	6	5	Ala-GCG	2	0	0	0	Glu-GAG	11	11	15	16	Gly-GGG	6	6	6	5

* the region analysed represents 356 amino acids colinear to the cytosolic GS.

TABLE 3.6B GS sequence codon usage and bias to G/C enriched third position codons. GS sequences used include pcGS-γ1 (FIG 3.5C), pcGS-α1, pcGS-β1 (Gebhardt *et al*, 1986) and pcGS-δ1 (Lightfoot *et al*, 1988) from *P. vulgaris*; pGS185 (Tingey *et al*, 1988), pGS341 (Tingey *et al*, 1987) and pGS299 (Tingey *et al*, 1988) from *P. sativum*; pGSA19 from *Medicago sativa* (Tischer *et al*, 1986); pGS1/15 from *Nicotiana plumbaginifolia* (Tingey *et al*, 1987a); and pBar1GS2 from *Hordeum vulgare* (J.Freeman, A.Marquez and B.G.Forde, pers.commun.). Percent codon usage is defined as the percentage of the 61 sense codons used for each sequence.

	% CODON USAGE	% BIAS TO G/C ENRICHED THIRD POSITION OF CODONS
pcGS-γ1	93.4	42.7
pcGS-β1	90.2	48.0
pcGS-α1	88.5	38.2
pcGS-δ1	91.8	36.0
pGS185	96.7	38.2
pGS299	91.8	41.9
pGS341	90.2	31.7
pGSA19	91.8	41.3
pGS1/15	93.4	48.0
pBar1GS2	93.4	63.7

An analysis of the amino acid compositions of the different encoded GS polypeptides identified little difference between plastid and cytosolic forms other than the former containing two additional cysteine residues (see TABLE 3.6A). This difference though could account for the plastid GS enzymes requirement of thiol reagents to maintain activity *in vitro* and its sensitivity to thiol reactive compounds (McNally *et al*, 1983).

To determine the divergence between each of the four *P. vulgaris* GS cDNAs and the other available full length higher plant GS sequences, pairwise percentage identities at both the nucleotide and the amino acid levels were calculated (TABLE 3.6C). The GS sequences used included three from *P. sativum*, pGS341 (Tingey *et al*, 1987a), pGS299 and pGS185 (Tingey *et al*, 1988); one from *Medicago sativa*, pGSA19 (Tischer *et al*, 1986); one from *Nicotiana plumbaginifolia*, pGS1/15 (Tingey *et al*, 1987b) and one from *Hordeum vulgare*, pBarlGS2 (J.Freeman, A.Marquez and B.G.Forde, pers. commun.). For the three plastid GS sequences (pcGS-δ1, pGS185 and pBarlGS2) which are longer at both N and C termini, a region colinear with the cytosolic GS pcGS-γ1 ORF was used (N.B. percentage identity figures quoted in the text refer to nucleotide sequence identity unless otherwise stated).

Within the *P. vulgaris* multigene family, all three cytosolic GS sequences were found to be only 70% identical to the plastid GS sequence, as similarly observed between *P. sativum* GS members (Tingey *et al*, 1988). Comparisons between the *P. vulgaris* cytosolic forms showed that pcGS-γ1 was more identical to pcGS-β1 (86%) than pcGS-α1 (79%) and in *P. sativum* pGS299 was approximately 80% identical to pGS341. Interestingly, comparisons between *P. vulgaris* and *P. sativum* GS sequences identified greater nucleotide sequence identity values of selected GS cDNAs between species than within the same species. For example the *P. vulgaris* pcGS-δ1 had greatest identity with the *P. sativum* pGS185 cDNA. Similarly, pcGS-α1 was 86% identical to the *P. sativum* pGS341 cDNA, whereas pcGS-β1 was most similar (approximately 85%) to the *P. sativum* pGS299 sequence. This observation suggested the presence of gene homologues between the *P. sativum* and *P. vulgaris* GS multigene families.

Comparisons to single members of incomplete GS multigene families (i.e. alfalfa and *N.plumbaginifolia*), identified 91% identity between

Table 3.6C % identity of GS nucleotide and amino acid sequences*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1) Pv pcGS- 1	100	85.6	78.8	69.7	68.9	78.5	82.4	85.2	79.4	68.5				
2) Pv pcGS- 1	89.6	100	80.5	70.2	69.6	78.9	84.6	87.5	80.2	69.3				
3) Pv pcGS- 1	85.7	87.6	100	70.8	70.9	86.1	79.0	81.9	80.4	69.3				
4) Pv pcGS- 1	77.8	76.9	78.1	100	89.9	70.7	69.8	70.3	69.8	76.0				
5) Ps pGS185	77.2	77.2	76.4	94.4	100	71.1	68.8	70.5	70.7	74.9				
6) Ps pGS341	85.7	85.6	92.4	78.9	77.0	100	79.3	81.1	78.9	67.6				
7) Ps pGS299	84.6	87.9	86.0	79.5	77.0	85.4	100	90.6	79.2	68.9				
8) Ms pGSA19	87.9	92.4	88.7	79.7	78.4	87.7	90.4	100	82.2	69.7				
9) Np pGS1/15	87.6	87.6	86.2	77.5	76.7	87.3	86.5	91.5	100	69.6				
10)Hv pBar1GS2	74.2	74.7	75.8	87.1	86.8	75.6	75.0	76.2	75.0	100				
11)Ch pGSC45	52.1	52.9	53.1	49.5	49.5	52.1	52.8	52.4	51.6	50.8	100			
12)Bj pBJ196A	42.4	41.8	42.1	41.1	39.8	43.1	42.8	42.0	41.5	40.7	40.4	100		
13)An pAn503	21.9	22.2	21.4	21.9	22.0	22.0	21.2	22.1	22.8	21.8	24.5	23.6	100	
14)St pJB8	20.0	19.6	17.7	18.3	19.5	19.2	18.3	21.4	20.7	20.6	23.4	23.2	55.6	100

* the sequences are pcGS- 1 (FIG 3.5C), pcGS- 1, pcGS- 1 (Gebhardt et al, 1986) and pcGS- 1 (Lightfoot et al, 1988) from P. vulgaris; pGS185 (Tingey et al, 1988), pGS341 (Tingey et al, 1987) and pGS299 (Tingey et al, 1988) from P. sativum; pGSA19 from Medicago sativa (Tischer et al, 1986); pGS1/15 from Nicotiana plumbaginifolia (Tingey et al, 1987a); pBar1GS2 from Hordeum vulgare (J.Freeman, A.Marquez and B.G.Forde, pers.commun.); pGSC45 from Chinese hamster (Hayward et al, 1986); pBJ196A from Bradyrhizobium japonicum (Carlson and Chelm, 1986); pAn503 from Anabaena 7120 (Tumer et al,1983); and pJB8 from Salmonella typhimurium (Janson et al, 1986).

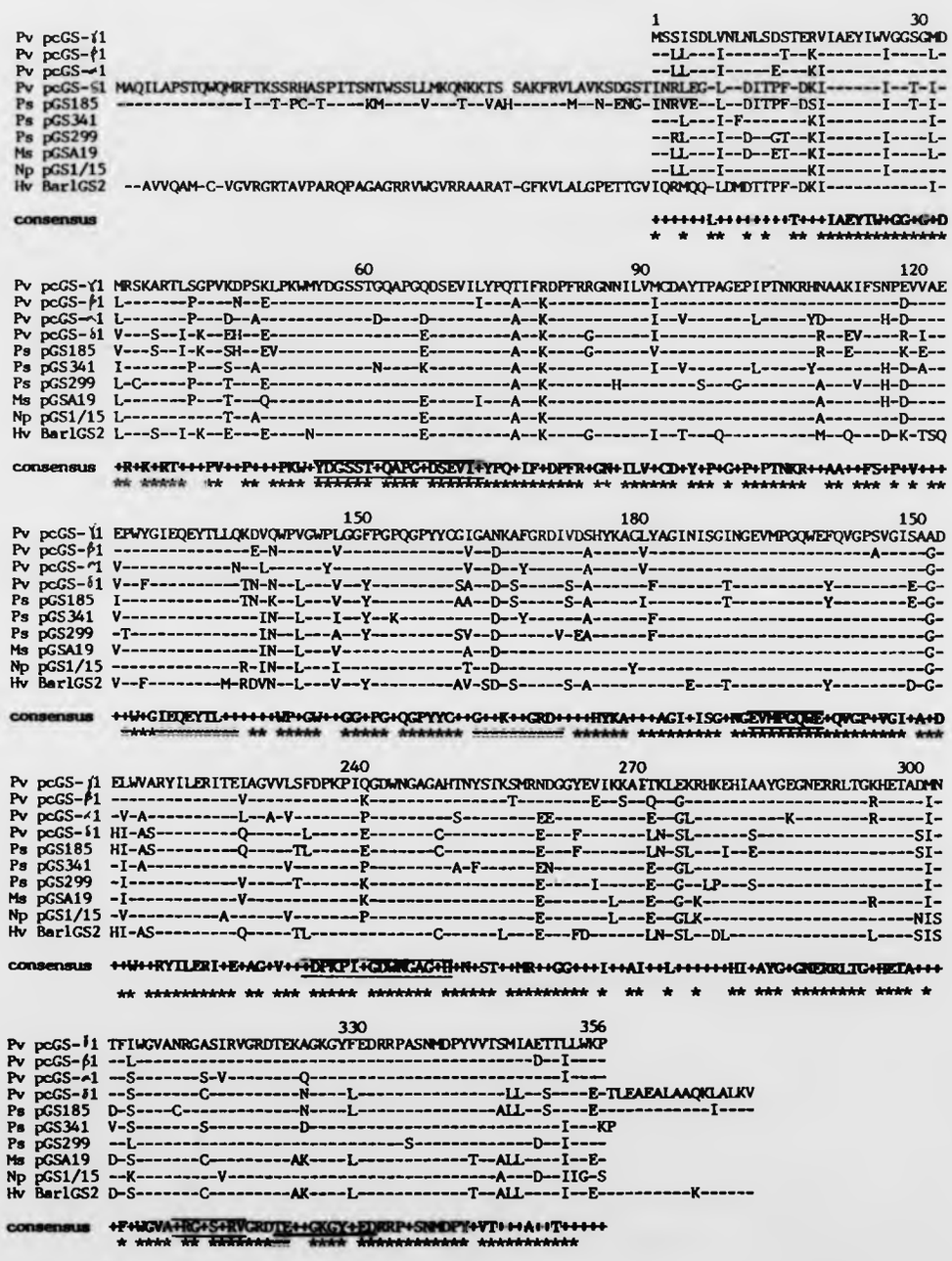
P. sativum pGS299 and the alfalfa GS, whereas pea pGS341 was only 81% similar to the alfalfa sequence. The *N.plumbaginifolia* cytosolic GS sequence however, exhibited no such preference to any *P. vulgaris* or *P. sativum* GS sequence, instead displaying approximately 80% and 70% identity to all cytosolic and plastidic GS sequences respectively. The monocot barley plastid GS sequence had approximately 69% identity to all cytosolic sequences, but also featured reduced identity (approximately 75%) to the pea and *P. vulgaris* plastid GS sequences.

Percentage identity comparisons at the amino acid level were extended to include the eukaryotic Chinese Hamster GS, pGSC45 (Hayward *et al*, 1986) and three bacterial GS seqences, *Bradyrhizobium japonicum* GS11, pBJ196A (Carlson and Chelm, 1986), and GS from *Anabaena* 7120, pAn503 (Tumer *et al*, 1983) and *Salmonella typhimurium*, pJB8 (Janson *et al*, 1986). pGSC45 was about 49-53% identical to all plant GS sequences. pBJ196A exhibited about 39-43% similarity to all eukaryotic GS, featuring no apparent preferential identity to either animal or plant, and plant cytosolic or plastid GS sequences. In contrast, pAn503 and pJB8 featured little similarity in comparison to either eukaryotic GS or pBJ196A sequences (less than 24%).

To establish which regions of the higher plant GS polypeptide are most highly conserved, all ten available higher plant GS primary amino acid sequences were aligned (FIG 3.6A). Within the pcGS-Y1 coding region, all plastid and cytosolic plant GS sequences are colinear. A consensus sequence, from all the GS sequences considered, featured 144 amino acid changes within the 356 amino acid coding region, of which 68 changes were structurally conservative (Miyata *et al*, 1979), resulting in an overall consensus identity value of 59.6%. A pairwise comparison of the N-terminal presequences from the three plastid GS cDNAs noted a 72% identity between pea and *P. vulgaris* sequences, but failed to identify homology between either of these presequences and the barley GS presequence. In contrast, the C-terminal extension of all three plastid GS sequences were highly conserved (approximately 94%).

The alignment of all ten plant GS sequences identified a high degree of sequence conservation at the primary amino acid sequence level. However, alignment of the divergent *S.typhimurium* and plant GS sequences could identify conserved regions (of importance) that can be related to the *S.typhimurium* three dimensional structure (Almassy *et al*,

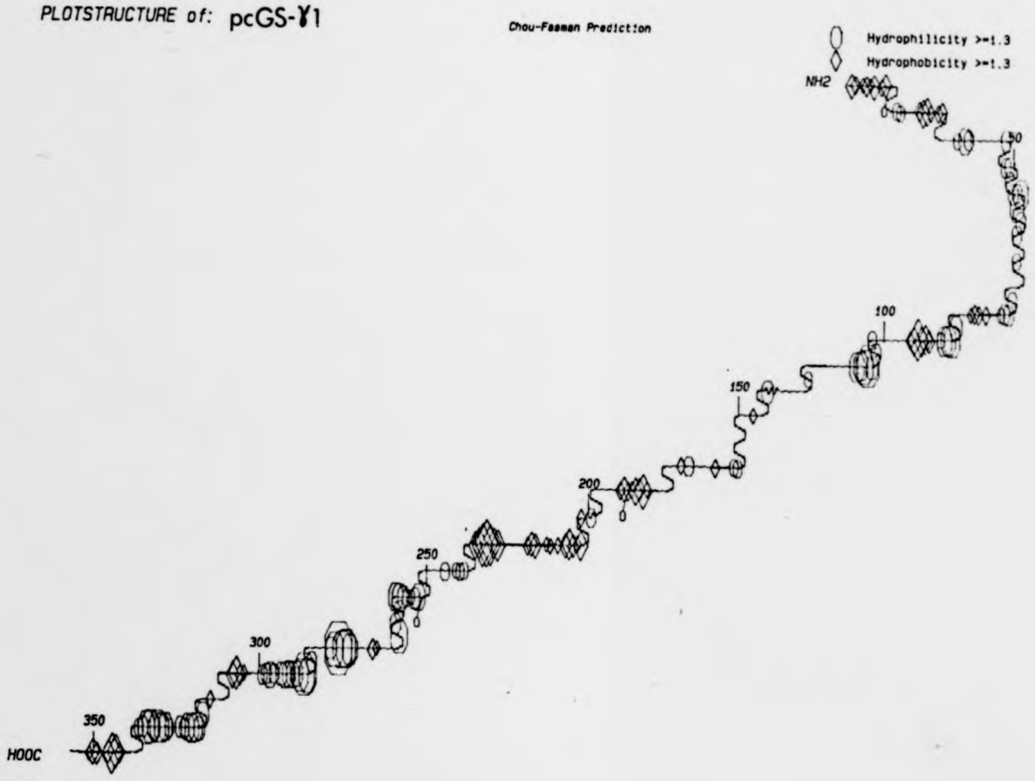
FIG 3.6A Comparisons of the amino acid sequences of higher plant GS polypeptides, deduced from cDNA sequences. These include pcGS-γ1 (FIG 3.5C), pcGS-α1, pcGS-β1 (Gebhardt *et al*, 1986) and pcGS-δ1 (Lightfoot *et al*, 1988) from *P. vulgaris*; pGS185 (Tingey *et al*, 1988), pGS341 (Tingey *et al*, 1987) and pGS299 (Tingey *et al*, 1988) from *P. sativum*; pGSA19 from *Medicago sativa* (Tischer *et al*, 1986); pGS1/15 from *Nicotiana plumbaginifolia (Tingey et al*, 1987a); and pBarlGS2 from *Hordeum vulgare* (J.Freeman, A.Marquez and B.G.Forde, pers.commun.). The sequence of pcGS-γ1 is shown in full and others only when they disagree. Consensus shows a sequence where all ten sequences agree. Note that pcGS-δ1, pGS185 and pBarlGS2 encode precursors to the plastid GS and all others sequences encode cytosolic GS polypeptides. Residues are numbered from the initiating pcGS-γ1 sequence methionine. * signifies structurally conserved residues, according to Miyata *et al* (1979). Residues underlined both top and bottom represent putative active site residues identified by Eisenberg *et al* (1987). Heavily underlined residues 323 to 331 represent a putative plant GS ATP binding site peptide according to Kim and Rhee (1988).

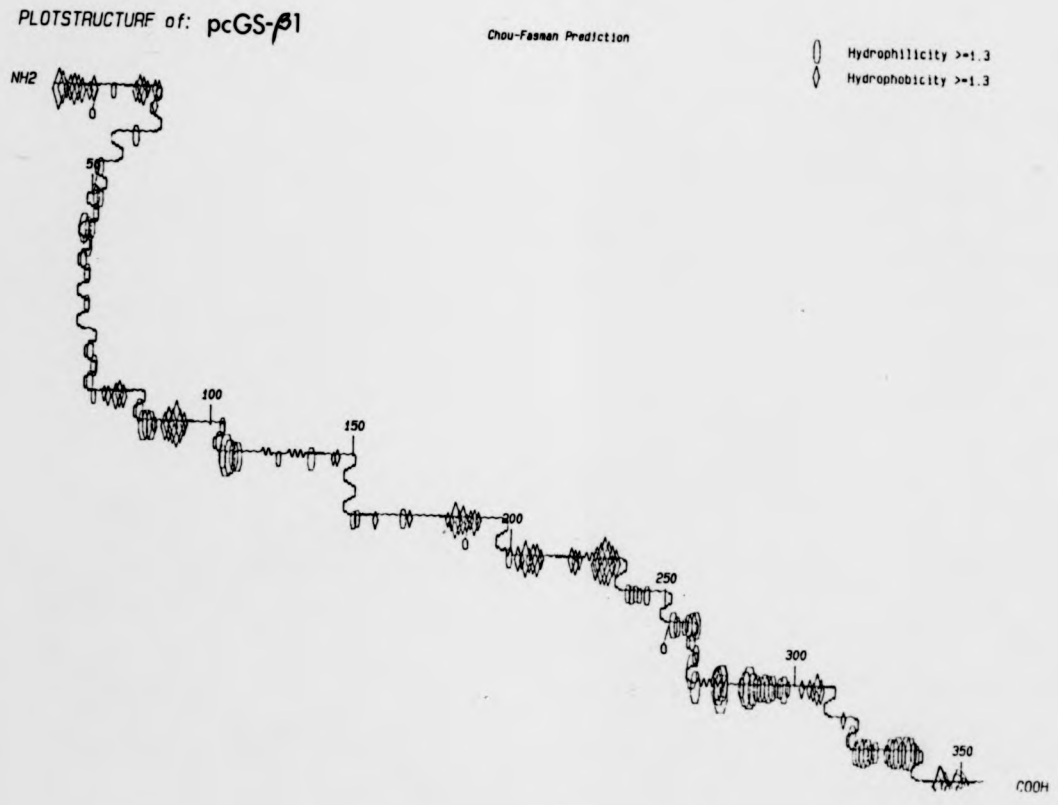


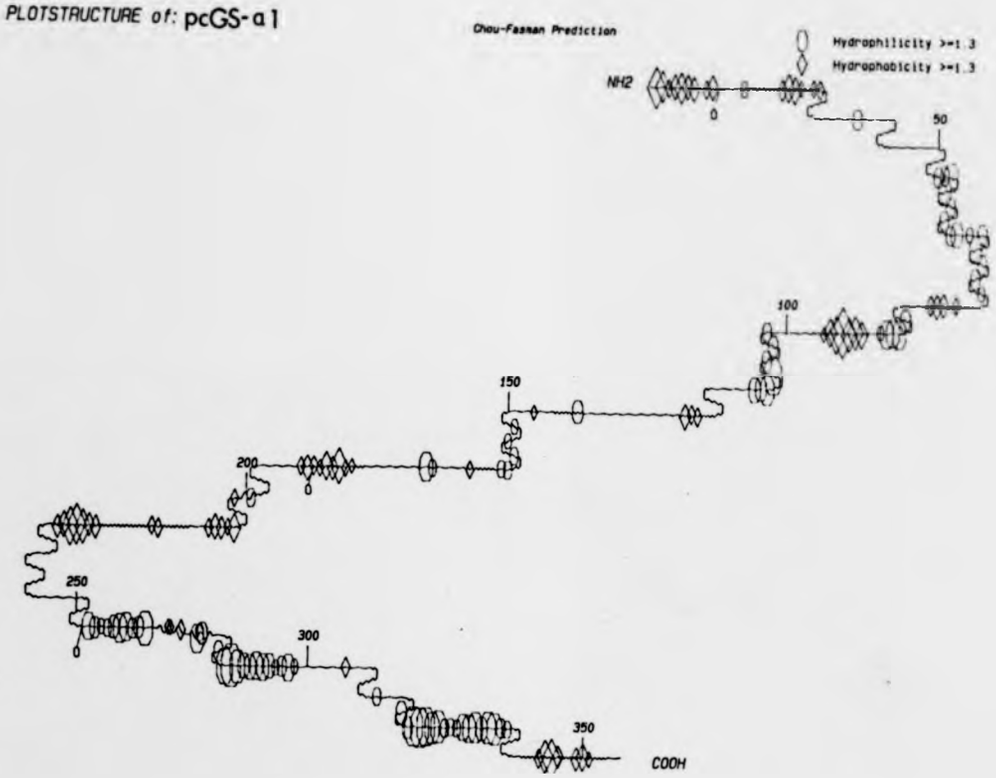
1986). Eisenberg *et al* (1987) have performed such an alignment procedure between the alfalfa and *S.typhimurium* GS sequences, and identified four conserved regions which, on comparison to the higher plant GS sequence alignment (see FIG 3.6A), falls within regions of good conservation. Eisenberg *et al* (1987) have proposed that this alignment is of functional significance, since the four conserved regions appeared to line the *S.typhimurium* GS active site cylinder (Almassy *et al*, 1986). However, hydrophobicity calculations (data not shown), and secondary structure prediction programmes, such as PREDICT and PLOTSTRUCTURE (which graphically presents the secondary structural predictions according to the Chou and Fasman rules) (data not shown), failed to identify structural similarities between the *S.typhimurium* and *P. vulgaris* GS primary amino acid sequences. In contrast, PLOTSTRUCTURE analyses of the four *P. vulgaris* GS polypeptides identified (with respect to the α-helical and ß-pleated sheet confirmations) a remarkably similar secondary structure and hence folding pattern, as would be expected for proteins of such high homology (Sweet and Eisenberg, 1983). Note that the shape of the polypeptides printed on the pages is of little significance in the secondary structure interpretations (FIG 3.6).

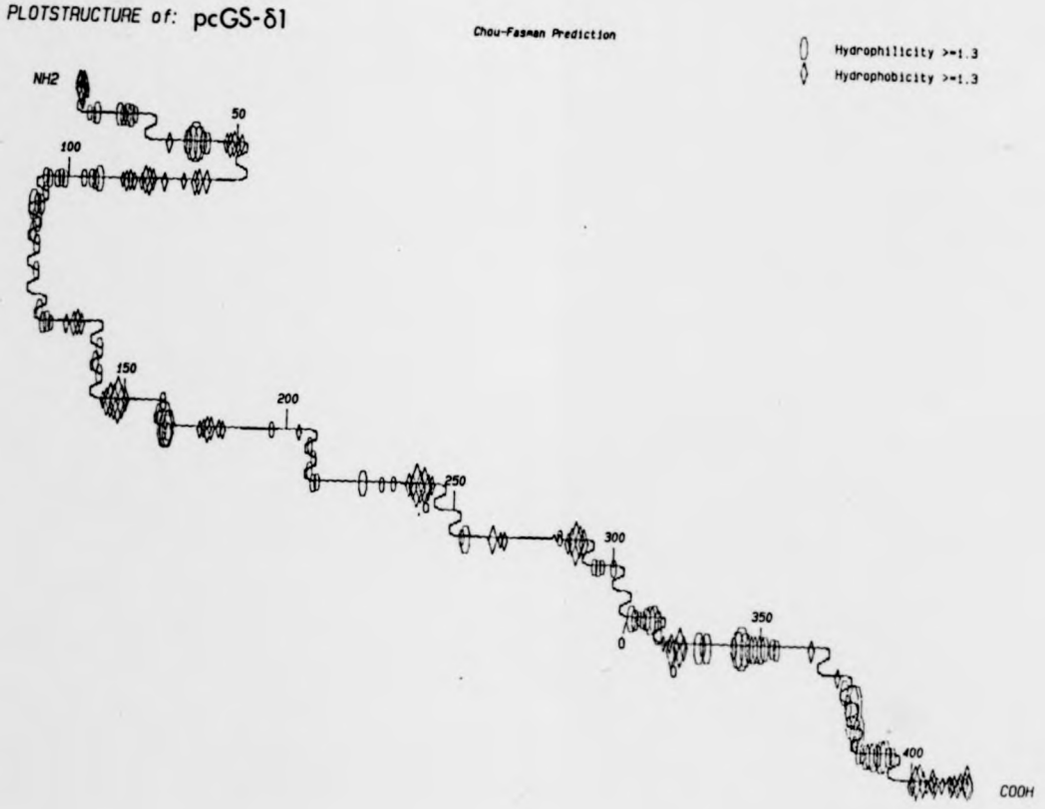
By using sequence alignment predictions in conjunction with bacterial GS X-ray crystallographic data and biochemical lines of evidence, specific functions can be assigned to several individual residues which appear conserved between bacterial and plant GS sequences. For example, the *S.typhimurium* and alfalfa GS aligned homology block I has been proposed to represent the C-terminal active site domain (Eisenberg *et al*, 1986), which contains an ASP residue postulated to function during catalytic confirmational changes within the active site (Almassy *et al*, 1986). The ASP is conserved between the *S.typhimurium* and all known plant GS sequences (corresponding to residue 57, see FIG 3.6A). The *S.typhimurium* TYR 179 residue, postulated to serve a communicative role between the GS active and binding sites (Almassy *et al*, 1986), is also conserved in plant GS sequences as TYR 159 (FIG 3.6A). Levine (1984) has identified an *E.coli* histidine residue (corresponding to *S.typhimurium* HIS 270), that was susceptible to GS enzyme inactivating oxidation, and this aligns with the conserved plant GS HIS 250 residue within homology block III (FIG 3.6C). Colanduani and Villafranca (1985) have suggested that an ARG residue is present at the bacterial GS enzyme active site. Sequence

FIG 3.6B Graphic representation of PLOTSTRUCTURE secondary structure predictions for the pcGS-α1, pcGS-ß1, pcGS-Y1, and pcGS-δ1 GS amino acid sequences according to the Chou-Fasman rules. α-helical structures are represented by a wavy line, whereas a pleated line pattern represents ß-pleated sheet formation. The pcGS-δ1 secondary structure prediction also includes the 57 and 16 amino acid N and C terminal extensions.


PLOTSTRUCTURE of: pcGS-Y1
Chou-Fasman Prediction
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
HOOC
50
100
150
200
250
300
350


PLOTSTRUCTURE of: pcGS-β1
Chou-Fasman Prediction
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
COOH


PLOTSTRUCTURE of: pcGS-a1
Chou-Fasman Prediction
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
0
50
100
150
200
0
250
300
350
COOH


PLOTSTRUCTURE of: pcGS-δ1
Chou-Fasman Prediction
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
400
COOH

alignment by Eisenberg *et al* (1987) has identified two conserved ARG residues (represented by ARG 312 and 317), both within homology block IV of the alfalfa GS sequence, which are conserved between all plant GS sequences (FIG 3.6A). In contrast, the *E.coli* LYS 47 residue, that reacted with an ATP analogue, possibly as an ATP binding site group (Pinkofsky *et al*, 1984), could not be aligned with any plant GS LYS residues. However, Kim and Rhee (1988) have recently reported the sequence of a yeast GS peptide containing a LYS residue specifically labelled with an ATP analogue, which, by sequence comparisons with the *P. vulgaris* pcGS-α1 and pcGS-ß1 GS sequences, aligned with the *P. vulgaris* GS residues 323 to 331. The labelled LYS residue corresponds to LYS 326 which is completely conserved with all the other plant GS sequences (FIG 3.6A). Secondary structure predictions of this region for all four *P. vulgaris* GS sequences (see FIG 3.6B), failed to identify the characteristic ß-pleated sheet-loop-α helix structure typical of known adenine nucleotide binding sites. However, this simple secondary structure analyses could fail to predict the true secondary structure of this region.

3.7 DISCUSSION

This chapter has described the isolation of a full length GS cDNA, initially termed F14, identified in a *P. vulgaris* root nodule cDNA library screened with the partial GS cDNA probe pcPvNGS-01 (Cullimore *et al*, 1984). Characterisation, initially by restriction mapping (FIG 3.2D) and subsequently by DNA sequence analysis (FIG 3.5C), confirmed that this GS cDNA is identical, in parts, to the partial cDNA pcPvNGS-01 (Cullimore *et al*, 1984) and to the *gln-Y* genomic clone (B.G.Forde, pers. commun.), and is also similar, but not identical to the three other *P. vulgaris* GS cDNAs previously isolated (Gebhardt *et al*, 1986; Lightfoot *et al*, 1988). The *P. vulgaris* GS multigene family is therefore suggested to consist of at least four expressed genes, in addition to a fifth GS gene identified on the pcPvNGS-01 homologous genomic clone (J.Turton and B.G.Forde, pers. commun.; T.Aldridge and J.V.Cullimore, unpubl. data), which is in agreement with southern blot analysis of *P. vulgaris* genomic DNA (Gebhardt *et al*, 1986; Lightfoot *et al*, 1988).

The relationship between each of the three cytosolic GS cDNAs pR-1, pR-2 (Gebhardt *et al*, 1986) and F14 (section 3.2), and the three previously identified cytosolic GS subunits, α, ß and ϒ (Lara *et al*, 1984) was resolved by comparing the two dimensional separation of each GS cDNAs *in vitro* synthesised polypeptide relative to the separation of authentic native GS subunits (FIG 3.3). Such analysis identified that the pR-2, pR-1 and F14 cDNA encoded polypeptides comigrated exactly with the native α, ß and ϒ GS subunits respectively, and the cDNAs were thus renamed pcGS-α1, pcGS-ß1 and pcGS-ϒ1 accordingly. These observations were in agreement with previous conclusions by Gebhardt *et al* (1986) based on the correlation of the differential organ expression of each cytosolic GS gene relative to the three cytosolic GS subunits. The genetic origin of the different GS polypeptides thus resides in different members of the GS multigene family encoding each polypeptide. In addition, the exact comigration of the *in vivo* and *in vitro* synthesised GS polypeptides suggests that each GS cDNA, including pcGS-ϒ1, firstly contain full length ORFs, and secondly, that their encoded polypeptides do not undergo post-translational modification, the latter being in agreement with previous observations by Padilla *et al* (1987). In contrast, the fourth GS cDNA clone pcGS-δ1 has previously been shown to encode a higher M_r nuclear-encoded precursor to the chloroplast GS polypeptide (Lightfoot *et al*, 1988); this precursor is post-translationally imported into the chloroplast and cleaved to produce the mature δ polypeptides. The observed correlation of the four *P. vulgaris* GS cDNAs with the previously identified α, ß, ϒ and plastid GS subunits (Lara *et al*, 1984), provided additional proof for a 4-5 gene member model for the *P. vulgaris* GS multigene family. Furthermore the results of Southern blot analyses of genomic DNA from the legume *P. sativum* (Tingey *et al*, 1987a), the Solanaceae *N.plumbaginifolia* (Tingey *et al*, 1987b) and the crucifer *Arabidopsis thaliana* (Peterman *et al*, 1987), and the isolation of a number of GS genomic clones (see introduction) all suggested that throughout higher plants GS is encoded by a small multigene family.

Many higher plant proteins are encoded by multigene families and these can generally be segregated into two categories. In one, individual members share very similar coding regions, their expression regulated in a very similar manner and they function to accomodate the need for large quantities of the encoded protein at a particular

developmental stage. The small subunit of ribulose bisphosphate carboxylase (Dean *et al*, 1986), chlorophyll a/b binding protein (Dunsmuir *et al*, 1984), seed storage proteins (Talbot *et al*, 1984) and leghaemoglobin (Brown *et al*, 1984) are all examples of such multigene families. In contrast, other multigene families encode related, but distinct gene products that are expressed differentially in terms of organ specificity and environmental stimuli. Both chalcone synthase (Ryder *et al*, 1987) and GS (Gebhardt *et al*, 1986), from *P. vulgaris*, provide such examples. Individual *P. vulgaris* GS genes and their products feature distinctive expression patterns (see section 1.5), subcellular compartmentation and sequence divergence (upto 30%). There is therefore great interest in studying the regulation, structure and evolution of these genes.

gln-Y mRNA has previously been shown to be expressed only in nodules, and not roots or leaves (Gebhardt *et al*, 1986) leading to suggestions that the *gln-Y* gene is regulated in a nodule specific manner. In addition, a comprehensive study of the *P. vulgaris gln-Y* promoter (B.G.Forde, pers. commun.) has suggested that similar, if not the same trans-acting factors involved in the regulation of the soybean *Lhb c3* gene (Jensen *et al*, 1988) are also involved in *gln-Y* gene regulation. However, analysis of mRNA from a wider range of *P. vulgaris* organs by RNase mapping with a pcGS-Y1 probe (FIG 3.4C), although confirming that *gln-Y* mRNA was present in nodules but not roots or leaves, further identified *gln-Y* expression in stems, petioles and green, but not white, cotyledons. In addition, this mRNA is present at widely different relative abundances, being found in stems and petioles at a 50- fold, and green cotyledons at a 10- fold lower level relative to *gln-Y* expression in root nodules. The observed extranodular expression was not a general feature of the leaky expression of other nodule specific *P. vulgaris* genes, since *Lhb* mRNA could only be detected in nodule tissue (FIG 3.4C, see section 3.4). This expression pattern instead is representative of the highly differential mode of regulation of the *gln-Y* gene, which appears more complex than originally anticipated, contrasting with the seemingly nodule specific *Lhb* gene regulation. In addition, the appearance of the Y polypeptide appears to be essentially transcriptional, since the Y polypeptide is detectable in all tissues expressing *gln-Y* mRNA, at levels reflecting the relative abundance of the mRNA.

The highly differential organ expression for the *P. vulgaris gln-γ* GS gene, is in marked contrast to the *P. sativum* 'nodule enhanced' GS cDNA pGS341. pGS341 mRNA is clearly present in roots and leaves, (Tingey *et al*, 1987a), although at a 20- fold lower abundance compared to nodules, and is also detectable at elevated levels in green cotyledons (E.Walker and G.Coruzzi, pers, commun.; see section 1.5).

The functional advantages for the differential organ pattern of expression of the *gln-γ* gene and its products are not immediately apparent. It is known that GLN is a major product of cotyledon nitrogen breakdown, assimilation and transport (Lignowski *et al*, 1971), and the *gln-γ* gene product could have an important assimilatory role during germination, especially since the γ polypeptide represented a major cotyledon GS subunit (FIG 3.4D). The evolution of such an expression pattern for *gln-γ* in nodules and other organs is of considerable interest. Nodule expressed genes could have evolved to meet the regulatory requirements of a rhizobially controlled nodule developmental programme (see section 1.4). This could either be achieved through the adaptation of the expression of a pre-existing gene to function in the symbiosis, such as the *Parasponia* globin gene (Bogusz *et al*, 1988), or involve, through gene duplication, the evolution of a nodule expressed multigene family member, such as the soybean nodule specific uricase gene (Nguyen *et al*, 1985). At present it is unknown whether the *gln-γ* nodule expression developed through an adaptive mechanism from a pre-existing *gln-γ* gene or by a duplication event from a closely related GS gene, such as *gln-β*. Sprent and Raven (1988) have suggested that the typical nitrogen rich composition of the legume seed arose as a selected response to the plants requirement for a large nitrogen reserve to invest in nodule differentiation. Such a proposal could suggest that *gln-γ* extranodular expression has evolved after the development of *gln-γ* nodule expression, and that it represented the adaptation of a nodule expressed gene's regulation to suit the requirements of cotyledon nitrogen metabolism. Such proposals for the development of the *gln-γ* expression pattern are at present highly speculative.

The identification of the factors controlling the *gln-γ* extranodular expression and the regions of the gene responsible for this, in comparison to the nodule trans and cis acting regulatory factors, clearly merits further investigation. Schell *et al* (1988) have reported that in addition to a nodule specific *trans* acting factor

binding the *Sesbania rostrata Lhbc3* promoter region, leaf nuclear factors bind to at least three other regions, thus suggesting that multiple *cis* and *trans* acting factors play a role in gene regulation.

The complete sequence determination of all four expressed *P. vulgaris* GS multigene family members has enabled an analysis of the similarity of the GS genes. Parameters such as codon usage and percentage nucleotide (or amino acid) sequence identities (see section 3.6) identified a high degree of similarity between the plastid and cytosolic GS sequences, thus leading to a proposed gene duplication based evolution for all members of the GS multigene family (Coruzzi *et al*, 1988; Lightfoot *et al*, 1988; Tingey *et al*, 1988). A similar analysis between the two *Zea mays* nuclear encoded cytosolic and plastid glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences identified major differences in codon usage. In addition, the plastid GAPDH shared a greater identity with a thermophilic GAPDH sequence, which lead to a proposed endosymbiotic mode of evolution for the plastid GAPDH gene, and subsequent transfer to the nucleus (Brinkmann *et al*, 1987).

It was observed that the *P. vulgaris gln-δ* gene was approximately 70% identical relative to the α, ß and ϒ GS sequences, whereas the three cytosolic GS genes shared an identity of at least 80%. Thus an evolutionary model featuring a plastid GS gene duplicating from an ancestral eukaryotic gene before the later divergence of the more closely related cytosolic genes could be postulated. The remarkably similar identity value of approximately 69% observed from homology comparisons between all available higher plant cytosolic GS and the divergent monocotyledon and dicotyledon plastidic GS gene sequences, supports the hypothesis that all plastid GS genes from higher plants arose from a single gene duplication event before the speciation of higher plants. Moreover, the widespread occurence of both cytosolic and chloroplastic GS genes (see section 1.5) gave further support to this hypothesis.

The presence of several genes encoding cytosolic GS in all higher plants studied to date suggests that multiple cytosolic GS genes may have arisen before the speciation of higher plants. Furthermore, Gebhardt *et al* (1986) have previously observed that the three *P. vulgaris* cytosolic GS coding regions had diverged to a similar extent to the *Adh 1* and *Adh 2* genes from *Z.mays*, which were thought to have

arisen by the duplication of a single gene at least 120 million years ago, and hence prior to the divergence of monocots and dicots (Dennis *et al*, 1985). Such a proposal is further supported by the observation that pairs of cytosolic GS genes are highly conserved between species. Each of the *P. sativum* cytosolic GS sequences are more similar to one or other of the *P. vulgaris* GS sequences than to each other. For example, pea pGS341 and *P. vulgaris* pcGS-α1 are 86% identical, pea pGS299 and *P. vulgaris* pcGS-β1 are 85% similar. In addition, the alfalfa GS sequence features a stronger identity to the pea pGS299 (about 91%) than to pGS341 (approximately 81%), whereas pea pGS299 and pGS341 were only about 80% identical. The high homology of pcGS-γ1 to the pcGS-β1 sequence (approximately 86%) could support its recent duplication and evolution as a nodule expressed gene, or alternatively reflect the incompleteness of the cloning and sequencing of both the *P. sativum* and alfalfa multigene families (see section 1.4). The lower identity exhibited between the pcGS-γ1 and pGS341 GS cDNAs (about 78%) was surprising considering their similar expression patterns (i.e. elevated levels in nodules and green cotyledons, and low levels in roots and leaves, E.Walker and G.Coruzzi, pers. commun.). Even proposed homologue pairings (by identity) featured differences in their individual expression patterns, such as the ubiquitously expressed pcGS-α1 and the nodule (and cotyledon) enhanced pGS341. This could reflect the differences between *P. vulgaris* and *P. sativum* in the development of the regulatory elements on the different gene homologues. Alternatively it may suggest that the observed stronger interspecies similarities may be coincidental. Further analysis of the number and similarity of the cytosolic genes in *P. sativum* and other plants would provide support or otherwise for the timing of the duplication of the cytosolic GS genes.

It is probable that some higher plant GS genes have arisen since speciation. For example, Coruzzi *et al* (1988) have identified an additional *P. sativum* GS gene that was highly similar to the pGS341 sequence, termed pGS132 (see section 1.5). Sequence analysis of their respective genomic clones (E.Walker and G.Coruzzi, pers. commun.) identified unbroken homology throughout the 5' untranslated and promoter regions until nucleotide -400, and then both genes retained little homology any further upstream, suggesting a recent duplicative/recombinational event. The close linkage of the *P. vulgaris*

gln-Y and the fifth GS sequence is also suggestive of a recent duplication.

The alignment of ten higher plant GS sequences (FIG 3.6A) has identified that all share a common colinear ORF featuring an overall identity of 59.5%, suggesting that the structure of the GS protein in higher plants is highly conserved. In contrast, the plastid GS N terminal presequences, that post-translationally target the GS protein to the chloroplast (Lightfoot *et al*, 1988), have diverged to a greater degree than the mature GS subunit primary sequences, such that the dicot GS presequences are 30% divergent and the monocot presequence shows little or no homology. Thus, when separate functions reside on a single molecule, as for plastid GS, the associated regions can apparently feature widely different rates of sequence divergence.

An extension of the percentage identity comparisons to other eukaryotic and prokaryotic GS primary amino acid sequences concluded that Chinese Hamster GS (Hayward *et al*, 1986) exhibited about 49-53% identity to plant GS, and thus probably arose from an ancesteral eukaryotic GS gene. In contrast, comparisons of the eukaryotic GS sequences to the *Anabaena* 7120 (Tumer *et al*, 1983) and *S.typhimurium* (Janson *et al*, 1986) prokaryotic GS sequences exhibited a very low identity (below 24%) suggesting that eukaryotic and prokaryotic GS were only distantly related.

The *B.japonicum* GSII sequence has been observed to display a greater similarity to plant rather than other prokaryotic GS sequences (Carlson and Chelm, 1986). Carlson and Chelm (1986) have suggested that the GSII sequence could have arisen, in this member of the *Rhizobiaceae* (a family noted for their close interactions with plants), by horizontal gene transfer during symbiotic interactions. However, identity comparisons with a wider range of GS amino acid sequences (section 3.6) has observed that *B.japonicum* GSII had no preferential identity to plant or Chinese Hamster GS. Furthermore, *B.japonicum* GSII displayed an equal identity to either plastid or cytosolic plant GS sequences, which is surprising since these genes are thought to have evolved prior to plant speciation and the development of the legume symbiosis. Thus it would appear likely that *B.japonicum* GSII did not arise from a horizontal gene transfer event, but is derived from a common progenitor of the eukaryotic GS sequence prior to the plant-animal evolutionary division

(such a conclusion assumes a similar evolutionary rate of sequence changes between prokaryotic and eukaryotic GS sequences). This conclusion is further supported by the occurrence of the GSII activity in both the gram negative *Rhizobiaceae* and the distinctive gram positive actinomycete *Frankia* (Edmands *et al*, 1987). In reference to the previous discussion and its conclusions, a speculative evolutionary pathway for both prokaryotic and eukaryotic GS genes is diagrammatically illustrated (FIG 3.7).

The structural and evolutionary relationship between prokaryotic and eukaryotic GS has been further examined by sequence alignment of the divergent GS sequences. Eisenberg *et al* (1987), using the alignment of the alfalfa GS and *S.typhimurium* GS primary sequences, have correlated the regions of strongest sequence conservation to the active site of the *S.typhimurium* GS structure (Almassy *et al*, 1986). Hence, as Eisenberg *et al* (1987) suggested, the plant and bacterial GS enzymes share a similar polypeptide geometry at their active sites. In addition, a number of residues noted for their biochemical importance in bacterial GS are conserved within the plant GS sequences. Thus, it appears that both prokaryotic and eukaryotic GS are related, albeit distantly, derived from a common ancesteral gene. Further X-ray crystallographic studies on the higher plant enzyme are clearly required to confirm the similarities between the bacterial primary sequence and moreover to reconcile these similarities with the differences in the quarternary structure of these two enzymes.

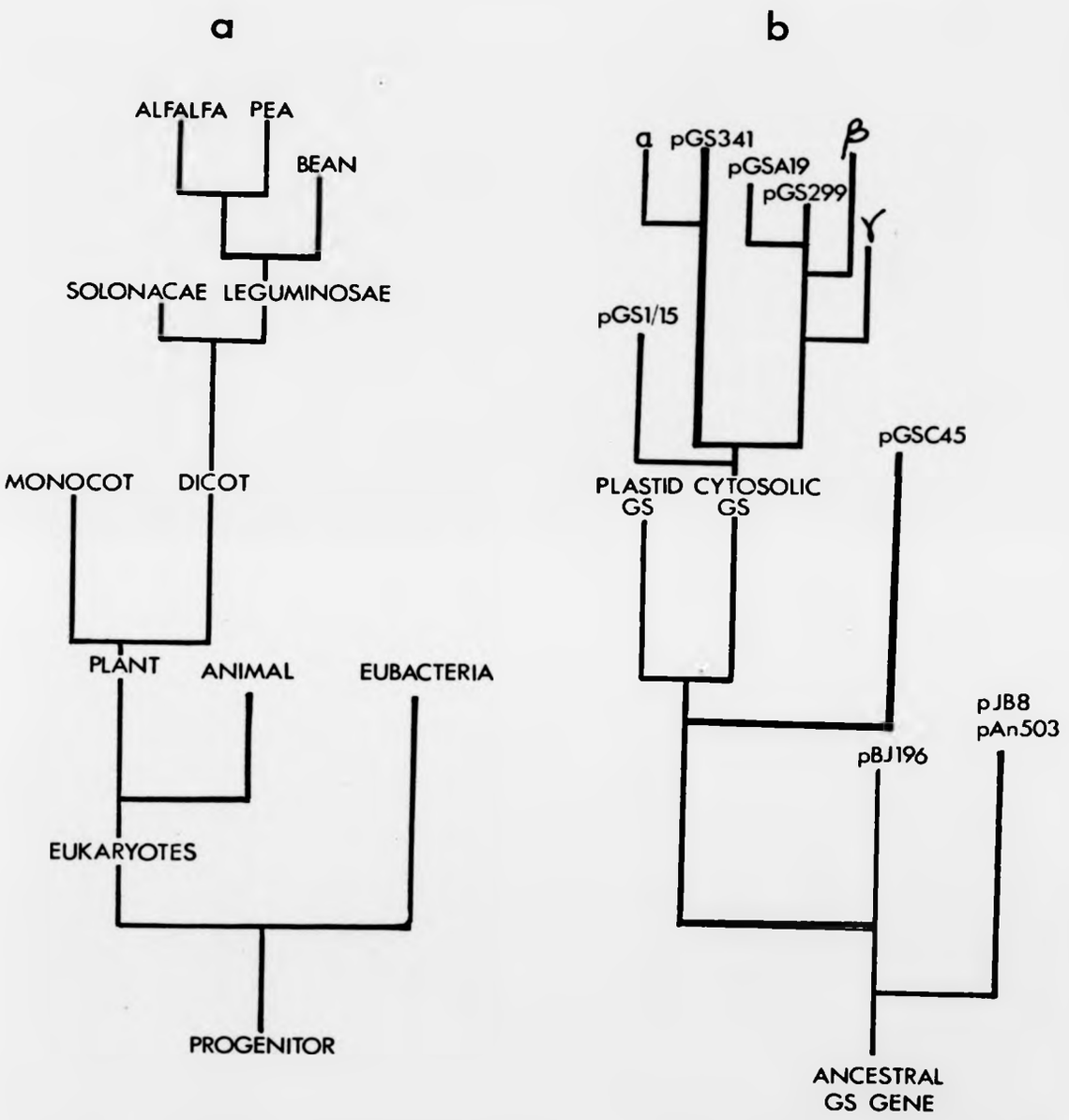


FIG 3.7 Speculative evolutionary pathway of a) the phylogenic relationships between selected groups of organisms; b) the characterised plant, animal and bacterial GS sequences (see text for details).

CHAPTER 4

ISOLATION AND SUBUNIT COMPOSITION OF NODULE AND PLUMULE GS ISOENZYMES

4.1 INTRODUCTION

GS has been studied extensively in plants (see Stewart *et al*, 1980, for a review). The nodule GS enzyme is both physically and kinetically similar to GS from other plant organs, consisting of eight GS subunits, M_r 39,000-45,000, with a native M_r 350,000-400,000 (see Cullimore and Bennett, 1988, for a review). Nodule GS appears, as does GS from other tissues, to be composed of a heterogeneous mixture of GS subunits (Lara *et al*, 1984; see Cullimore and Bennett, 1988, for a review). The heterogeneity of GS polypeptides raises questions about the assembly of the octameric enzyme. Do individual GS polypeptides only assemble with like subunits, hence in a homologous fashion, to produce a variety of homo-octamers, or alternatively, is a heterologous mixing of unlike subunits possible, to produce a variety of hetero-octamers? The assembly model could be of some physico-chemical importance, as the *S.typhimurium* GS active site is located between adjacent subunits (Almassy *et al*, 1986), and this structural feature is extrapolated to also occur within the plant GS enzyme (Eisenberg *et al*, 1987).

P. vulgaris root nodules contain two major cytosolic GS polypeptides, termed γ and β (Lara *et al*, 1984), whereas pea root nodules have been observed to feature five GS polypeptides (Tingey *et al*, 1987). Hence *P. vulgaris* root nodule GS provides a simple model to study GS subunit mixing during enzyme assembly. Cullimore *et al* (1983) identified two GS isoenzymes, separable by conventional ion exchange chromatography (IEC), termed GSN1 and GSN2. GSN1 was present in nodules, but not roots and leaves, whereas GSN2 appeared similar to the root isoform, GSR. Lara *et al* (1984) have examined the GS subunit compositions of the two nodule isoenzymes, identifying that GSN1 was mainly composed of the γ GS polypeptide, with a little β, and GSN2 *vice versa*. In contrast, Cullimore (1985) observed that GSN1 contained an equimolar mixture of γ and β, and GSN2 contained β alone.

Robert and Wong (1986) have examined *P. vulgaris* nodule GS, using a GS activity and Coomassie staining procedure of crude nodule extracts separated by native PAGE. The authors identified two major nodule GS staining activity bands, also termed GSN1 and GSN2, the latter having a faster electrophoretic mobility relative to GSN1. The GSN1 activity band was present in nodules but not roots, whereas the GSN2 activity band had a similar mobility to the root activity band, and thus appeared

analogous to the GS isoenzymes identified using IEC techniques (Cullimore *et al*, 1983). However, the native PAGE technique could further resolve the GSN1 activity band into eight subcomponent bands, but the GSN2 band remained a single species. Robert and Wong (1986) concluded that the nine GS activity bands represented all nine mixing possibilities of two subunits within the GS octamer, and thus suggested a heterologous model of assembly. In addition, changes were observed in the GSN1 activity band's electrophoretic mobility during nodulation, which the authors correlated to the *P. vulgaris* cultivar chosen and its stage of development. Cultivars were segregated into three categories on the basis of GSN1 activity band mobility. Category one cultivars exhibited no change in GSN1 band mobility during nodule development; category two cultivar nodules featured a rapid change in GSN1 electrophoretic mobility three weeks after inoculation, resulting in GSN1 and GSN2 activity band fusion; the category three nodule GSN1 activity band displayed a gradual fusion with GSN2 during nodulation. Robert and Wong (1986) postulated that the increased mobility of the GSN1 activity band was representative of changes in the relative ϒ to ß GS subunit ratios during nodulation, which would affect the relative levels of the nine mixing forms, with a preference to form ß rich hetero-octamers.

In addition to ϒ and ß GS subunits, low levels of α subunit synthesis has been reported in *P. vulgaris* root nodules (Padilla *et al*, 1987), and plastid GS polypeptides have been detected in nodules of other legumes, such as pea (Tingey *et al*, 1987).

The above observations require that a comprehensive study of the subunit structure of both the major and minor *P. vulgaris* root nodule GS isoenzymes should be made, taking advantage of the advances in ion exchange fast liquid protein chromatography (IEC FPLC) and molecular techniques. This section describes the results of such experiments to consider the subunit composition of the separated GS isoenzymes, and experiments designed to investigate the changes in the GS isoenzymes during nodule development and in different cultivars. The question of GS subunit assembly is not limited to nodule tissue. Multiple GS polypeptides have been identified in other *P. vulgaris* organs (Lara *et al*, 1984), and a comparative study of GS isoenzymes in the developing leaf (plumule) is presented.

4.2 *P.vulgaris* NODULE GS ISOENZYMES AND THEIR SUBUNIT COMPOSITION

A crude extract was prepared from three week old *P. vulgaris* cv. Tendergreen root nodules and separated by IEC FPLC. Eluted fractions were assayed for both transferase (GSt) and semi-biosynthetic (GSs) GS activities. Four GS activity peaks were resolved (FIG 4.2A) which featured differences in their relative GSt and GSs values, termed the T:S ratio (Table 4.2). This result suggests that there are kinetic variations between each GS activity peak. When the above procedure was performed in triplicate, GSt activity profiles only varied within 5% of each other (data not shown) hence confirming the procedure was completely reproducible. In addition, when each of the four activity peaks were desalted and then rerun separately, each peak produced a single activity peak, eluting at its original gradient position (FIG 4.2A).

The GS subunit composition of each separated activity peak was determined by one and two dimensional gel electrophoresis, followed by western immunodetection (as described in chapter 3). One dimensional analysis, which separates the γ, δ and, $\beta + \alpha$ GS subunits, identified that the GS activity peak I consisted of γ and $\beta + \alpha$; peak II was composed of equimolar γ and $\beta + \alpha$; peak III contained $\beta + \alpha$, whereas peak IV ran as a doublet, similar in mobility to the leaf plastid GS control (see FIG 4.2B). To resolve whether β or α compose the three cytosolic GS isoforms, two dimensional separation and immunodetection of GS subunits was necessary (FIG 4.2C). Total nodule extracts contained two major cytosolic GS subunits, namely γ and β and a minor quantity of the α subunit. Analysis of the individual isoforms identified that peak I was composed mainly of γ and a little β subunit, peak II contained equimolar γ and β polypeptides, whereas peak III consisted of mainly β with a trace of the α subunit. Peak IV was found, as suggested from figures 4.2B and 4.2C, to consist of two GS subunits which appeared distinct in both molecular weight (M_r 44,000) and isoelectrofocusing positions relative to the three cytosolic GS polypeptides, and is concluded to consist of the plastid GS subunits.

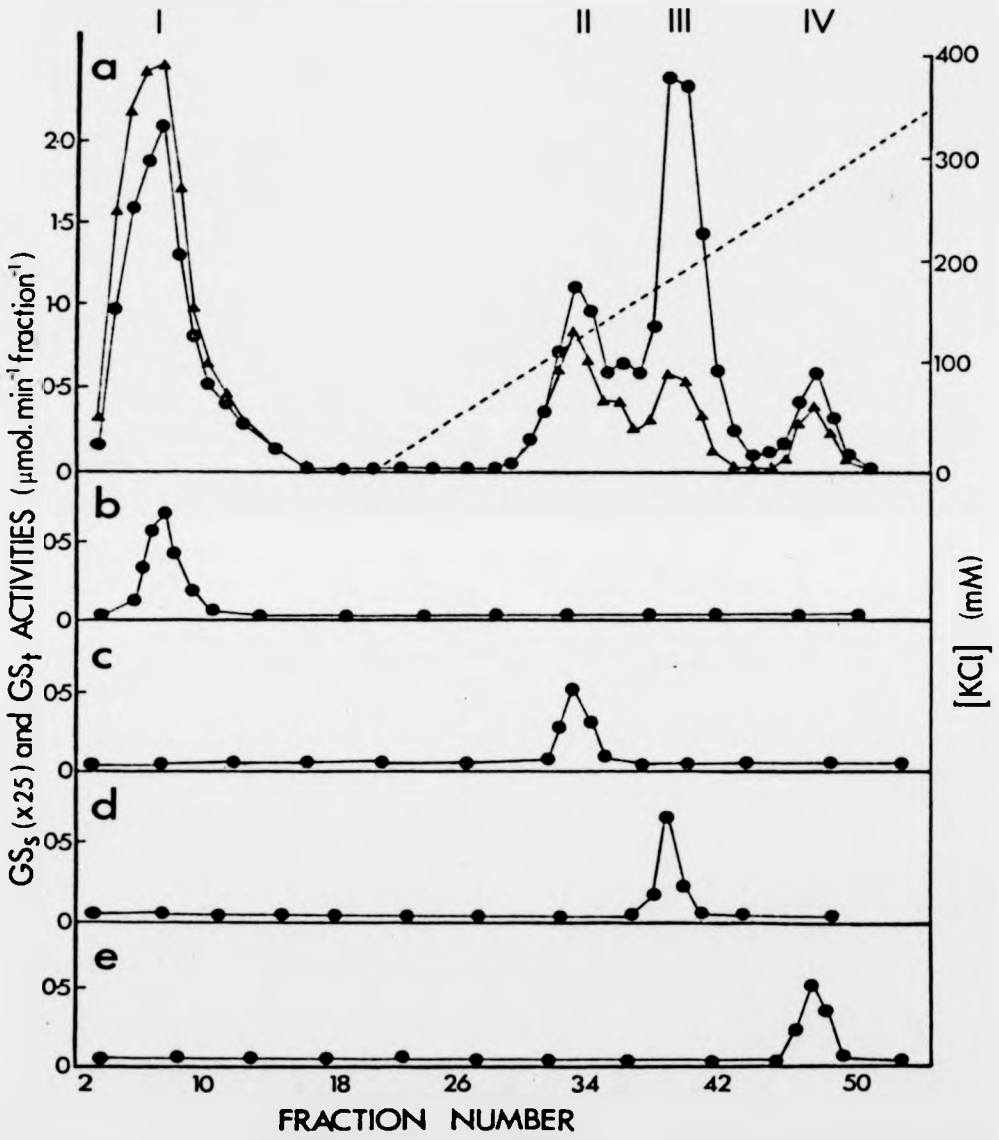


FIG 4.2A GSs (▲) and GSt (●) activity measurements of a crude nodule extract (a) fractionated by IEC FPLC, and the individual nodule GS activity peaks (I, II, III and IV respectively) (b-e), desalted and rechromatographed.

Table 4.2 GSs:GSt activity ratios for the nodule GS isoforms separated by IEC FPLC.

PEAK NUMBER	T:S ACTIVITY RATIO
I	20
II	36
III	100
IV	45
(Chloroplastic GS)	45

N.B. GSs and GSt activity values for each nodule GS isoform were obtained from peak activity fractions (see FIG 4.2A). The chloroplastic GS T:S activity ratio value was similarly obtained from GSs and GSt activity measurements of an IEC FPLC separated crude extract from 14 day old *P. vulgaris* leaf extracts.

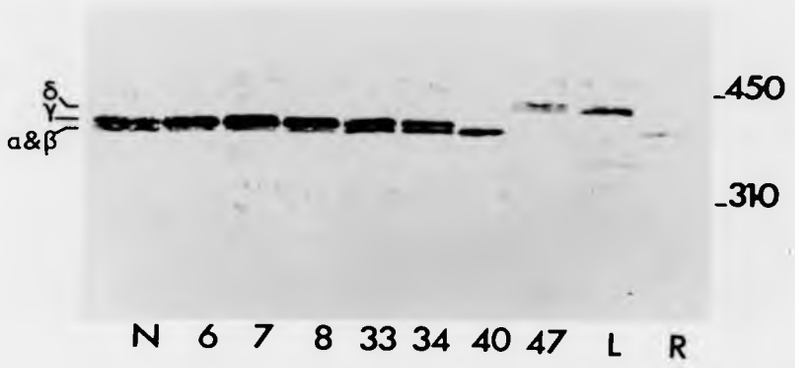


FIG 4.2B Western immunodetection of the nodule GS isoforms polypeptide composition. 250 nmoles min^{-1} of GSt activity from crude nodule (N), leaf (L) and root (R) samples, and nodule IEC FPLC fractions 6, 7, 8 (GS activity peak I), 33, 34 (peak II), 40 (peak III) and 47 (peak IV), were separated by one dimensional SDS-PAGE, western blotted and GS polypeptides visualised with peroxidase labelled anti-GS antiserum. Molecular weight markers are given in Kd.

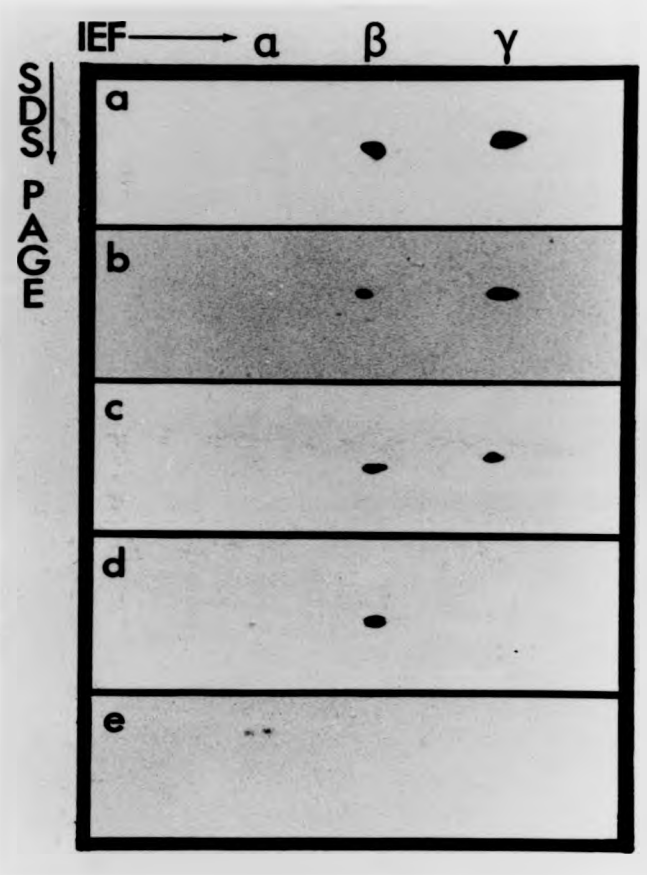


FIG 4.2C Two dimensional separation and western immunodetection of nodule GS isoforms, to determine their GS polypeptide compositions. Samples containing 250 nmoles min^{-1} of GSt activity include a crude nodule extract (a) and fractions from the IEC FPLC separated nodule GS activity peaks I, II, III and IV (b-e).

4.3 ANALYSIS OF CULTIVAR SPECIFIC VARIATION OF *P.vulgaris* ROOT NODULE GS ISOENZYMES

To determine if the cultivar specific variation in the electrophoretic mobility of the GSN1 activity band observed by Robert and Wong (1986) could be detected using IEC FPLC (as previously described), root nodule samples were analysed from three *P. vulgaris* cultivar categories. These included the group one cultivar Bush Blue Lake 274, group two cultivar Cascade and group three cultivar Red Kidney. Nodules were harvested at 14, 21 and 28 days after inoculation with *R.leguminosarum bv. phaseoli*, under similar growth conditions to those described by Robert and Wong (1986).

Nodule extracts were analysed by IEC FPLC and fractions assayed for GSt activity. Four major GS activity peaks were resolved by IEC FPLC at each developmental stage in all three *P. vulgaris* cultivars studied (see FIG 4.3A-C). The activities correlated in elution positions to GS activity peaks I to IV as described in section 4.2. Throughout the study of each cultivar, the peak III activity remained constant providing a relative guide to changes in activity peaks I, II and IV. All cultivars exhibited an increased peak II, relative to peak I, activity during nodulation, with an associated broadening of both peaks. Bush Blue Lake 274 nodules featured the highest peak II activity, being approximately double that detected in cv. Red Kidney and three times that of cv. Cascade. Bush Blue Lake 274 also contained two minor activity peaks between peaks I and II, which were barely detectable in the other two cultivars.

4.4 *P.vulgaris* DEVELOPING PLUMULE GS ISOENZYMES. SUBUNIT COMPOSITION AND ASSEMBLY

GS isoenzymes were examined, by IEC FPLC (as previously described), using plumule tissue dissected from overnight soaked *P. vulgaris* seeds. Two GSt activity peaks were detected (FIG 4.4A) consisting of a minor peak (fraction 39 to 42) and a major peak activity (fractions 44 and 45). Two dimensional electrophoresis and subsequent immunodetection (as previously described) identified that the crude plumule extract contained both ß and α GS subunits, and GS activity fraction 40 and 44 were composed only of the ß and α subunits respectively (FIG 4.4B).

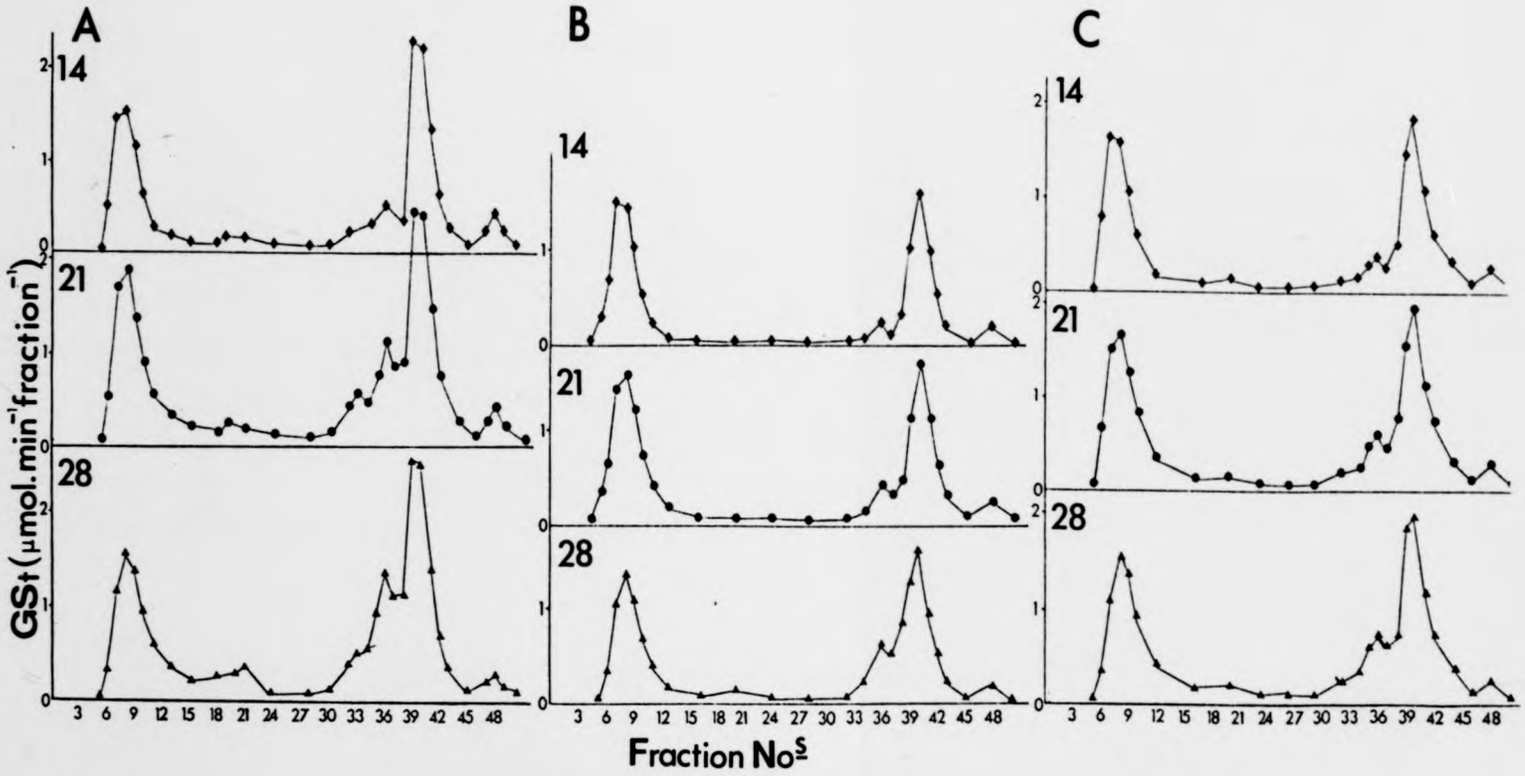


FIG 4.3 Determination of *P. vulgaris* cultivar variations in nodule GS isoform composition using IEC FPLC. GSt activity measurements of crude nodule extracts, fractionated by IEC FPLC, were prepared from nodules of *P. vulgaris* cultivars (A) Bush Blue Lake 274, (B) Cascade, (C) Red Kidney, 14, 21 and 28 days after inoculation.

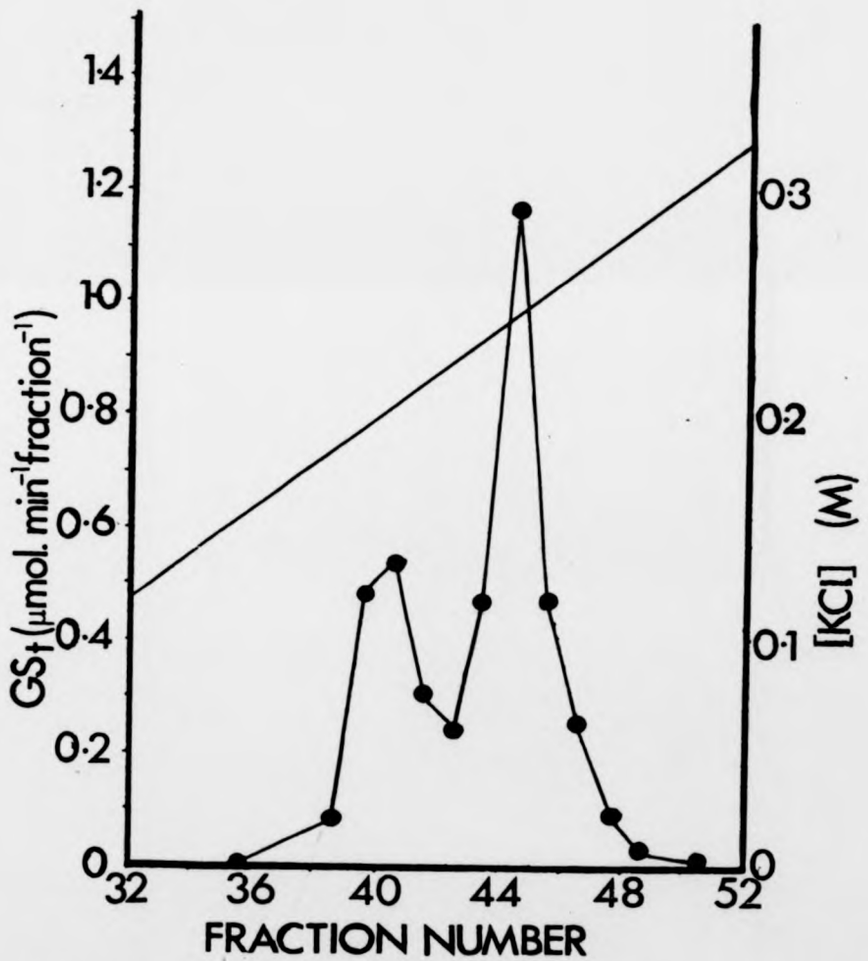


FIG 4.4A GSt activity measurements of a crude imbibed plumule extract fractionated by IEC FPLC.

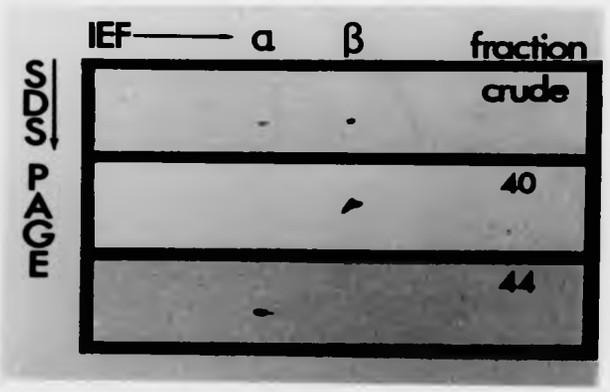


FIG 4.4B Two dimensional separation and western immunodetection of imbibed plumule GS isoforms, to determine their GS polypeptide compositions. Samples include a crude imbibed plumule extract and IEC FPLC separated GSt activity fractions 40 and 44.

Similar such IEC FPLC analysis of plumule tissue from two day germinated seeds (FIG 4.4C), identified a complex pattern of GS isoforms consisting of a major broad peak (fractions 39 to 45) containing at least two activity peaks, and a minor peak (fraction 47). Two dimensional GS subunit analysis of the fractions 40, 42 and 44 identified ß, α + ß, and α compositions respectively (FIG 4.4D). The minor peak, fraction 47, represented a plastid GS isoform, from subunit analysis (data not shown).

An experiment was designed to determine whether the mixing of the α and ß GS subunits at the two day germinated stage was an extraction artefact, rather than representative of the GS isoenzymes present *in vivo*. Since homo-octameric α and ß isoforms can be extracted from imbibed tissue (FIG 4.4A and 4.4B), if the subunit mixing seen in two day old plumule tissue is produced *in vitro*, then it could be due to a mixing factor present in the extract of the two day old plumule tissue. To assay for such a mixing activity, a two day germinated tissue extract was prepared and treated with anti-GSN1 antiserum and protein A Sepharose, resulting in the immunoprecipitation of over 80% of sample GSt activity on centrifugation. The sample supernatant (with GS removed) was then mixed with imbibed plumule tissue, the tissue homogenised in the supernatants presence, and the resulting extract analysed by IEC FPLC. The GSt profile (FIG 4.4E) appeared identical to the imbibed tissue activity profile (FIG 4.4A). Thus a GS disassembly/reassembly factor in the two day germinated plumule tissue could not be detected using the above procedure (see discussion).

4.5 DISCUSSION

This chapter has described the use of high resolution ion exchange fast protein liquid chromatography (IEC FPLC) to characterise GS isoforms from crude *P. vulgaris* nodule and plumule tissue extracts. The results appeared to reflect the true *in vivo* composition of the GS isoforms, and not represent extraction artefacts, since the technique was both reproducible and individual nodule GS isoforms eluted as single activity peaks at the same gradient position on rechromatography (FIG 4.2B). Moreover, attempts designed to detect remixing of GS subunits during plant tissue extraction failed to identify any changes in GS isoform composition (section 4.4).

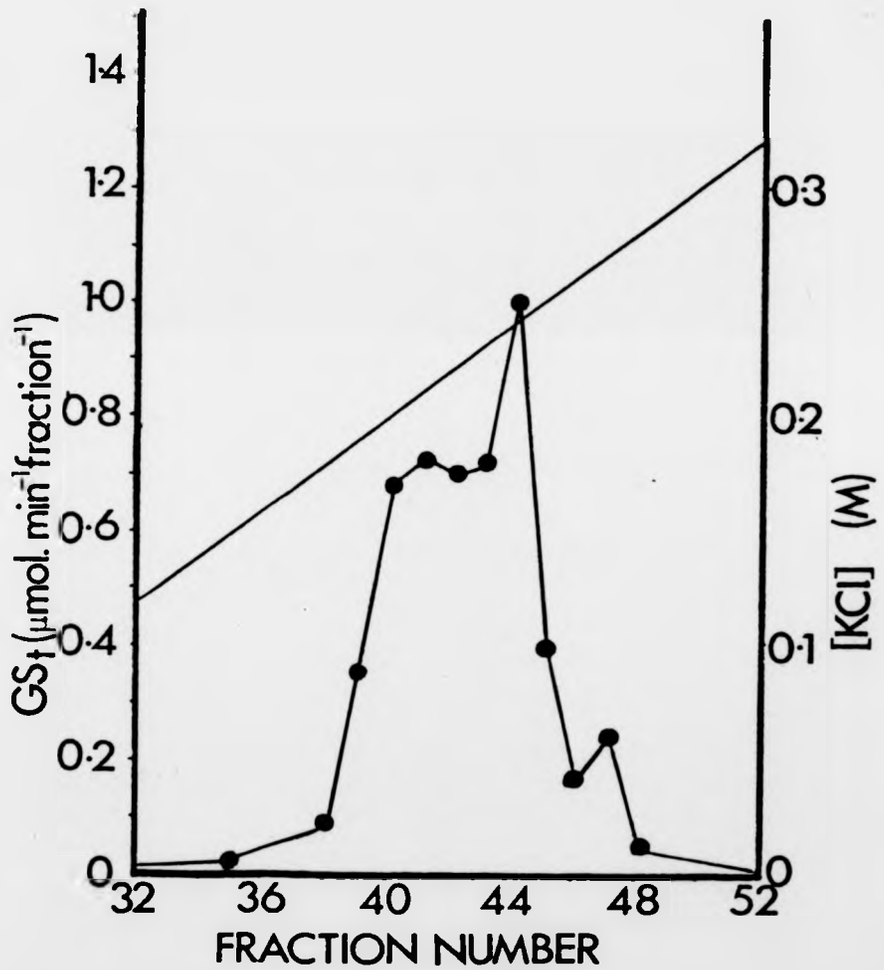


FIG 4.4C GSt activity measurements of a crude two day germinated plumule extract fractionated by IEC FPLC.

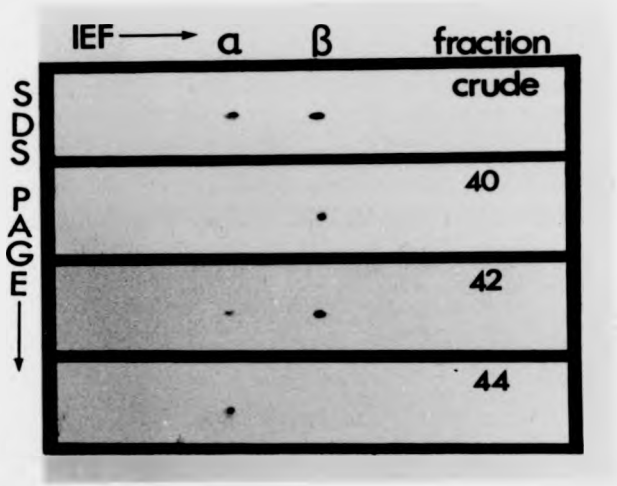


FIG 4.4D Two dimensional separation and western immunodetection of two day germinated plumule GS isoforms, to determine their GS polypeptide compositions. Samples include a crude two day old germinated plumule extract and IEC FPLC separated GSt activity fractions 40, 42 and 44.

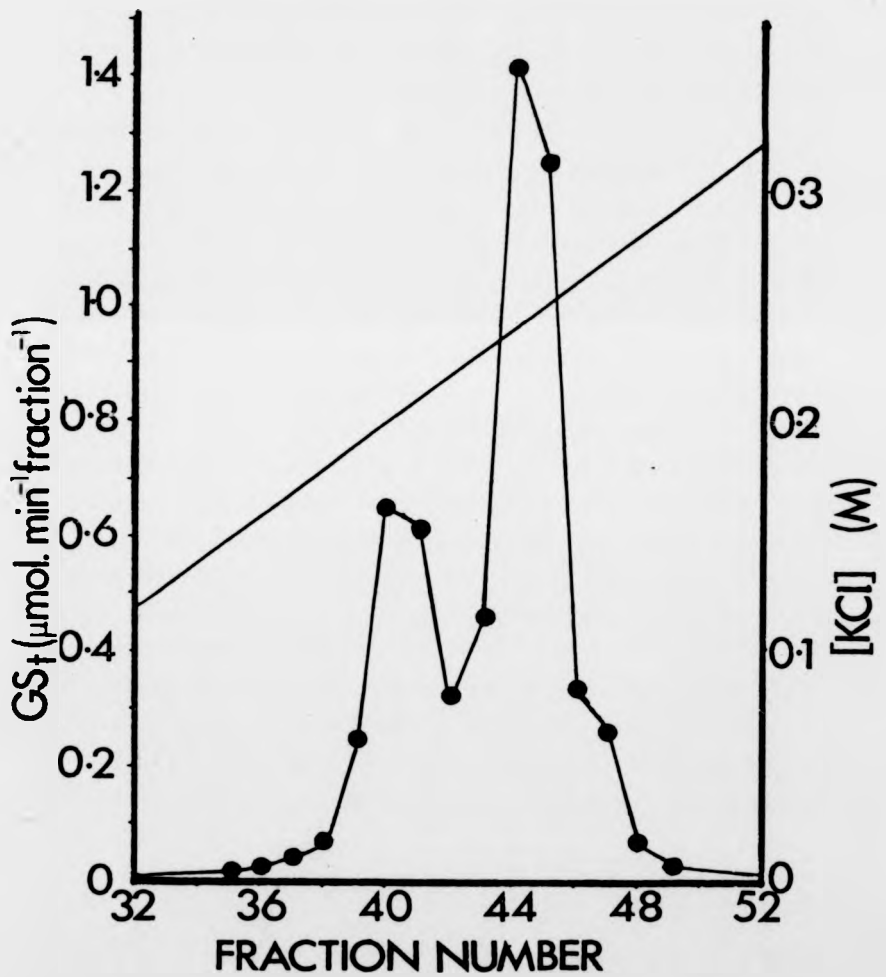


FIG 4.4E GSt activity measurements of imbibed plumule tissue extracted in the presence of a two day germinated plumule supernatant containing, through immunoprecipitation, less than 20% normal GS activity, and separated by IEC FPLC (see text for details).

P. vulgaris cytosolic GS subunits (α, ß and ϒ) appear able to assemble into GS isoenzymes in both a homologous and heterologous manner, in which α_8 and $ß_8$, and $(\alpha + ß)_8$ and $(ϒ + ß)_8$ isoforms have been identified. In nodule extracts, IEC FPLC have separated three cytosolic GS activities, composed of, mainly ϒ, ϒ + ß and ß GS subunits. This observation is in close agreement with studies by Robert and Wong (1986) who appear to have further resolved all nine possible ϒ and ß GS subunit mixing forms within the octameric enzyme. Thus it appears that all three *P. vulgaris* cytosolic GS subunits are able to mix, perhaps randomly, to produce hetero-octameric GS isoforms.

Robert and Wong (1986) have also observed the non-random mixing of the ϒ and ß nodule GS subunits, preferentially forming the ß octamer relative to the other eight ϒ and ß subunit mixing combinations. If the mixing of ϒ and ß GS polypeptides is truly random, the hetero-octamers would be in greater abundance than the homo-octameric $ϒ_8$ and $ß_8$ isoforms. Robert and Wong (1986) argued that this inconsistancy reflected the ß polypeptide's greater tendency to assemble with itself, rather than with ϒ. Alternative explanations could include the partial spatial separation of the ϒ and ß GS subunits through differences between *gln-ϒ* and *gln-ß* cellular expression patterns in which *gln-ϒ* and *gln-ß* are coexpressed within a common cell type, and *gln-ß* is also expressed in an additional cell type. This model is supported by cytochemical studies on nodulated transgenic plants transformed with *gln-ß* and *gln-ϒ* promoter GUS gene fusions (B.G.Forde *et al*, submitted). *gln-ϒ* driven GUS activity is localised in the nodule infected cell zone, whereas *gln-ß* regulated GUS activity is present in both the nodule infected and cortical regions. Alternatively, the non-random mixing of ϒ and ß GS subunits could reflect differences in the temporal expression of the *gln-ß* and *gln-ϒ* genes within the same nodule cell type. Examination of the changes in the individual *gln-ß* and *gln-ϒ* GS mRNAs during nodulation (section 5.2) has identified a constitutive *gln-ß* expression in contrast with a transient induction of *gln-ϒ* expression, which could support a temporal expression model. An additional explanation could involve both temporal and spatial expression models. Similar interpretations can be used to explain the changes in the subunit composition of the plumule GS isoenzymes of germinating *P. vulgaris* plants. Initially, overnight soaked plumule tissue contained both α_8 and $ß_8$ GS isoforms. However, within two days of

germinating, plumule tissue contained $(\alpha + \beta)_8$ heterologous isoforms (FIG 4.4A and C). Studies on the mixing and assembly of the products of other closely related plant genes have reached similar conclusions (see Gottlieb, 1982, for a review). For example, maize germinating endosperm express two sucrose synthetase genes, which encode kinetically differing subunits (Echt and Chourey, 1985), whose assembly has highlighted the role of temporal and spatial gene expression in determining isoform composition (Chourey *et al*, 1986). Furthermore, Chourey *et al* (1986) have speculated that the multiple sucrose synthetase isoenzymes could impart a greater physiological adaptability in relation to sucrose metabolism in developing seedlings, as they undergo the transition from dependence on stored endosperm to photosynthetically derived energy sources. Likewise, changes in the GS isoforms present in the developing plumule and nodule organs may provide physiological advantages with respect to nitrogen metabolism. Lara *et al* (1983) have correlated the increased GS activity during nodulation with the induction of the GSN1 isoform, suggesting that GSN1's specific function is to assimilate dinitrogen fixed NH_4^+, since the GSN2 activity is too low to perform this.

The detection of three nodule cytosolic GS isoforms by FPLC is in agreement with previous observations using conventional IEC (Cullimore *et al*, 1982;1983). FPLC nodule GS activity peaks II and III had similar T:S ratio values to the previously identified GSN1 and GSN2 isoforms (Cullimore *et al*, 1983). The authors also observed a third GS activity that eluted on the KCl gradient void, as found for the FPLC GS activity peak I. However, Cullimore *et al* (1983) noted that on rechromatography, over 80% of the void GS activity coeluted with the GSN1 isoform, in contrast with the FPLC peak I activity which reproducibly eluted on the void volume. The GS subunit compositions of the FPLC nodule isoforms are seen to differ. Peak I is composed of mainly γ but also a little β GS subunit; peak II features equimolar amounts of both γ and β GS polypeptides; whereas peak III contains mainly β with traces of α subunit. Such a description of nodule GS isoform subunit compositions is not in agreement with previous observations by Lara *et al* (1984), which may reflect the above noted differences between the conventional and FPLC IEC techniques employed to separate the GS isoforms. Lara *et al* (1984) have identified that the GSN1 and GSN2 isoforms were composed of mostly γ (and a little β) GS polypeptide, and vice versa,

respectively. In contrast, Cullimore (1985) observed, also using conventional IEC techniques, that GSN1 and GSN2 contained equimolar mixtures of γ and β, and β alone respectively, which was in agreement with the GS subunit compositions of FPLC separated nodule isoforms (section 4.2). However, the identification of the α polypeptide in the FPLC GS peak III (section 4.2) but not GSN2 (Cullimore, 1985), probably reflects the use of a two dimensional, rather than one dimensional procedure, which can separate the α and β GS subunits. Previously it was thought that the GSN2 isoform, although sharing chromatographic, immunological and kinetic characteristics with the root GS isoform, GSR, differed in its subunit composition, with the identification of the α subunit in roots but not in nodules (Lara *et al*, 1984). The subsequent detection of α subunit in nodules by Padilla *et al* (1987) and in this study, and its specific association with the FPLC GS activity peak III (homolgous to GSN2), concluded that GSN2 and GSR were also similar in containing both α and β, although the proportions of the two subunits may differ.

When the *P. vulgaris* cultivar and developmental stage specific changes in γ and β mixing patterns, observed by Robert and Wong (1986) (see section 4.1 for a description), were studied using FPLC IEC, no such differences could be detected. All examples of the three cultivar categories featured a typical four peak nodule GS activity profile, previously identified in section 4.2, throughout nodulation. There was little change in subunit mixing during nodulation except a general decrease in FPLC GS activity peak I and an increase in peak II, with an associated broadening of both activity peaks. Throughout nodule development the GS activity peak III remained constant for each of the cultivars. The greatest change in subunit mixing was seen for cv. Bush Blue Lake 274 which generated an additional two minor activity peaks during later nodulation, eluting between GS activity peaks I and II, probably representing γ rich β hetero-octamers. This pattern of increasing β subunit mixing in later developmental stages could reflect changes in the relative abundance of γ and β mRNA and thus, their GS subunits (as described in section 5.2). The inability to detect developmental changes in nodule GS γ and β subunit mixing for individual *P. vulgaris* cultivars, as observed by Robert and Wong, could be due to the differences between the plant growth conditions or the separative techniques employed. Robert and Wong (1986) were able to detect all

nine ϒ and ß GS subunit mixing possibilities, whereas the IEC FPLC procedure employed in this chapter resolved, at most, five cytosolic GS isoforms. Thus, slight changes in subunit mixing, identified by Robert and Wong (1986), would remain undetected using IEC FPLC.

The identification of a plastid GS activity and polypeptides in *P. vulgaris* root nodules is in agreement with the detection of *gln-ð* mRNA in nodules (Lightfoot *et al*, 1988; Bennett *et al*, submitted). A previous *P. vulgaris* root nodule subcellular fractionation study (Awonaike *et al*, 1981) detected a minor plastid located GS activity. Furthermore, a recent nodule organellar fractionation study has successfully detected the plastid GS isoform associated with nodule plastids (Chen and Cullimore, pers. commun.). There have also been reports of a plastid GS activity in pea roots (Miflin, 1974; Vezina *et al*, 1987) contrary to other observations in pea, bean, barley and rice (Oaks and Gadal, 1979; Suzuki *et al*, 1981), suggesting that the plastid GS activity may have a role in non-green tissue nitrogen metabolism. Nodule plastid GS runs as two GS polypeptides, when separated by two dimensional gel electrophoresis (FIG 4.2C), in contrast with etiolated and green leaf plastid GS that are composed of three and four GS polypeptides respectively (Lara *et al*, 1984). These observations may reflect differences in presequence processing or post-translational modification to the plastid GS precursor occurring in nodule and leaf plastids.

CHAPTER 5

THE REGULATION OF EXPRESSION OF GS IN ROOT NODULES OF *PHASEOLUS VULGARIS* L.

5.1 INTRODUCTION

All previous studies of changes in *P. vulgaris* GS during nodulation have featured the separate analysis of GS mRNAs, polypeptides and isoenzymes (Lara *et al*, 1983; Gebhardt *et al*, 1986; Padilla *et al*, 1987). The following section describes a complete integrative study of the changes in the expression of the major GS mRNAs, their polypeptides and isoenzymes in relation to nodule development. In addition, the regulation of nodule GS in relation to dinitrogen fixation and its product, NH_4^+, has been examined by growing nodules in an atmosphere of 80% Argon: 20% Oxygen ($Ar:O_2$).

5.2 CHANGES IN GS mRNAs, POLYPEPTIDES AND ISOENZYMES DURING *P.vulgaris* NODULE DEVELOPMENT

Seedlings of *P. vulgaris* cv. Tendergreen were inoculated with *Rhizobium leguminosarum bv. phaseoli* and nodules harvested at different times of development. Note that at 5, 8 and 10 days after inoculation the top 5 cm of the root systems were harvested as the nodules were too small to be picked individually.

Tissue samples were assayed for GS transferase (GSt) and semibiosynthetic (GSs) activities in conjunction with NADH-GOGAT and nitrogenase acetylene reduction measurements (see Chen and Cullimore, 1988). GS activity measurements were expressed both as per mg soluble protein and per g fresh weight (FIG 5.2A and B). GSs activities, expressed using either parameter, featured an initial increase at day 10, followed by a substantial increase at day 12, continuing to rise until day 18, representing (relative to the day 8 sample), either a 5- or 13- fold increase overall if expressing the activity as per mg soluble protein or per g fresh weight respectively. The GSs activity subsequently decreased approximately 50% by day 24. In contrast, the GSt activities showed an overall 5- fold and 1.5- fold increase from days 8 to 16 when expressed as per gram fresh weight or per mg soluble protein respectively. The large increases in GSt and GSs activities between days 10 to 12 must be attributed in part to harvesting individual nodules at day 12, compared to isolated root systems at days

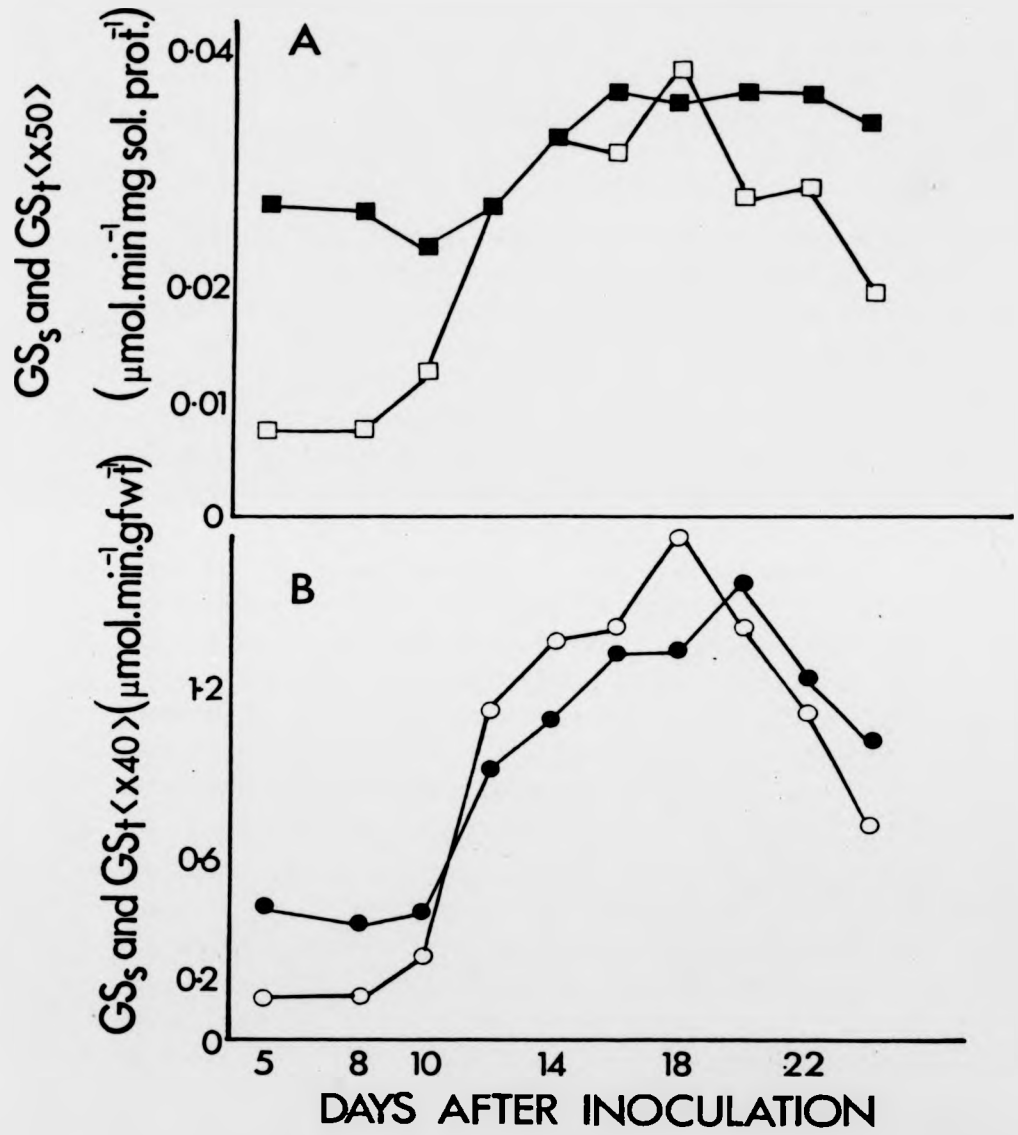


FIG 5.2A Changes in GS synthetase (GSs, □) and transferase (GSt, ■) specific activities (per mg soluble protein) during nodule development.

FIG 5.2B Changes in GS synthetase (GSs, ○) and transferase (GSt, ●) activities (per g fresh weight) during nodule development.

5 to 10. However, due to root tissue possessing a high GSt: GSs activity ratio and a low protein content per g fresh weight (Cullimore, 1982), the GSs activities expressed per g fresh weight describes the greatest induction of GS activity during nodulation. This reason may reflect the limited induction of GS observed by Padilla *et al* (1987) who only observed a 1.7- and 2.7-fold increase in GSs and GSt activity values during a comparable nodule developmental study for *P. vulgaris*.

Crude tissue extracts were analysed by IEC FPLC (as described by section 4.2) to determine the GS isoforms present at each nodule developmental stage. Initially, at days 5 and 8, two GSt activity peaks were identified (FIG 5.2C). The major activity peak represented a ß octameric isoform, whereas the minor peak eluted at a position similar to the α isoform (see section 4.4). At day 10 the minor α activity decreased and a new GSt activity was detected eluting on the void volume representing the ϒ rich isoform. Day 12 featured a rapid increase in the ϒ rich isoform and the appearance of the ϒß hetero-octameric isoform. Day 14 exhibited an increase in all 3 isoform activities and the development of the nodule plastid GS activity (see section 4.2). At day 16, the ϒ rich isoform had reached its maximal value and subsequently decreased, particularly between days 22 and 24. In contrast the ϒß hetero-octameric isoform became a more significant GSt activity. Approximately constant plastid and ß GSt activities were detectable throughout this later nodulation period.

The GSt activity assay was used in the analysis of nodule isoforms because of its sensitivity. However, it has been shown that ϒ rich, ϒß, ß and plastid GS isoforms display different GSt: GSs activity ratios of 20, 36, 100 and 45 respectively (see section 4.2, Table 4.2). Using these values the GSs activity for each isoform during nodule development was calculated as a percentage of the total GSs activity (per mg soluble protein) (FIG 5.2D). The increase in nodule GS activity at day 10 occurred concomitantly with the induction of the ϒ rich isoform. By day 16 the ϒ rich and ϒß isoforms represented 71 and 14% of the total GSs activity respectively, and comparable to previous estimations of 84% for the ϒß hetero-octameric GSN1 isoform (Lara et al, 1983). After day 16 the ϒß isoform gained greater kinetic importance as the ϒ rich isoform decreased in activity value. The plastid GS activity was present throughout later stages of nodulation, albeit at low levels.

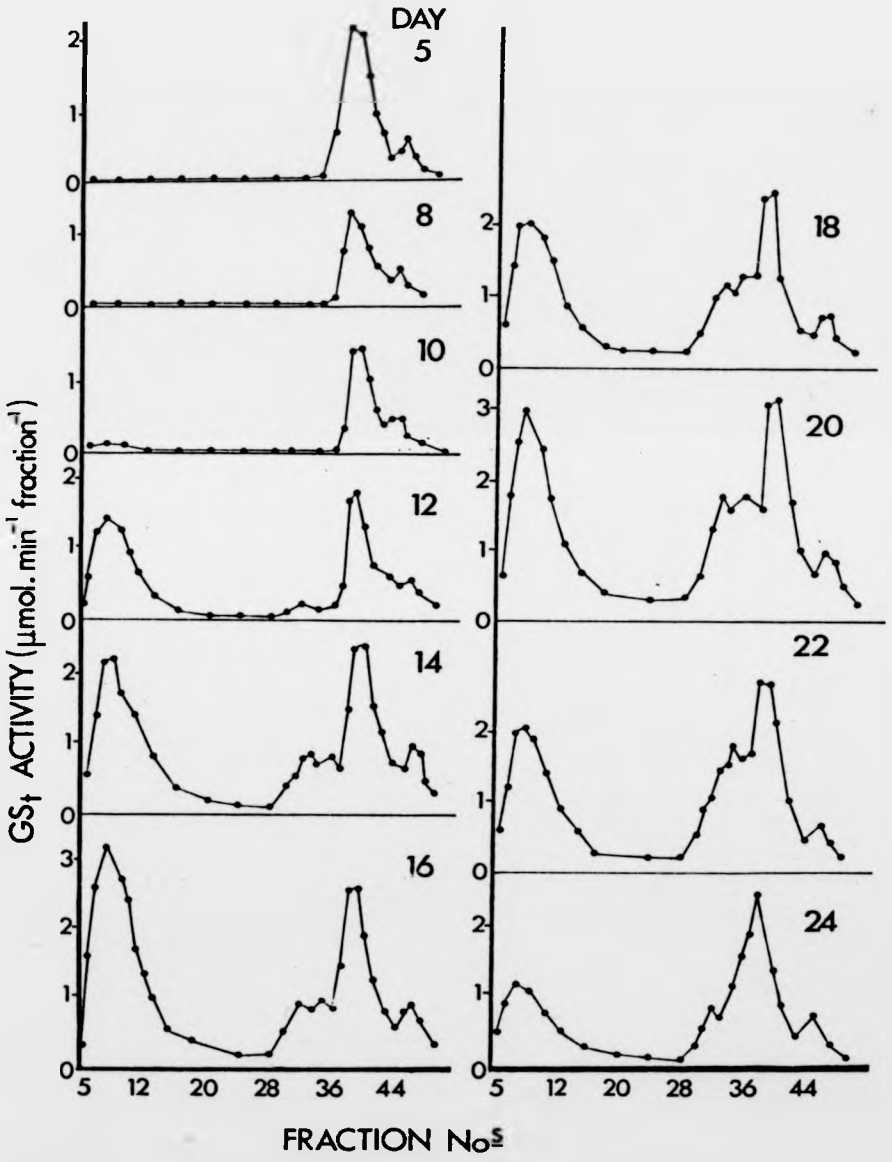


FIG 5.2C Changes in GS isoform composition during nodule development. Crude nodule extracts containing equal quantities of soluble protein were fractionated by IEC FPLC and assayed for GSt activity.

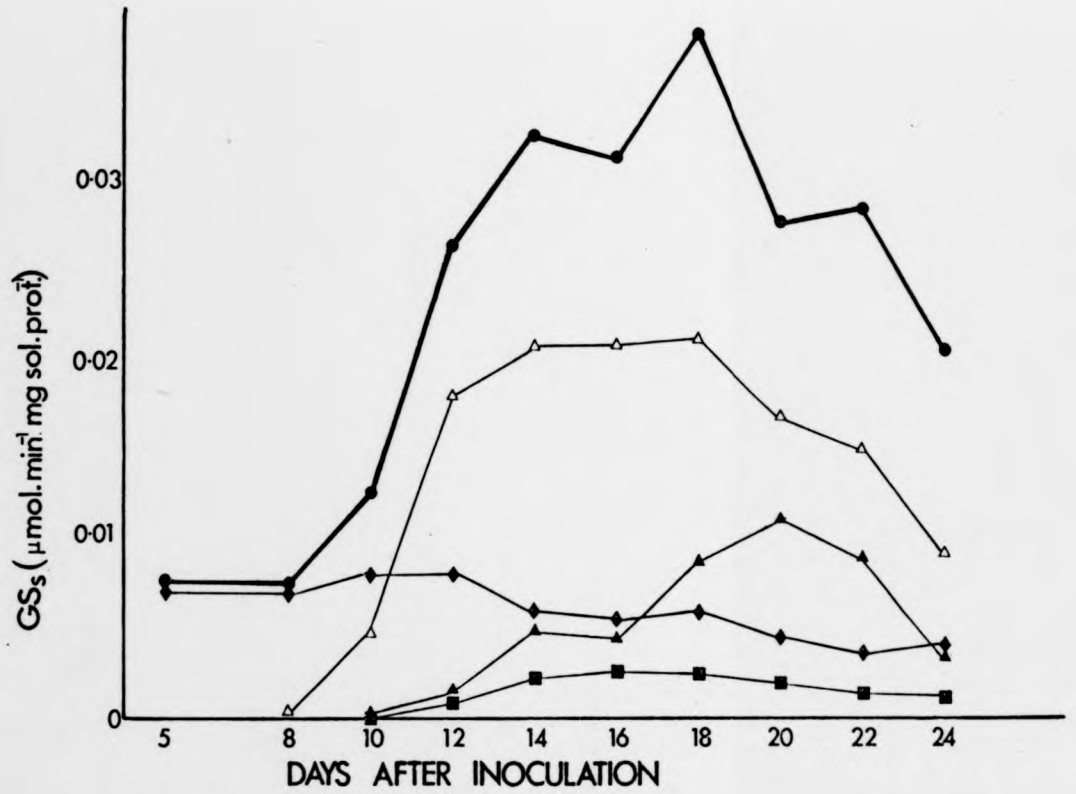


FIG 5.2D Changes in the relative importance of the individual nodule GS isoforms with respect to total nodule GSs activity. GSt activity values obtained for the individual GS isoforms at different stages of nodulation (see FIG 5.2C), were converted to GSs activity values using the transferase to synthetase activity ratios values determined in chapter 4 (see Table 4.2). Total nodule GSs activity (-●-); γ rich nodule GS isoform (-△-); γß isoform (-▲-); ß isoform (-◆-); and plastid GS isoform (-■-).

Polypeptide analysis of the nodule ϒ and β GS subunits using a one dimensional SDS-PAGE immunodetection technique (as described in section 3.4), loading samples containing an equal quantity of GSt activity, showed that although the β GS subunit was initially the major GS subunit, the ϒ polypeptide became the predominant nodule GS subunit (FIG 5.2E). ϒ was first detectable, at very low levels at day 8, becoming approximately equimolar with β at day 10 and reaching its maximum abundance relative to the β subunit at day 12. From days 14 to 22, ϒ gradually decreased in relative abundance. 2-D immunodetection (as described in section 4.2) confirmed that the ϒ and β polypeptides were the only major GS subunits throughout nodulation (data not shown).

Each tissue sample was also analysed for *gln-ϒ*, *gln-β* and *Lhb* mRNA (FIG 5.2F) using an RNase protection procedure (as previously described in section 3.4) with probe-ϒB, probe-β and probe-Lhb (see materials and methods, section 2.64). Both *gln-ϒ* and *Lhb* mRNA were first detectable at day 8, increasing in abundance until day 12, and then gradually decreasing about 3- fold by day 22. In contrast, the *gln-β* mRNA levels remained approximately constant throughout nodule development. The abundance of *gln-ϒ* and *gln-β* mRNA at day 12 were estimated from the standard curves to be about 25 and 15 pg per µg total RNA respectively, whereas the p*Lhb*-1 homologous mRNA was approximated from the intensities of the autoradiograph to be in at least a 100- fold higher abundance than *gln-ϒ* mRNA.

5.3 NODULE GS REGULATION UNDER $Ar:O_2$ AND $N_2:O_2$ ATMOSPHERIC GROWTH REGIMES

Seedlings of *P. vulgaris* cv. Tendergreen were inoculated with *Rhizobium leguminosarum bv. phaseoli* R4962. Two days after inoculation (day 2) individual developing plants were sealed into a total of 12 air tight containers and their root systems subsequently grown under either an 80% : 20% $N_2:O_2$ (5 pots in total) or an 80% : 20% $Ar:O_2$ atmosphere (7 pots) (see materials and methods, section 2.1 for further details). Throughout the experiment sample pots growing under an $Ar:O_2$ regime were periodically monitored for contaminating dinitrogen. All such pots contained no more than 0.4% dinitrogen at any one time. Experiments were undertaken on samples between days 15 and 19 after inoculation to

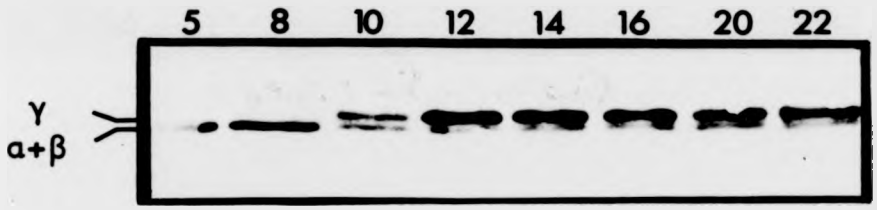


FIG 5.2E Western blot to show changes in the relative abundances of the different GS polypeptides during nodule development. A sample containing an equal GSt activity of 250 nmoles min^{-1} from each time point was separated by one-dimensional SDS-PAGE, western blotted and detected by peroxidase labelled anti-GS antiserum.

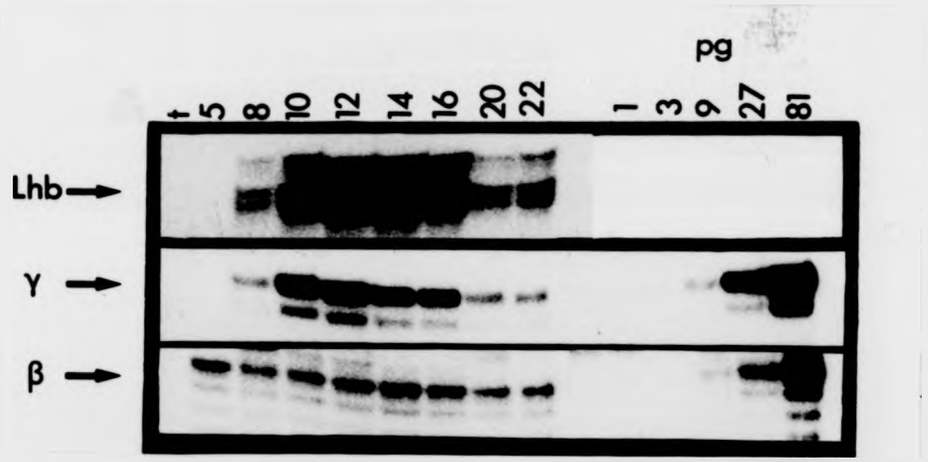


FIG 5.2F Changes in the abundances of mRNA of the *gln-ß*, *gln-γ* and *Lhb* genes during nodule development. Yeast tRNA (t) and samples of total RNA isolated from nodulating root systems (days 5, 8 and 10) and isolated nodules (days 12, 14, 16, 20 and 22) were analysed by RNase protection. 1 µg samples were analysed with the *Lhb* probe, and 2 µg samples with the γ and ß probes. A calibration curve for quantifying the GS mRNAs were set up using different amounts (1, 3, 9, 27 and 81pg) of *in vitro* synthesised (m) RNAs. The *Lhb* autoradiograph was exposed for a fifty fold shorter period.

remove the separate effects of GS induction prior to day 15 and nodule senescence at day 20 onwards (see section 5.2), and thus instead observing the physiological effects of the nitrogen supply on the levels of nodule proteins. At day 15 one N_2:O_2 and one Ar:O_2 pot were harvested. Two N_2:O_2 pots were also switched to an Ar:O_2 atmosphere, and *vice versa*, to investigate the short term effects of dinitrogen removal or addition. In addition, two Ar:O_2 pots were supplied with an NH_4^+ solution applied to the upper nodulated root system. At day 16, single pots from N_2:O_2 and Ar:O_2 grown, N_2:O_2 to Ar:O_2 and Ar:O_2 to N_2:O_2 switches, and NH_4^+ supplied Ar:O_2 samples were harvested. Similarly the remaining pots were harvested at day 19. Note that at day 19, some samples showed signs of nodule senescence. Ar:O_2 and N_2:O_2 to Ar:O_2 switched samples featured a slight green nodule colouration and similarly the NH_4^+ supplied Ar:O_2 sample exhibited a brown nodule discolouration. In contrast the N_2:O_2 and Ar:O_2 to N_2:O_2 switched samples displayed a good red nodule colouration. Tissue samples were analysed for their total GS transferase (GSt) activity (FIG 5.3A), and their activities expressed as per g fresh weight. At day 15 the N_2:O_2 sample gave a 50% higher GSt activity relative to the Ar:O_2 grown sample. The day 16 N_2:O_2 and N_2:O_2 to Ar:O_2 switch samples initially featured an increased GSt activity. However, between days 16 to 19 the N_2:O_2 to Ar:O_2 sample exhibited a steep decline in GSt activity, whereas the N_2:O_2 samples value continued to rise. Between days 15 to 19 the Ar:O_2 samples GSt value dropped steadily. In contrast the switch from an Ar:O_2 to a N_2:O_2 regime resulted in an immediate and sustained rise in activity until day 19. The addition of NH_4^+ to Ar:O_2 grown nodulated root systems resulted in an overall substantial decrease in activity particularly between days 16 and 19.

The GS isoforms were analysed by IEC FPLC, using samples containing an equal fresh weight of nodule tissue, as previously described in chapter 4. Control samples grown under a N_2:O_2 regime initially featured 3 of the 4 isoenzymes described in section 4.2 (i.e. the γ rich, β and plastid isoforms) (FIG 5.3B). Between days 15 and 16 all three GS isoform activities increased significantly and the γ rich isoform continued to do so until day 19 when further isoforms containing mixtures of both γ and β subunits were generated. Samples transferred from a N_2:O_2 to an Ar:O_2 atmosphere at day 15, contained a GS activity profile similar to the 16 day N_2:O_2 control sample (FIG 5.3C). However

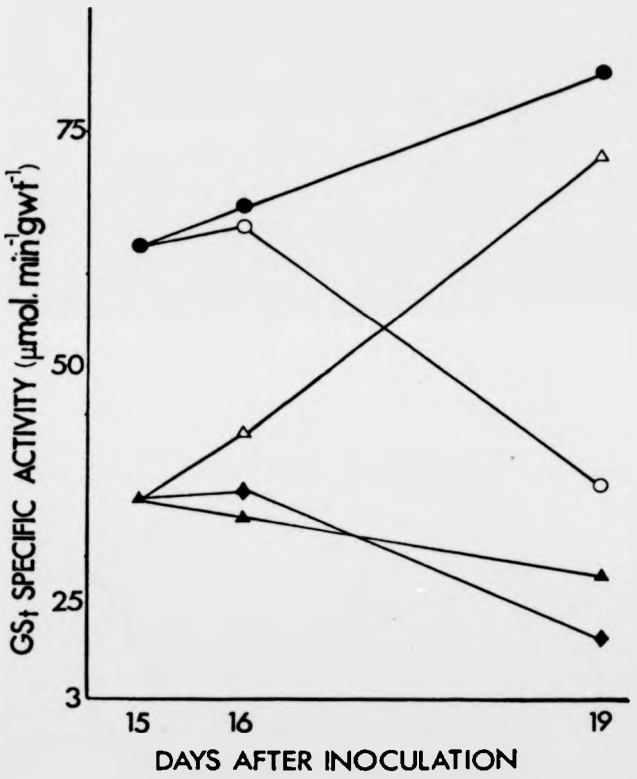


FIG 5.3A Changes in GSt activity of nodules grown under a variety of physiological conditions. Samples were either grown in N_2:O_2 (●), or switched from N_2:O_2 to Ar:O_2 at day 15 (○), or Ar:O_2 (▲), or switched from Ar:O_2 to N_2:O_2 at day 15 (△), or Ar:O_2 grown nodules were treated with NH_4^+ (◆).

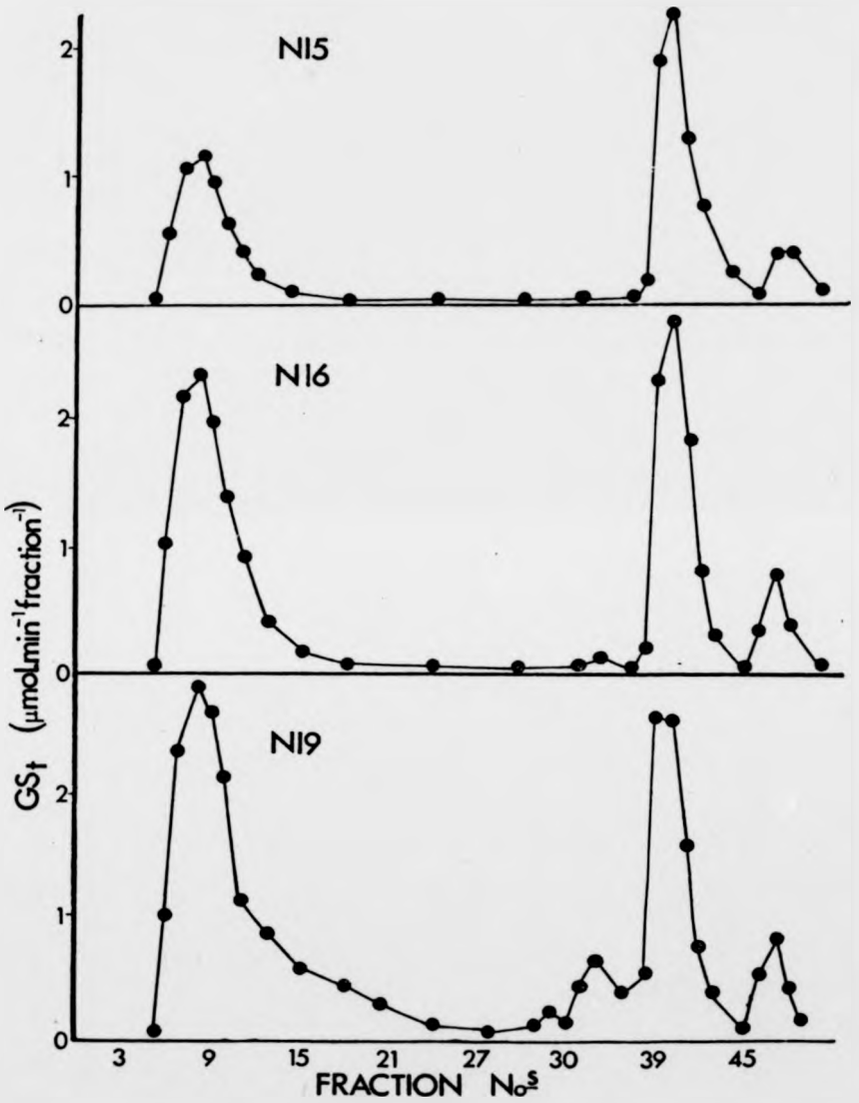


FIG 5.3B Changes in the GS isoenzyme composition of nodule samples grown under a N_2:O_2 atmosphere.

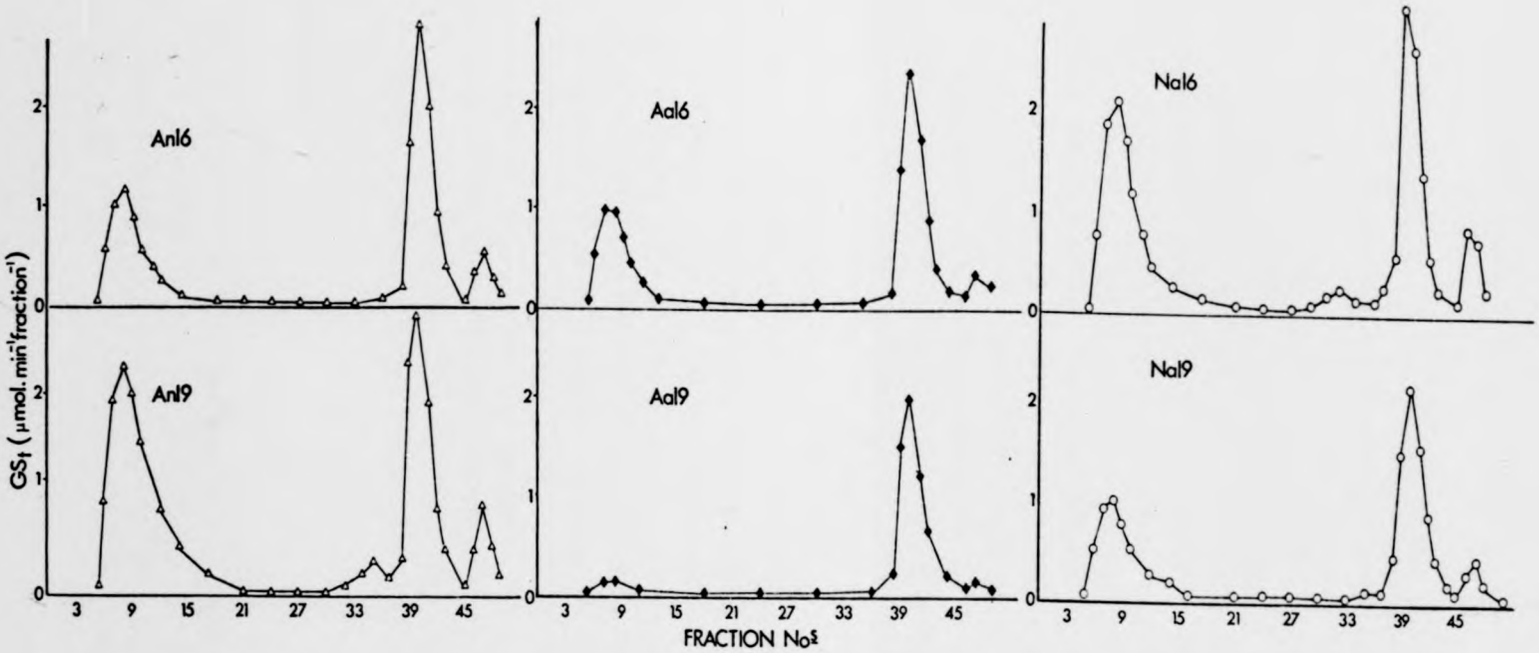


FIG 5.3C GS isoenzyme composition of day 16 and 19 day nodules either switched switched (at day 15) from $Ar:O_2$ to $N_2:O_2$ (An), or grown in $Ar:O_2$ and treated with NH_4^+ (Aa), or switched from $N_2:O_2$ to $Ar:O_2$ (Na).

at the later day 19 stage the sample exhibited reduced levels of all 4 isoforms. The γ rich and plastid GS isoforms were particularly reduced (approximately 50%) whereas the β isoform displayed a smaller activity decrease (about 30%). Samples grown under an $Ar:O_2$ regime throughout nodulation contained only 3 of the 4 nodule isoforms, with no γβ isoforms observed (FIG 5.3D). The low γ rich and plastid GS activities remained approximately constant, and the β isoform decreased 20%. When nodulated root systems were transferred from an $Ar:O_2$ to a $N_2:O_2$ atmosphere at day 15, by day 16 all three isoforms had increased approximately 40% (FIG 5.3C). This rise in activity continued until day 19 which represented an overall 2- to 3- fold increase for the γ rich and plastid GS isoforms respectively, compared to the day 15 sample. In addition, at day 19 the appearance of the γβ rich hetero-octamer was observed. Treatment of $Ar:O_2$ grown nodulated root systems with an NH_4^+ solution resulted in a 90% decrease in the γ rich and plastid GS activities by day 19, whereas the β isoform activity had only decreased by approximately 20% (FIG 5.3C).

Polypeptide analysis of the γ and β GS subunits using a one dimensional SDS-PAGE immunodetection technique (FIG 5.3F) observed that at day 15 both $Ar:O_2$ and $N_2:O_2$ samples featured similar γ and β subunit abundances. However, between days 15 and 19, the $N_2:O_2$ control sample exhibited an increased abundance of both β and particularly γ, whereas both GS subunits levels were reduced in $Ar:O_2$ grown samples and the addition of NH_4^+ failed to increase, if not further decreased, the level of both subunits at later stages. In contrast, the switch of growth regime from $Ar:O_2$ to $N_2:O_2$ resulted, although not immediately, in a substantial increase in γ and β subunit abundance, whereas the reverse switch produced a progressive reduction of both GS polypeptides.

5.4 DISCUSSION

This chapter has described the regulation of GS in the nodule. During nodule development the induction of *gln*-γ gene expression, first detected eight days after inoculation (FIG 5.2F) occurs at least one day prior to the onset of dinitrogen fixation (see Chen and Cullimore, 1988), as previously described by Padilla *et al* (1987). In addition, *gln*-γ gene induction occurs concomitantly with the appearance of a *Lhb*

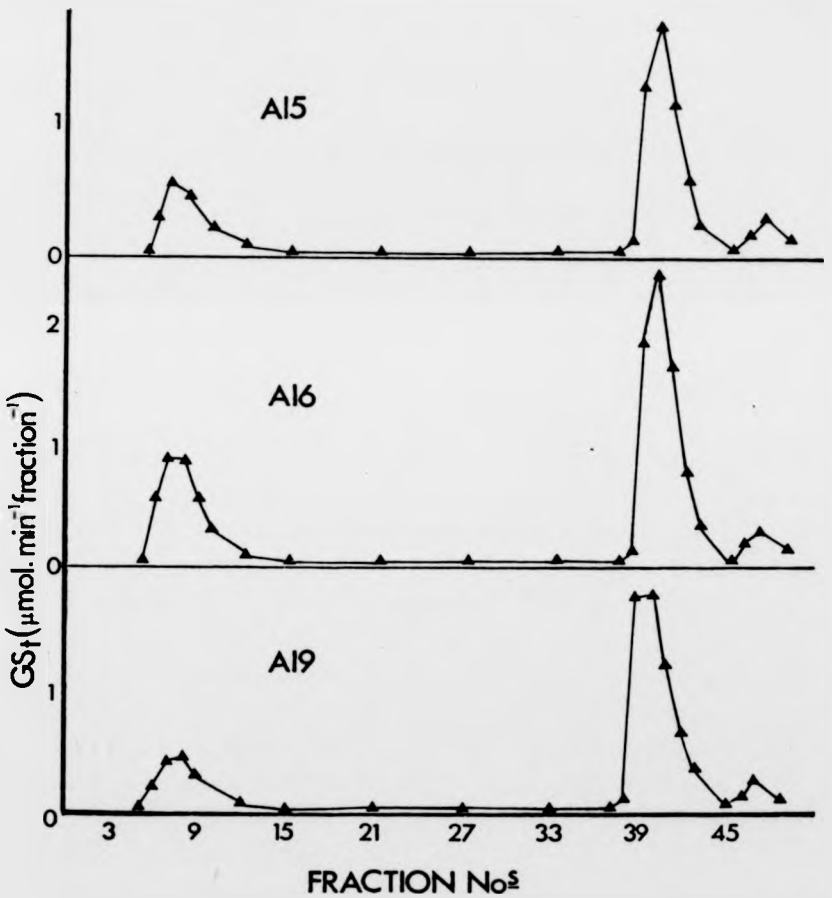


FIG 5.3D Changes in the GS isoenzyme composition of nodule samples grown under an $Ar:O_2$ atmosphere.

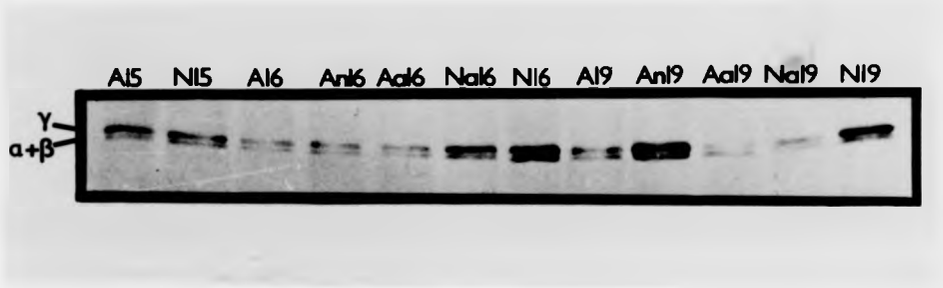


FIG 5.3E Western blot to show changes in the relative abundances of the different GS polypeptides during growth under different physiological conditions. 100 µg of soluble nodule protein from 15 day $Ar:O_2$ (A15) and $N_2:O_2$ (N15) grown samples; 16 day $Ar:O_2$ (A16), $Ar:O_2$ to $N_2:O_2$ switched (An16), $Ar:O_2$ treated with NH_4^+ (Aa16), $N_2:O_2$ to $Ar:O_2$ switched (Na16) and $N_2:O_2$ (N16) grown samples; and 19 day $Ar:O_2$ (A19), $Ar:O_2$ to $N_2:O_2$ switched (An19), $Ar:O_2$ treated with NH_4^+ (Aa19), $N_2:O_2$ to $Ar:O_2$ switched (Na19) and $N_2:O_2$ (N19) grown samples, were separated by one-dimensional SDS-PAGE, western blotted and probed with anti-GS antiserum.

mRNA, and therefore with a variety of *P. vulgaris* nodulin genes (Campos *et al*, 1987; Sanchez *et al*, 1987), as well as the plastid GS mRNA (J.V.Cullimore in Bennett *et al*, submitted). Both *gln-γ* and *Lhb* genes are transiently induced, which is in agreement with the expression patterns of other *P. vulgaris* nodulins studied (Campos *et al*, 1987; Sanchez *et al*, 1987). In contrast, the abundance of *gln-β* mRNA, as previously observed by Gebhardt *et al* (1986), remains constant during nodulation (FIG 5.2F), whereas the *gln-α* mRNA is at a greater abundance in the young nodulating root systems compared to mature nodules (Bennett *et al*, submitted). Therefore, the GS genes are not coordinately regulated in nodules, and their transcripts accumulate to different abundances. However, the *gln-γ* and *Lhb* genes (as well as the *gln-δ* mRNA [Bennett *et al*, submitted]), appear to be regulated in a similar manner, which contrasts with the differences in regulation between the soybean GS and *Lhb* genes (Sengupta-Gopalan and Pitas, 1986; Hirel *et al*, 1987; see section 1.5).

The abundance of *gln-γ* mRNA and the appearance of the γ polypeptide have been monitored in separate investigations (see Gebhardt *et al*, 1986; Padilla *et al*, 1987), and the two events are hence difficult to correlate. However, this study (section 5.2) has directly correlated the increase in nodule GS activity to the induction of *gln-γ* gene expression, the concomitant detection of the γ polypeptide and the appearance of the γ GS isoform. This correlation suggests that the initial synthesis of γ is largely under transcriptional, and not translational, control.

Padilla *et al* (1987) have proposed that the developmental induction of the γ mRNA and the variable proportion of the GS subunits could explain the formation of different GS isoforms during development. It is feasible that the relative ratios (and cellular distribution) of *gln-γ* and *gln-β* mRNAs during nodulation determined the formation of the IEC FPLC separable γ and γβ isoforms. The initial induction and high level expression of the *gln-γ* gene would result in the preferential formation of the γ rich isoform, whereas later, the decrease in *gln-γ* relative to *gln-β* mRNA abundances, would lead to the increased activity of the γβ hetero-octameric isoforms. This expression model describes the observed pattern of changes of the nodule GS isoforms during nodulation (FIG 5.2C and D), and can also be used to interpret the

changes in nodule GS isoforms during growth under $Ar:O_2$ growth conditions (FIG 5.3B, C and D; J.V.Cullimore, pers.commun.).

Chen and Cullimore (1988) have identified two nodule NADH-GOGAT isoenzymes, termed NADH-GOGAT I and NADH-GOGAT II. The NADH-GOGAT II activity appears to be nodule specific, and is developmentally expressed with similar induction kinetics to the GS γ isoform, suggesting that the NADH-GOGAT II and *gln*-γ genes are regulated in a similar manner, as were other *P. vulgaris* nodulin genes (Campos *et al*, 1987; Sanchez *et al*, 1987).

The role of dinitrogen and its fixed product, NH_4^+, in the regulation of nodule GS, has been examined by growing nodulated *P. vulgaris* roots under an 80% argon: 20% oxygen atmosphere. In a previous such study using nodulated cowpea plants, Atkins *et al* (1984b) could detect an induction of GS activity concomitant with nitrogenase and *Lhb* expression. In contrast, Hirel *et al* (1987) observed, measuring changes in total soybean *Lhb* and GS mRNAs, that GS mRNA remained at a similar abundance to uninoculated roots. In *P. vulgaris* $Ar:O_2$ grown nodules, reduced levels of nodule GS activity (FIG 5.3B), GS polypeptides (FIG 5.3F) and GS mRNA (J.V.Cullimore, pers.commun.) were detected, relative to the $N_2:O_2$ control nodules. However, the ability to detect *gln*-γ mRNA, γ polypeptide and γ isoforms, in the absence of dinitrogen, meant that dinitrogen fixation did not provide the primary signal for the induction of the *gln*-γ gene. This conclusion is in agreement with the ability to detect *gln*-γ gene expression two days prior to dinitrogen fixation (Padilla *et al*, 1987; section 5.2), and the presence of the γ isoform in *P. vulgaris* nodules inoculated with *Fix*$^-$ mutants of *R.leguminosarum bv. phaseoli* (Lara *et al*, 1983). Instead, studies using a variety of rhizobial mutants that block nodulation at a number of developmental stages, have correlated the induction of nodule GS expression (Sengupta-Gopalan and Pitas, 1986; Dunn *et al*, 1988; section 1.5) with infection thread formation and intracellular bacterial release (see section 1.4 for further discussion). However, a product of dinitrogen fixation may be involved in the magnitude and/or maintenance of the plant nodule GS expression. This can be examined by transferring $N_2:O_2$ grown nodules to an $Ar:O_2$ regime, thus inhibiting bacteroid mediated dinitrogen fixation. $N_2:O_2$ to $Ar:O_2$ switched samples feature reductions in nodule GS activity (FIG 5.3A), GS polypeptides (FIG 5.3C) and mRNA levels (J.V.Cullimore, pers.commun.), which is particularly

marked for the *gln*-Y gene products. To directly determine if the reduction in *gln*-Y gene products in $Ar:O_2$ grown plants is related to the removal of NH_4^+, an extranodular source of NH_4^+ was provided to established nodulated roots grown under an $Ar:O_2$ regime. Such treatment resulted in an accelerated nodule senescence and decrease of GS activity (FIG 5.3A), GS polypeptides (FIG 5.3C) and mRNA (J.V.Cullimore, pers.commun.), relative to $Ar:O_2$ grown control nodules. Therefore, the addition of an extranodular source of NH_4^+ is insufficent to induce nodule GS expression. Groat and Vance (1982) have concluded, after failing to detect increases in GS and NADH-GOGAT activities of Fix^- mutant alfalfa nodules treated with NH_4^+ or nitrate, that the increases in these two activities during normal nodulation is not related to the increased levels of NH_4^+ coming from dinitrogen fixation. However, it is possible that the addition of an extranodular NH_4^+ source fails to mimic the true physiological affects of bacteroid NH_4^+ production, in which the synthesis of NH_4^+ in a localised area of differentiated nodule cells could enhance the expression of these genes. Such a localised increase of NH_4^+ concentration (or another product of dinitrogen fixation) within the nodule can be obtained by transferring $Ar:O_2$ grown nodules to a $N_2:O_2$ atmosphere. $Ar:O_2$ to $N_2:O_2$ switched nodules feature a substantial increase, relative to $Ar:O_2$ controls, in nodule GS activity (FIG 5.3A), GS polypeptides (FIG 5.3C) and mRNA (J.V.Cullimore, pers.commun.), which is particularly pronounced for *gln*-Y gene products. Thus, the provision of nitrogen in this manner is sufficent to enhance the expression of nodule GS, suggesting that NH_4^+ (or a product of dinitrogen fixation) has a role in regulating the magnitude or maintenance of this expression. Atkins *et al* (1984b) have likewise proposed a role for NH_4^+, after similar experiments in cowpea, postulating that the role of bacteroid mediated NH_4^+ synthesis is not just one of providing a nitrogen source for plant growth. The authors propose that NH_4^+ has a role in the maintenance of an effective symbiosis, such as maintaining bacteroid and peri-bacteroid membrane permeability through its continued excretion, enabling solute exchange between plant and bacterial partners.

CHAPTER 6

THE RECOMBINANT SYNTHESIS OF THE α, ß AND ϒ GS POLYPEPTIDES IN *ESCHERICHIA COLI*

6.1 INTRODUCTION

GS has been best studied in enteric bacteria, and is present as a single GS gene, encoding a subunit of M_r 52,000, that assembles into a dodecameric enzyme. The enzyme is highly regulated, through covalent modification by adenylylation, resulting in a greater sensitivity to the effects of feedback inhibition (see Reitzer and Magasanik, 1987, or section 1.6 for a review). In contrast, our knowledge of plant GS enzyme regulation is comparatively limited. The octameric plant enzyme is not subject to adenylylative modification (Kingdon, 1974), instead it appears to be regulated through allosteric modification by small metabolites, such as amino acids and nucleotides (Stewart *et al*, 1980). Studies on plant GS are further complicated by the presence of multiple GS isoforms, consisting of a heterogeneous mixture of GS subunits, encoded by small multigene families (see McNally and Hirel, 1983; Cullimore and Bennett, 1988; section 1.5 for reviews). Multiple GS genes, subunits and heterogeneous isoforms could provide regulatory advantages and/or enable individual subunits to evolve characteristics ideally suited to the physiological conditions of the GS enzymes cellular and subcellular site of expression. For example, the plant plastid GS with its green tissue site of expression and increased activity under illuminated conditions (Hirel and Gadal, 1980) is ideally suited to assimilate NH_4^+ released during photorespiratory conditions. The importance of the multiple plant cytosolic GS genes is less clear. The differential organ expression of each of the *P. vulgaris* GS *gln-α*, *gln-β* and *gln-γ* genes has been identified (Gebhardt *et al*, 1986; section 5.2), but provides little information concerning the properties of the individual GS gene products. Attempts to purify the individual homo-octameric isoenzymes is complicated by the ability of GS subunits to mix and produce hetero-octameric isoforms (Lara *et al*, 1984; chapter 4). A simple system, expressing selected single plant GS genes, would provide homo-octameric isoforms of each individual cytosolic GS subunit, enabling the analysis of their individual properties. Lozoya *et al* (1988) has examined the catalytic differences of two parsley 4-coumarate:CoA ligase isoforms through the expression of two near full length 4-coumarate:CoA ligase cDNAs in the heterologous host, *Escherichia coli*. Likewise, the availability of the full length GS α, β and γ cDNAs (Gebhardt *et al*, 1986; section 3.2) enables the recombinant expression of plant GS in *E.coli* (see Gatenby, 1987, for a review).

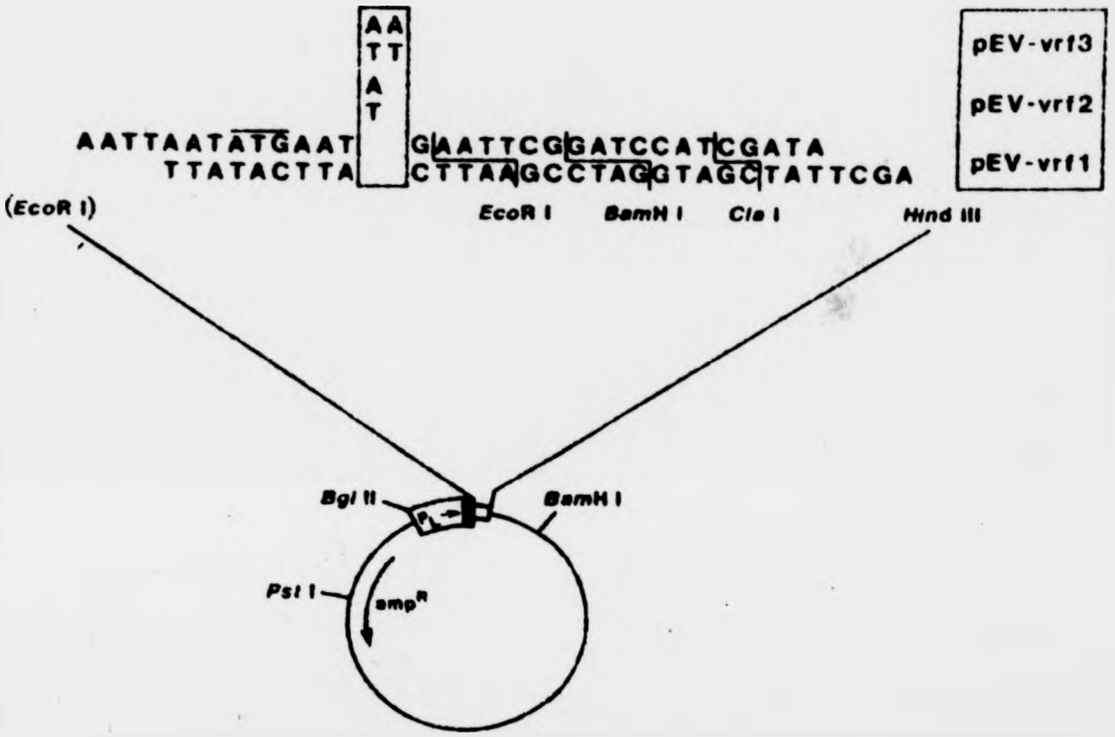


FIG 6.2A Restriction map and coding sequence surrounding the initiation codon of the pEV recombinant expression vector. *EcoRI*, *BamHI* and *ClaI* restriction sites, and hence positions of DNA insertion, are provided in all reading frames by expression plasmids pEV-vrf1, 2 and 3. The ATG start codon is overlined, and the placement of the additional frameshift A (pEV-vrf2) or AA (pEV-vrf3) bases are indicated.

DasSarma *et al* (1986) have demonstrated the synthesis, assembly and kinetic activity of the product of an alfalfa GS cDNA recombinantly expressed in *E.coli*, and furthermore, achieved the complementation of an *E.coli glnA* mutant with the plant GS gene.

The following describes the cloning, expression and complementation studies performed in a λP_L *E.coli* expression system using the three *P. vulgaris* cytosolic GS cDNAs, and discusses the results obtained with respect to alfalfa GS and other recombinant gene expression studies performed in *E.coli*.

6.2 CLONING AND ISOLATION OF RECOMBINANT GS EXPRESSION CONSTRUCTS

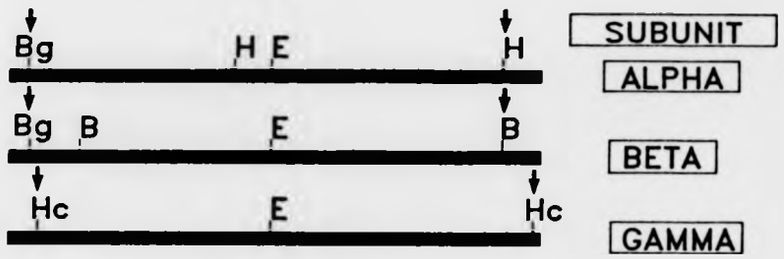
The three *P. vulgaris* cytosolic GS cDNAs pcGS-α1, pcGS-β1 and pcGS-γ1 were expressed in the pEV expression vector system (Crowl *et al*, 1985) which uses the strong *E.coli* bacteriophage λP_L promoter to drive recombinant expression (see Caulcott and Rhodes, 1986, for a review). The λP_L based expression system provided a very strong promoter that is negatively controlled by a repressor protein, coded by the λcI gene. A temperature sensitive mutant of the λcI gene, termed λcI857, which at 42°C results in an inactive repressor, repressor retains its activity at 30°C. Thus, λP_L promoter is regulated by a simple change of temperature in a host λcI857 gene copy either on the chromosome or on a low copy number compatible plasmid. In addition, the pEV vectors contain all necessary bacterial transcriptional and translational signals. These include an ATG initiation codon embedded within a synthetic model Shine and Dalgarno 5' mRNA and coding sequence, designed, from computer analysis, to maximise expression. The DNA fragments to be expressed could be inserted within the first 10 amino acids of the coding sequence which contains *EcoR1*, *BamH1* and *Cla1* restriction sites, provided in all three reading frames (FIG 6.2A). Thus the pEV expression vectors provided the ideal characteristics necessary to express all three *P. vulgaris* GS cDNAs in *E.coli*.

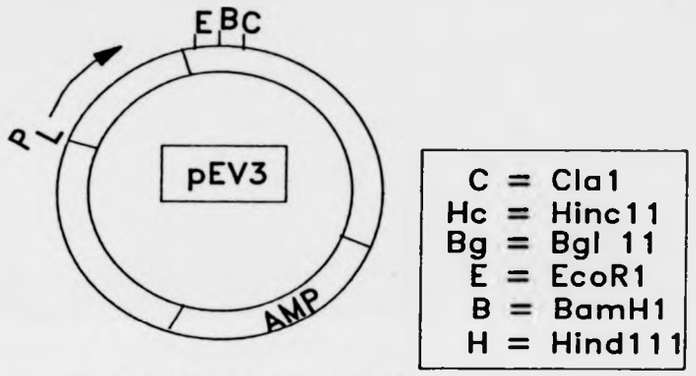
The provision of a vector derived ATG initiation codon is a necessary prerequisite for recombinant expression since the restriction sites within each of the three GS cDNAs (pcGS-α1, pcGS-β1, pcGS-γ1) limited the use of their own initiating methionines during manipulation,

and hence the GS cDNAs were cloned as translational fusions as follows (see FIG 6.2B and section 2.59 for further details). A 1300 bp pcGS-α1 *BglII-HindIII* fragment was cloned into the *BamHI* site of pEV3 via a three way ligation involving a *BamHI-HindIII* adaptor fragment. An 1400 bp pcGS-β1 *BglII-BamHI* fragment was cloned into the *BamHI* site of pEV3. A 1400 bp pcGS-γ1 *HincII* fragment was cloned into the T4 polymerase *filled in ClaI* site of pEV3. All manipulations should result in the *in frame* fusion of expression vector and plant GS cDNA derived coding sequences (FIG 6.2B). In addition, all plant GS coding residues removed during cloning should be replaced with an equal number of vector derived residues. Although this provided a useful spacing function, the newly fused residues differed in sequence, replacing serine and other non-polar amino acids with asparagine and lysine charged residues (FIG 6.2B).

The isolation of the pcGS-α1 and pcGS-β1 recombinant expression vectors, termed pcGS-Eα and pcGS-Eβ respectively, were performed in the host strain MM294, containing a wildtype prophage cI repressor, enabling bacterial manipulations at 37°C. *E.coli* strain MM294 was separately transformed with ligations containing the pEV3 and 1300 bp pcGS-α1, and pEV3 and 1400 bp pcGS-β1 DNA fragments. 300 putative pcGS-Eα and pcGS-Eβ clones were screened by colony hybridisation, using the radiolabelled pcGS-α1 1300 bp *BglII-HindIII* fragment, to identify pEV3 recombinant plasmids containing GS DNA inserts. Positively hybridising clones were subsequently restriction mapped to identify the correct GS insert orientation (see FIG 6.2C). The pcGS-γ1 expression plasmid, termed pcGS-Eγ, was isolated using an alternative strategy. Initially, the pEV3 and pcGS-γ1 ligated DNA fragments were transformed into the *E.coli* strain, RR1 (containing a plasmid-borne cI857 repressor gene), grown at 30°C (on solid medium), then temperature induced for two hours at 42°C. pcGS-Eγ recombinants expressing GS antigen were identified (clones 3 and 13), using a colony immunodetection scheme (data not shown) (see section 2.9 for further details). Subsequent restriction mapping of several colonies showed that those staining positively and negatively for GS antigen had correctly and incorrectly orientated pcGS-γ1 DNA inserts respectively (see FIG 6.2D).

I





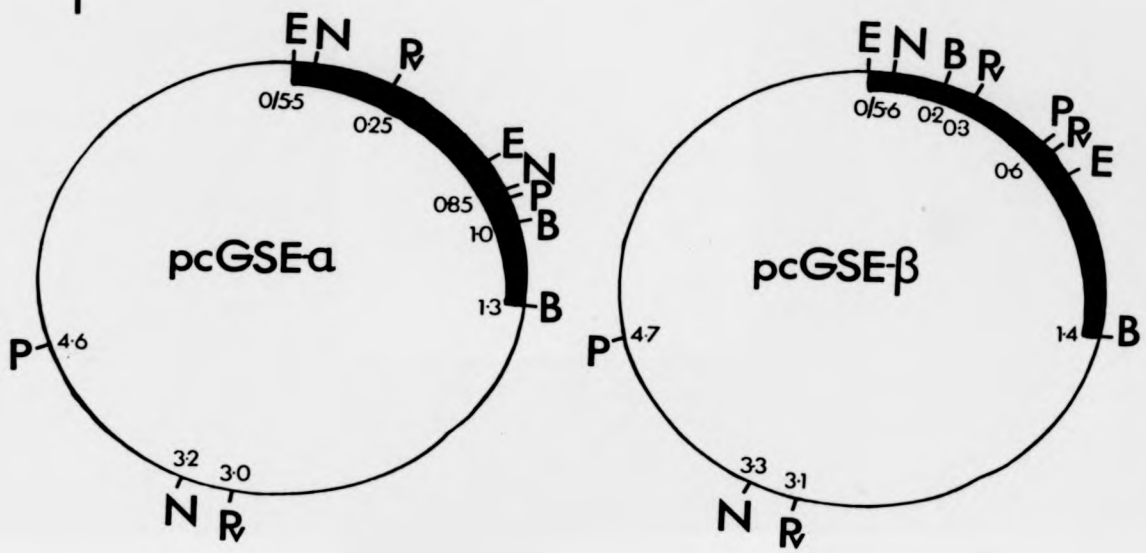
II

```
pEV3      M   N   K   N   S   D   P   S   I
          ATG AAT AAG AAT TCG|GAT CCA TCG ATA
pcGS-α1   M   S   L   L   S   D   L   I   N
          ATG TCT TTG CTT TCA|GAT CTC ATC AAC
pcGS-ß1   M   S   L   L   S   D   L   I   N
          ATG TCG CTG CTC TCA|GAT CTC ATC AAC
pcGS-Y1   M   S   S   I   S   D   L   V   N
          ATG TCA TCA ATC TCC GAT CTT GTT|AAC

pcGS-Eα   M   N   K   N   S   D   L   I   N

pcGS-Eß   M   N   K   N   S   D   L   I   N

pcGS-EY   M   N   K   N   S   D   P   S   V
```

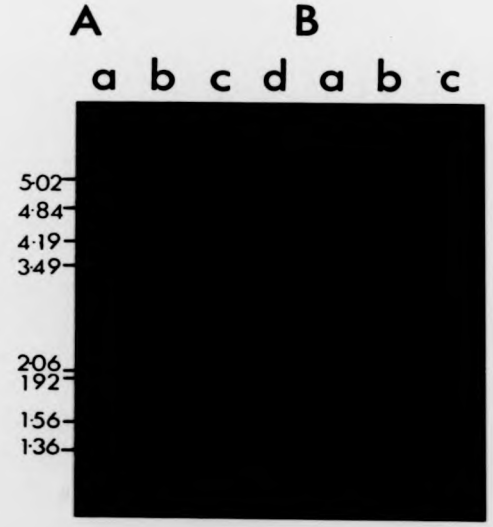
FIG 6.2B Cloning strategy for the recombinant expression of the α, ß and Y GS polypeptides. Restriction sites used (marked with arrows) (I), and resulting in in frame translational fusions between the pEV3 and pcGS-α1, pcGS-ß1 and pcGS-Y1 cDNA sequences (II), to give pcGS-Eα, pcGS-Eß, and pcGS-EY respectively (see text for details).

FIG 6.2C Restriction map analysis of pcGS-Eα and pcGS-Eß expression vector constructs. (I) Anticipated restriction map of the pcGS-Eα and pcGS-Eß expression vectors, containing the 1.3 Kbp *BglII- HindIII* pcGS-α1 and 1.4 Kbp *BglII-BamHI* pcGS-ß1 fragments respectively (thickened black line), in the correct orientation. E signifies *EcoRI*; N, *NdeI*; P, *PstI*; Pv, *PvuII*; and B, *BamHI*. pcGS-Eα plasmid DNA containing a correctly orientated pcGS-α1 insert would on, for example, *PvuII* restriction endonuclease digestion, release 2.8 and 2.7 Kbp fragments. pcGS-Eß plasmid DNA containing a correctly orientated pcGS-ß1 insert would on, for example, *PvuII* restriction endonuclease digestion, release fragments of 0.3, 2.5 and 2.8 Kbp. (II) Plasmid DNA from pcGS-Eß (A) and pcGS-Eα (B) recombinant clones were restricted with *PstI* (a), *PvuII* (b), *BamHI* (c) and *NdeI* (d), and the fragments electrophoretically separated on a 1% agarose gel. The restricted fragment pattern of pcGS-Eß (A) and pcGS-Eα (B) plasmid DNA are in agreement with the anticipated restriction map for the pcGS-Eα and pcGS-Eß recombinant expression vectors (I). Molecular size markers (given in Kbp) were provided by λ DNA restricted with *EcoRI* and *HindIII*.

I


E
N
Rv
0·155
0·25
E
N
P
B
0·85
1·0
pcGSE-α
1·3
B
P
4·6
3·2
3·0
N
Rv
E
N
B
Rv
0·156
0·2
0·3
P
Rv
E
0·6
pcGSE-β
1·4
B
P
4·7
3·3
3·1
N
Rv

II


A
B
a b c d a b c
5·02
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4·19
3·49
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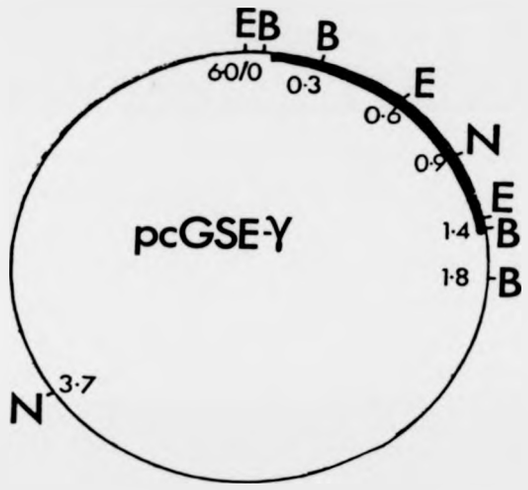
1·56
1·36

I



II

FIG 6.2D Restriction map analysis of pcGS-Eγ expression vector constructs. (I) Anticipitated restriction map of the pcGS-Eγ expression vector, containing the 1.4 Kbp *HincII* fragment (thickened black line), in the correct orientation. E signifies *EcoRI*; N, *NdeI*; and B, *BamHI* restriction sites. pcGS-Eγ plasmid DNA containing a correctly orientated pcGS-γ1 insert would on, for example, *BamHI* restriction endonuclease digestion, release 4.2, 1.1, 0.4 and 0.3 Kbp fragments, whereas an incorrect orientation would result in 4.2, 1.1 and 0.6 Kbp fragments. pEV3 vector without an insert would release 4.2 and 0.4 Kbp fragments *BamHI* restriction map analysis of putative pcGS-Eγ recombinants (II) identified that plasmid DNA from clones 3 and 13 gave the correct BamHI restriction map anticipated for the pcGS-Eγ recombinant expression vector (I). Molecular size markers (given in Kbp) were provided by λ DNA restricted with *EcoRI* and *HindIII*.

6.3 EXPRESSION OF pcGS-Eα AND pcGS-Eβ IN E.coli

pcGS-Eα and pcGS-Eβ, together with the pEV3 expression vector and a similar λP_L based expression plasmid, pAS1.EH80 (known to express an influenza antigen at high levels, see Ferguson *et al*, 1985), were transformed separately into the *E.coli* strain N5151. N5151 is known to contain a λ lysogen encoding the cI857 temperature sensitive repressor, and is noted for its high expression of foreign recombinant proteins (Ferguson *et al*, 1983). The four separate plasmid-containing forms of N5151 were initially grown in liquid culture at 30°C (under antibiotic selection) until cultures reached an OD_{600} of approximately 0.6 units. Expression was initiated by temperature induction, through transferring cultures to 42°C for a 2 hour period, and control samples for each recombinant were retained at 30°C.

Initially, samples grown at 30°C and 42°C were simply analysed by Coomassie staining of SDS-PAGE electrophoresed total *E.coli* cell protein, to detect the overexpression of the recombinant products. The pAS1.EH80/N5151 extract was observed to overexpress its 40 Kd product only at 42°C, thus suggesting that λP_L expression was efficently repressed at 30°C and induced at 42°C (data not shown). However no overexpressed recombinant GS protein was detectable by Coomassie staining in either pcGS-Eα and pcGS-Eβ temperature induced samples. To detect a lower level of recombinant GS product expression, the more sensitive immunodetective western blotting technique was employed. Total *E.coli* protein extracts from pcGS-Eα, pcGS-Eβ and pEV3 samples grown at 30°C and 42°C were separated by SDS PAGE, immunoblotted and probed with the anti-GS antibody. The resulting immunostained blot (FIG 6.3A) identified GS antigen in both the pcGS-Eα and pcGS-Eβ temperature induced samples, but not in the (repressed) 30°C or pEV3 samples. Both the recombinant α and β GS subunits appeared to be expressed at a similar level and have a M_r of 39,000, comparable with the plant GS controls.

To determine if the pcGS-Eα and pcGS-Eβ recombinant GS products were enzymically active and to ascertain the best method of extraction, temperature induced pcGS-Eα/N5151 cells were either French pressed or sonicated, centrifuged, and soluble and the insoluble fractions were then assayed for GSt activity and soluble protein (Table 6.3). Results

FIG 6.3A Western immunodetection of recombinantly expressed α and ß GS polypeptides. Native root (R) and leaf (L) samples containing 250 nmoles min^{-1} of GSt activity, and 100 µg of total *E.coli* protein from samples containing pcGS-Eß, pcGS-Eα and pEV3 at 30°C (a) and 42°C (b) were separated by SDS-PAGE, western blotted and probed with *P. vulgaris* root nodule GS antiserum. The size of molecular weight markers are shown in Kd.

Table 6.3 GS activity determination for the recombinantly expressed α gene product. Extracts of the temperature induced *E.coli* strain N5151 were obtained either by French pressing or sonication, separated into soluble (A) and insoluble (B) fractions by centrifugation, and assayed for the GSt activity and total protein released.

		GSt (µmol./min/mg sol. prot.)	total protein (mg)
French pressing	A	0.257	40
	B	0	30
Sonication	A	0.081	110
	B	0	30

indicated that French pressing released less soluble protein but more GSt activity than sonication, and that the insoluble pellet contained no GS enzyme activity. Furthermore, the results suggested that measuring the GSt activity of the recombinant GS product, could provide an ideal parameter to ascertain the best time length for temperature induction, to achieve maximal, active, GS product expression. Thus, time course studies for recombinant GS activities during temperature induction were performed for pcGS-Eα/N5151, pcGS-Eß/N5151 and pEV3/N5151. Cultures were grown at 30°C until reaching an OD_{600} of 0.6, divided into two and the two halves grown at 30 and 42°C respectively. Samples were taken at 60 minute intervals for over a five hour period (FIG 6.3B). Both pcGS-Eα and pcGS-Eß *E.coli* extracts exhibited increased GS activity within an hour of temperature induction, achieving maximal GS activities between two to three hours, then decreasing over 40% by the fourth hour. In contrast, the pEV3 30°C and 42°C, together with the pcGS-Eα and pcGS-Eß 30°C samples featured no such increase in GS activity. However, the GSt activity achieved by pcGS-Eα was approximately seventeen times higher than the comparable pcGS-Eß sample. To determine if the reason for such low recombinant ß relative to α GSt activity was due to product insolubility, pcGS-Eα, pcGS-Eß and pEV3 N5151 *E.coli* extracts were separated into soluble and insoluble fractions, electrophoresed by SDS-PAGE and GS immunodetected (as previously described) using the anti-GS antibody. The result (FIG 6.3C) indicated that the pcGS-Eα product was mainly soluble, whereas the pcGS-Eß recombinant GS was mainly insoluble. However, the expression of active soluble GS ß enzyme was sufficient to enable further physical characterisation.

Crude pcGS-Eα and pcGS-Eß N5151 *E.coli* extracts were analysed by IEC FPLC, as described in chapters 4 and 5 (FIG 6.3D). Native *P. vulgaris* α and ß GS isoforms, extracted from plumule tissue were also run (see section 4.4), to serve as a comparison to the recombinant α and ß GS isoforms IEC characteristics. Both the pcGS-Eα and pcGS-Eß *E.coli* extracts contained two IEC gradient GSt activity peaks eluting at fractions 42 and 60, and 35 and 60 respectively, whereas pEV3/N5151 extracts contained a single GSt activity peak eluting at fraction 60. The fractions 60 GSt activity appeared to represent the endogenous N5151 host strain GS activity, based on its presence in all three N5151 recombinant strains and the inability to immunodetect a plant GS product in pEV3/N5151 extracts. In contrast, the recombinant pcGS-Eα and

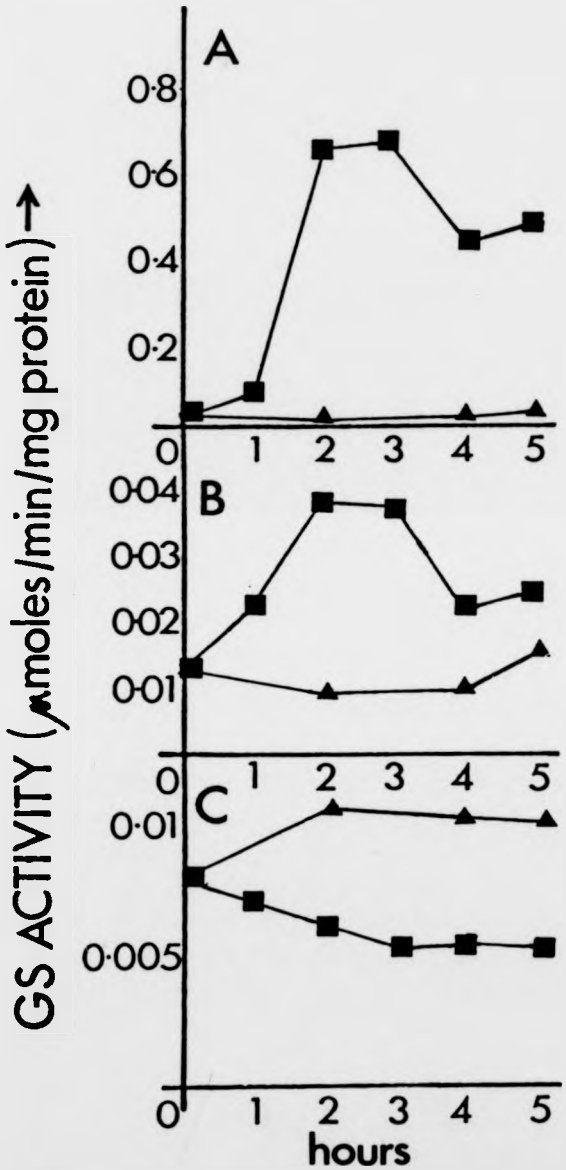


FIG 6.3B Measurement of the changes in GSt activity of N5151 soluble extracts during growth at 30oC (▲) and 42oC (■), containing pcGS-Eα (A), pcGS-Eβ (B) and pEV3 (C) recombinant plasmids.

FIG 6.3C Western immunodetection of recombinantly expressed soluble (a) and insoluble (b) α and β GS polypeptides. Native root (R) and leaf (L) samples containing 250 nmoles min^{-1} of GSt activity, and 100 µg of soluble *E.coli* protein (a), together with an equal fraction of the insoluble pellet (b), from samples expressing pcGS-Eβ, pcGS-Eα and pEV3, were separated by SDS-PAGE, western blotted and probed with *P. vulgaris* root nodule GS antisera. The size of molecular weight markers are shown in Kd.

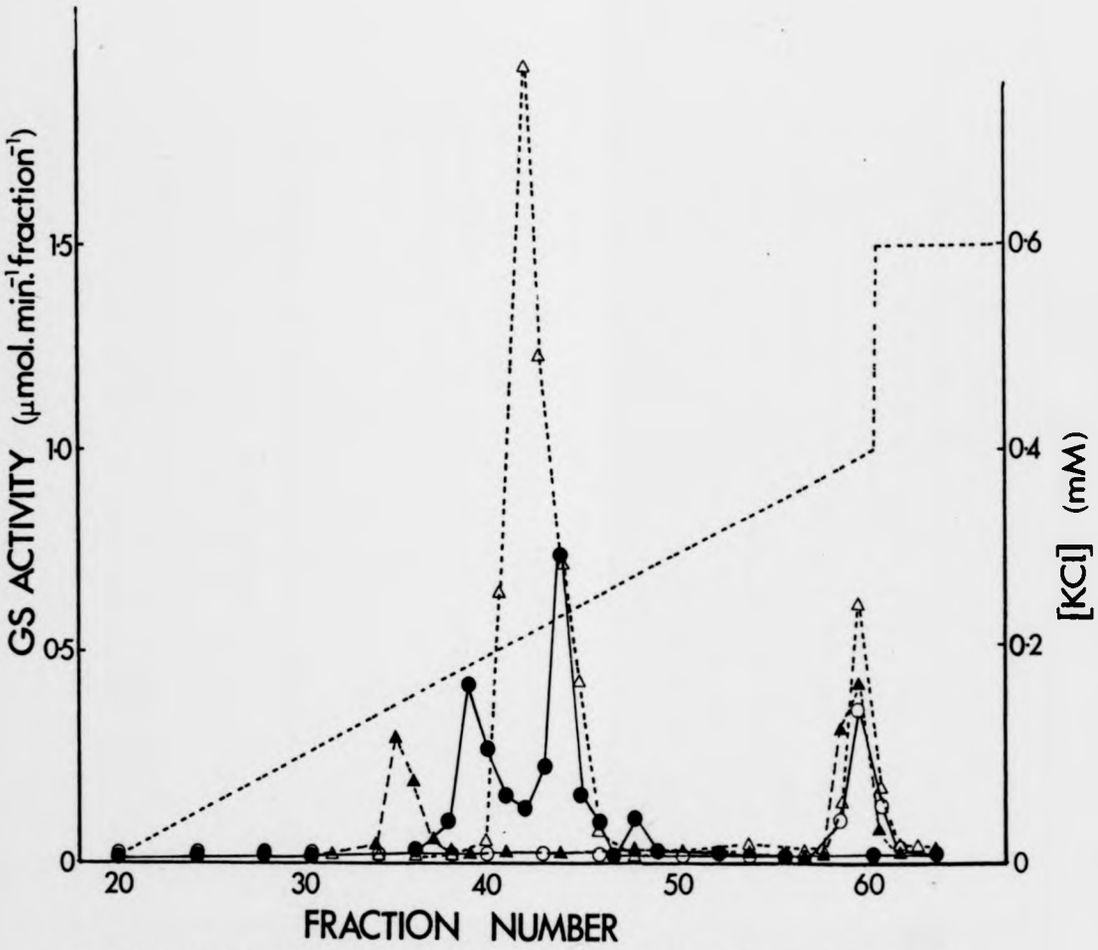


FIG 6.3D IEC FPLC analysis of plumule and recombinantly expressed α and ß GS activities. Soluble *E.coli* extracts from N5151 samples expressing pcGS-Eα (△), pcGS-Eß (▲) and pEV3 (○) plasmids, and a native (●) *P. vulgaris* imbibed plumule extract, were fractionated by IEC FPLC and assayed for GSt activity.

pcGS-Eβ activity peaks eluted in a relatively similar manner to the native *P. vulgaris* α and β plumule isoforms. However, both recombinant products eluted four fractions before their respective native isoforms, which may indicate some increase in the positive charge of the recombinant isoforms.

Further physical analysis of the pcGS-Eα, pcGS-Eβ and pEV3 N5151 *E.coli* extracts by FPLC gel filtration, determined native M_r of GS of 312,000, 380,000 and 500,000 respectively (FIG 6.3E). It was a possibility from gel filtration that the broad eluting GS activity peak for the pcGS-Eβ/N5151 extract was a composite of both β and *E.coli* GS isoforms. In order to further resolve the recombinant and endogenous host GS activities, individual GS isoforms were first separated by IEC FPLC, prior to gel filtration. Such partial purification of the recombinant α and β GS isoforms, together with the separated α and β plumule isoforms, enabled a direct correlation between recombinant and native α and β M_r (FIG 6.3F). Both recombinant and native α GS isoforms had native M_r of approximately 380,000, whereas the native and recombinant β isoforms had values of about 380,000 and 320,000 respectively, with no broadening or apparent shoulders from the recombinant GSt activity elution profile typical for crude N5151 extracts.

6.4 EXPRESSION OF ALL THREE *P. vulgaris* CYTOSOLIC GS cDNAs IN AN *E.coli glnA* MUTANT

Recombinant GS expression studies were switched from the N5151 host strain to the ET8894 *glnA* deletion mutant (MacNeil, 1981). ET8894, provided a number of useful advantages, such as working with a heterologous system free of any residual host GS activity. In addition, it provided an opportunity to demonstrate the true biological activity of the recombinant GS, through classical genetic complementation studies. Furthermore, the change of host strain might improve the recombinant β subunit solubility. The recombinant GS subunits could be expressed in ET8894 in two different manners. Firstly, expression could be temperature inducible in the presence of a plasmid-borne cI857 repressor (see section 6.1). Secondly, constitutive expression could be

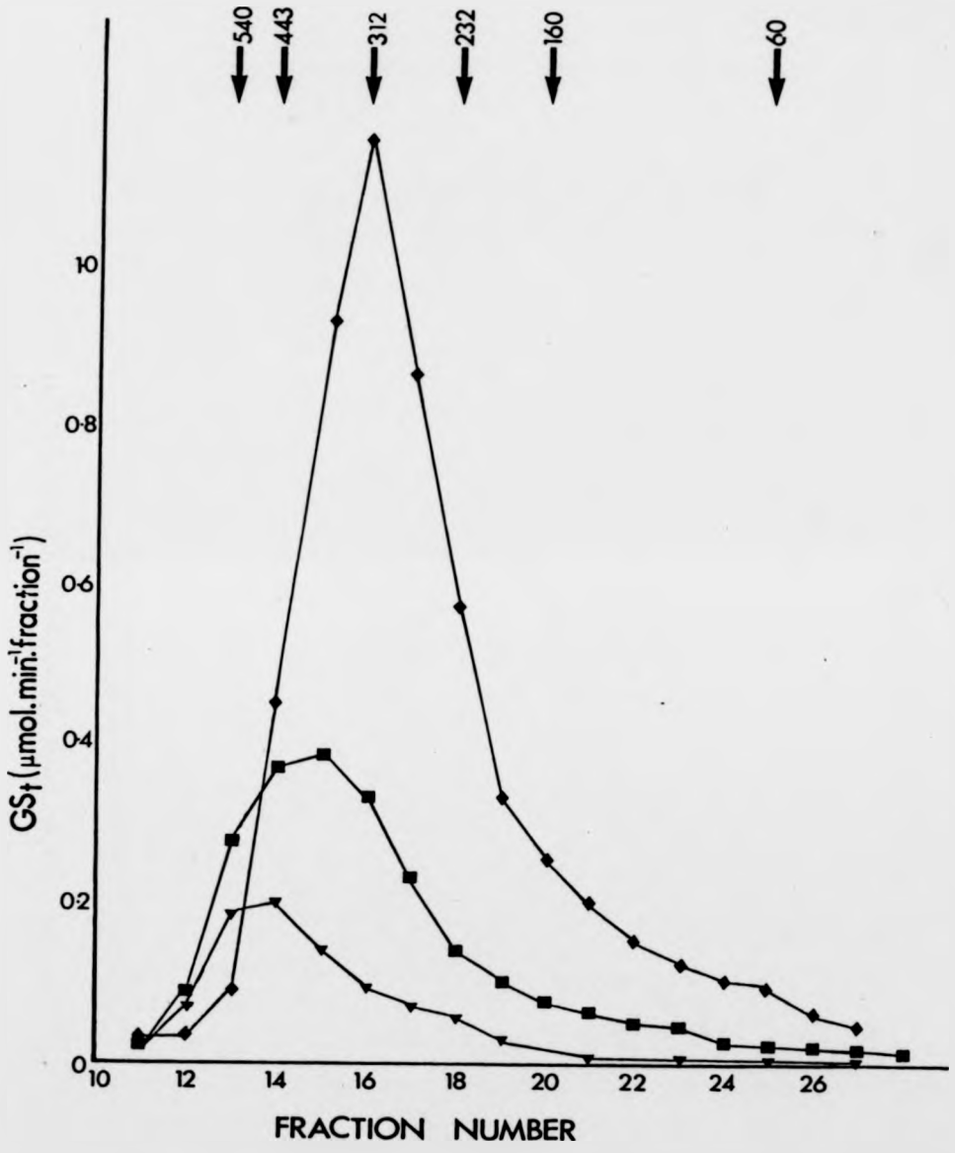


FIG 6.3E Native M_r determination of the recombinantly expressed α and ß GS activities. FPLC gel filtration analysis and GSt activity measurements of soluble extracts of N5151 expressing pcGS-Eα (◆), pcGS-Eß (■) and pEV3 (▼) plasmids. M_r markers used include ß-galactosidase (540,000), ferritin (443,000), catalase (232,000), alcohol dehydrogenase (160,000) and haemoglobin (60,000). In addition, the position of elution of root GS is also shown (estimated M_r 312,000).

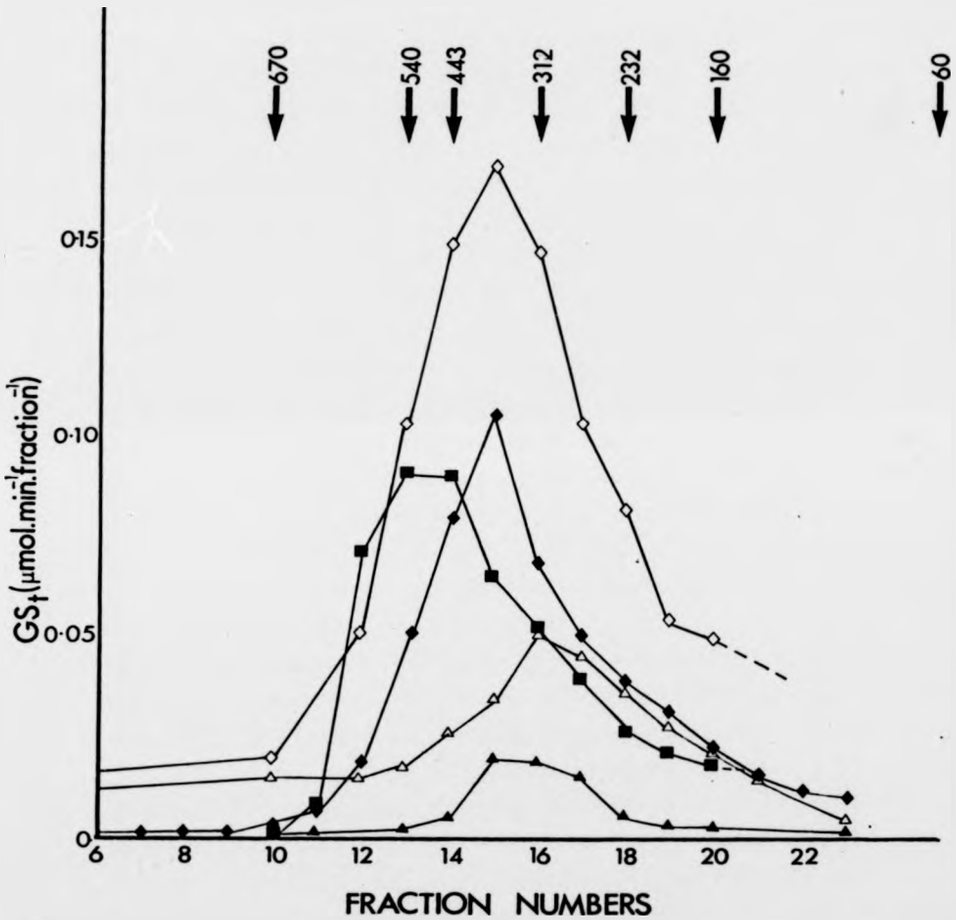


FIG 6.3F Native M_r determination of plumule and recombinantly expressed α and ß GS isoforms. FPLC gel filtration analysis and GSt activity measurements of IEC FPLC semi-purified recombinant α (◆), ß (▲) and pEV3 (■), and native *P. vulgaris* plumule α (◇) and ß (△) isoforms. M_r markers used include thyroglobulin (670,000), ß-galactosidase (540,000), ferritin (443,000), catalase (232,000), alcohol dehydrogenase (160,000) and haemoglobin (60,000). In addition, the elution position of root GS is also shown (estimated M_r 312,000).

achieved at lower growth temperatures (below 37°C) in the absence of the c1857 repressor.

To determine the ability of the three *P. vulgaris* GS expression plasmids, pcGS-Eα, pcGS-Eβ and pcGS-Eγ, to complement the ET8894 *glnA* mutation, each expression plasmid was transformed separately into ET8894. In addition, both pEV3 and pDC1 plasmids (a pBR325 based vector containing the *Methylococcus capsulatus (Bath) glnA* gene, D.Cardy, pers.commun.) plasmids were also transformed into ET8894, providing negative and positive controls respectively. Transformants were plated out onto M9 minimal media with or without a GLN supplement, thus providing non-stringent and stringent selective conditions respectively, and incubated at 37°C. Complementation (defined as the ability of the ET8894 strain to grow on a minimal media without the GLN supplement) was observed for pcGS-Eα and pcGS-Eγ, together with the positive control, pDC1. pEV3 transformed ET8894 was unable to grow without a GLN supplement, indicative of the inability to complement the *glnA* lesion. In contrast, no pcGS-Eβ recombinants could be isolated with or without the GLN supplement. The transformation and selection procedure was repeated at 30°C (FIG 6.4A) following the independent observation that ricin A chain was more soluble when expressed at 30°C rather than 37°C (O'Hare *et al*, 1987). No pcGS-Eβ recombinants could be isolated at this lower growth temperature, and it thus appeared unlikely that stable pcGS-Eβ transformants could be obtained in the absence of the cI857 repressor. However, the change in incubation temperature to 30°C (or even 25°C) achieved a noticeably faster growth rate (on solid media) for the pcGS-Eα and pcGS-Eγ ET8894 recombinants, whereas pDC1 (encoding a thermophilic GS enzyme) featured a lower growth rate (FIG 6.4B). Complementation of the ET8894 host by the pcGS-Eα, pcGS-Eγ and pDC1 expression vectors was further examined on minimal M9 media using a variety of NH_4Cl concentrations and the alternate nitrogen source, arginine, performed at 25°C, 30°C and 37°C incubation temperatures (Table 6.4A). Optimum complementation of ET8894 by pcGS-Eα and pcGS-Eγ was achieved at 30°C, irrespective of the nitrogen source or NH_4Cl concentration used, whereas pDC1 achieved a higher growth rate at 37°C. In addition, the efficiency of the complementation of ET8894 by pcGS-Eα, pcGS-Eγ and pDC1, depended on the choice of nitrogen source, and the concentration of NH_4Cl in the minimal media. A lower complemented growth was achieved by pcGS-Eα, pcGS-Eγ and pDC1 using

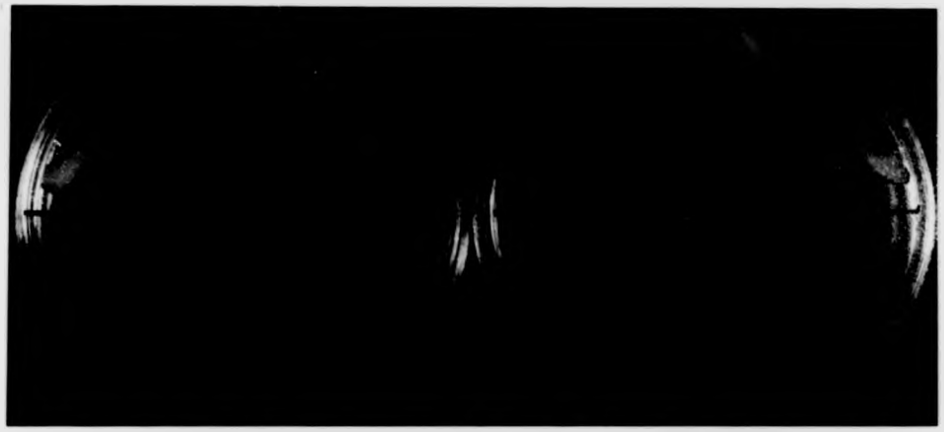


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FIG 6.4A Complementation of an *E.coli glnA* auxotroph by two recombinantly expressed *P. vulgaris* GS cDNAs. The *E.coli glnA* auxotroph ET8894 containing either pcGS-Eα (A), pcGS-Eγ (B), pDC1 (C) or pEV3 (D), was grown on M9 minimal medium (with 5 mM NH_4^+) with (+) or without (-) a glutamine supplement, incubated at 30°C.

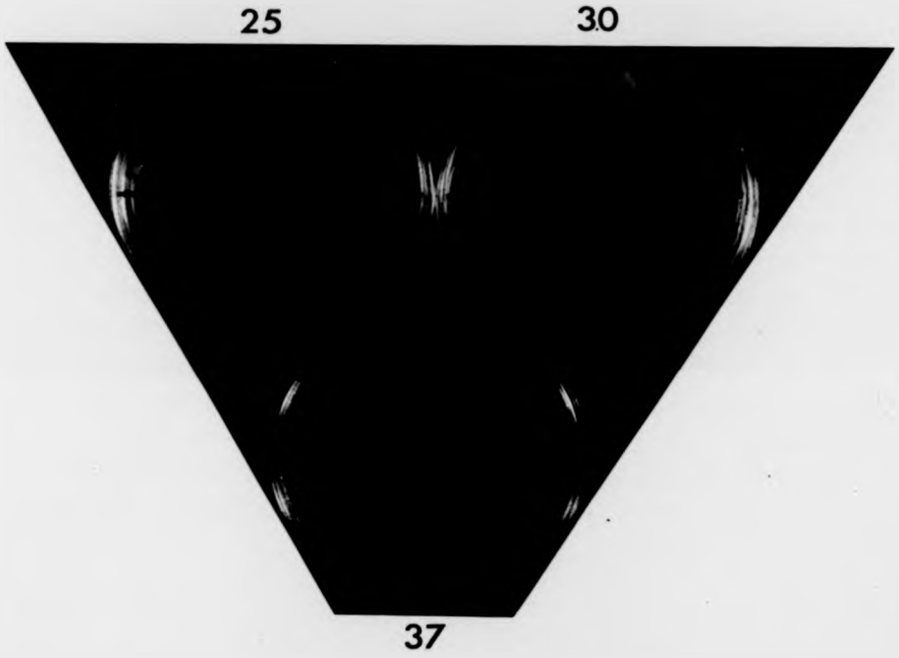

FIG 6.4B The effect of temperature on the complementation of an *E.coli glnA* auxotroph by two recombiantly expressed *P. vulgaris* GS cDNAs. The *E.coli glnA* auxotroph ET8894 containing either pcGS-Eα (A), pcGS-Eγ (B), pDC1 (C) or pEV3 (D), was grown on M9 minimal medium (with 5 mM NH_4^+) without a glutamine supplement, incubated at 25°C, 30°C and 37°C.

Table 6.4A Relative growth of pcGS-Eα, pcGS-Eϒ and pDC1 complemented *E.coli glnA* strain ET8894 at a variety of incubation temperatures and nitrogen sources. Relative rates of complementation by pcGS-Eα (A), pcGS-Eϒ (B), pDC1 (C) and pEV3 (D) were described by a scale of 1 to 5, (5 representing the highest growth rate) and + for very low but detectable growth, or - for undetectable growth. Complementation was performed on M9 minimal media containing either 0.2% arginine (ARG) or a variety of NH_4Cl concentrations (mM), in the absence of a glutamine supplement, at a 25, 30 or 37 C incubation temperature.

	INCUBATION TEMPERATURE (^{o}C)											
NITROGEN SOURCE	25				30				37			
	A	B	C	D	A	B	C	D	A	B	C	D
NH_4Cl												
0.1	1	1	+	-	2	2	1	-	-	-	2	-
0.5	2	2	+	-	3	3	1	-	-	+	2	-
5.0	2	3	2	-	4	5	3	-	-	1	5	-
20.0	2	3	2	-	5	5	4	-	-	1	5	-
ARG	1	1	+	-	2	2	1	-	-	1	2	-

arginine rather than NH4+Cl minimal media nitrogen sources. Optimal complementation of ET8894 by pcGS-Eα, pcGS-EΥ and pDC1 was achieved at 5 mM NH_4Cl media concentrations. Lower NH_4Cl concentrations resulted in a noticeably lower growth, although complementation of ET8894 by pcGS-Eα and pcGS-EΥ (at 30°C and 25°C), and pDC1 (at all temperatures), was detectable down to a 0.1 mM NH_4Cl concentration.

When all three *P. vulgaris* recombinant GS expression vectors and pEV3 were separately transformed into an ET8894 strain containing a plasmid-borne cI857 temperature repressor gene at 30 C, pcGS-Eß recombinants could be obtained at transformation frequencies comparable with pcGS-Eα, pcGS-EΥ and pEV3 plasmids. To obtain recombinant expression each of the ET8894 c1857 recombinant plasmid containing strains were temperature induced (as previously described) for two hours. The changes in the GSt activity of the pcGS-Eα/ET8894 c1857 strain was examined over a four hour time course, collecting samples at 40 minute intervals (FIG 6.4C). Temperature transfer in the pcGS-Eα/ET8894 c1857 strain was observed to induce the recombinant α GS activity in a manner identical to that using the N5151 host. In contrast, at 30°C no GSt activity could be detected due to the ET8894 *glnA* deficient genetic background. Calculations could thus be made of the specific activity of each GS recombinant product in ET8894 (TABLE 6.4B). pcGS-Eα featured about an eight fold higher GSt specific activity value than pcGS-Eß and pcGS-EΥ soluble extracts. Examination of the solubility of the individual GS recombinant products after such a temperature induced expression, using a one dimensional SDS PAGE and immunodetective procedure (see section 6.3 for details), identified that the pcGS-EΥ, as well as the pcGS-Eα and pcGS-Eß expression constructs synthesised GS subunits of the correct molecular weight (FIG 6.4D). However, GS recombinant products were observed to be mostly insoluble in pcGS-Eß/ET8894 cI857 extracts, approximately 50% insoluble for pcGS-EΥ, and mostly soluble for pcGS-Eα.

Examination of each soluble recombinant GS enzymes native M_r by FPLC gel filtration (as previously described in section 6.3), determined that the soluble α, ß and Υ recombinant GS subunits assembled into a GSt active oligomeric form, of native M_r 270,000, 270,000 and 230,000 respectively (see FIG 6.3E). Analysis of each of the recombinant GS enzymes chromatographic characteristics using IEC FPLC (as described in

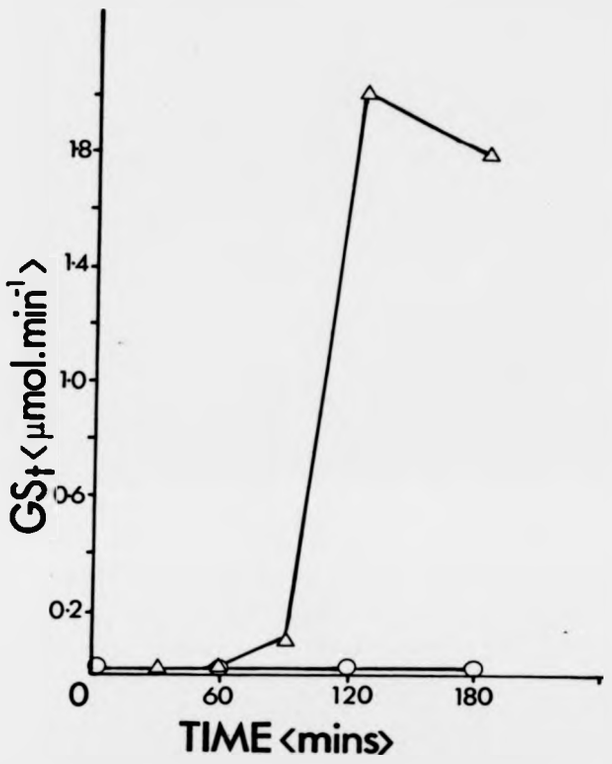

FIG 6.4G Measurement of the changes in GSt activity of soluble extracts of ET8894 expressing pcGS-Eα, during growth at 30°C (○) and 42°C (△).

Table 6.4B GS activity determination for the recombinantly expressed α, ß and ϒ gene products. Soluble extracts of *E.coli* strain ET8894 expressing the pcGS-Eα, pcGS-Eß and pcGS-Eϒ plasmids (after a two hour 42°C temperature induction period) were obtained by French pressing and centrifugation, and assayed for GSt activity and soluble protein released.

	GSt (μmol./min/mg sol. prot.)
α	0.135
ß	0.017
ϒ	0.013

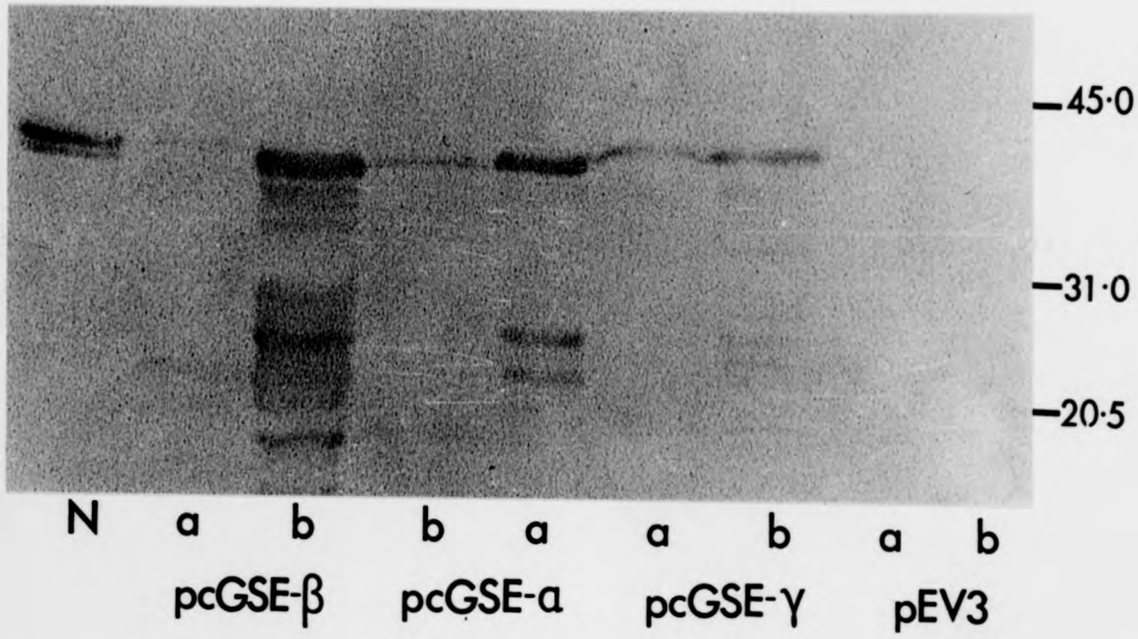

FIG 6.4D Western immunodetection of recombinantly expressed soluble (a) and (b) insoluble α,β and γ GS polypeptides. Native nodule (N) and leaf (L) samples containing 250 nmoles min^{-1} of GSt activity, and 100 µg of soluble *E.coli* protein (a), together with an equal fraction of the insoluble pellet (b), from ET8894 samples expressing pcGS-Eβ, pcGS-Eα, pcGS-Eγ and pEV3, were separated by SDS-PAGE, western blotted and probed with peroxidase labelled anti-GS antiserum. The size of molecular weight markers are shown in Kd.

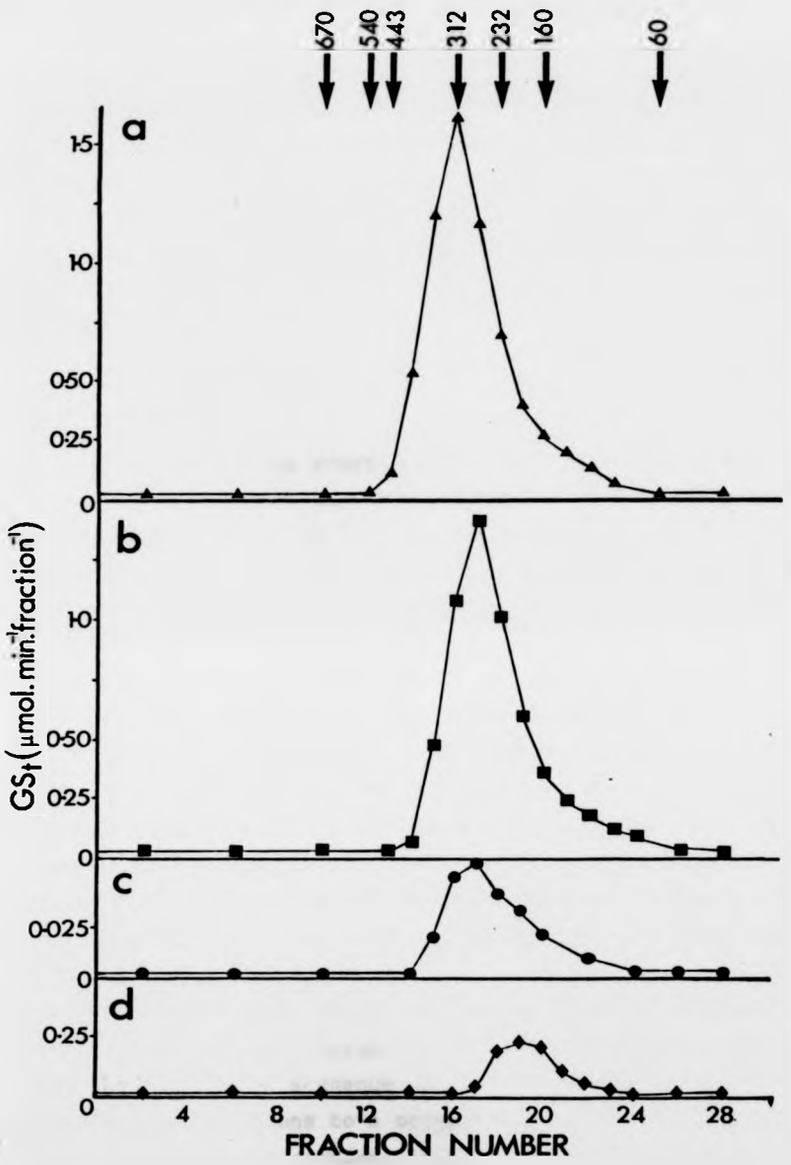

FIG 6.4E Native M_r determination of the recombinantly expressed α, β and γ GS activities. FPLC gel filtration analysis and GSt activity measurements of native *P. vulgaris* root GS (α), and soluble *E.coli* extracts expressing pcGS-Eα (b), pcGS-Eβ (c) and pcGS-Eγ (d) plasmids. M_r markers used include thyroglobulin (670,000), β-galactosidase (540,000), ferritin (443,000), catalase (232,000), alcohol dehydrogenase (160,000) and haemoglobin (60,000).

section 6.3), identified that all temperature induced ET8894 cI857 *E.coli* extracts contained only one GSt activity peak (FIG 6.4F). The recombinant α and ß GS isoforms synthesised in temperature induced ET8894 featured, as in N5151, slight differences in elution position relative to their native isoforms. In contrast, the recombinant Y isoform eluted, as does the native Y isoform, on the void volume of the column.

6.5 DISCUSSION

This chapter has described the separate expression of all three *P. vulgaris* cytosolic GS cDNAs using the pEV expression system in the heterologous host, *E.coli*. *E.coli* expressing each of the three recombinant GS cDNA expression vectors (pcGS-Eα, pcGS-Eß and pcGS-EY) directs the synthesis of recombinant α, ß and Y GS subunits of the correct size, and soluble extracts contain GS transferase activity (Table 6.3 and 6.4). Examination of the solubility of the recombinant GS products, identified a surprising variation in the behaviour of the individual GS polypeptides. The pcGS-Eα recombinant α subunit appears mainly soluble, the pcGS-EY Y GS polypeptide has intermediate solubility, whereas the pcGS-Eß ß subunit appears mainly insoluble. The reason for such distinctive behaviour by the products of the highly similar *P. vulgaris* GS sequences is unknown. Gatenby (1984) and Bradley *et al* (1986) have also observed differences in the solubility of the recombinant products of the closely related maize and wheat RUBISCO large subunit genes, when expressed in *E.coli*. Stubbs *et al* (1986) have postulated that the insolubility of a recombinantly expressed pea lectin was probably due to sequence changes at the N-terminus. Although N terminal modifications to a polypeptide could lead to aberrant folding and changes in confirmation, it would be unlikely that the differences of recombinant GS product solubility would be due to the N-terminal modification of the ß GS subunit, since identical changes to the α subunit have little apparent effect on protein solubility. In a recent review, Kane and Hartley (1988) have concluded there is little relationship between product insolubility and the choice of promoter, host strain, level of recombinant product expression or product hydrophobicity. Instead the authors suggested other potential reasons, including gene product charge. Saito *et al* (1987a and b) have observed

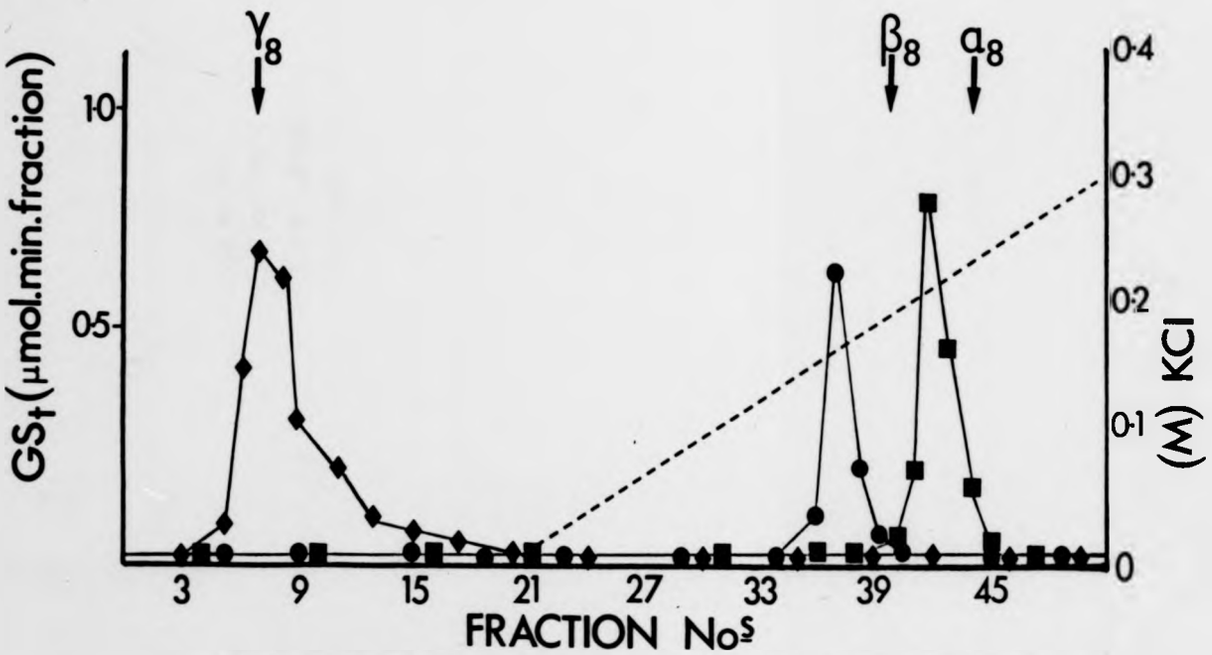

FIG 6.4F IEC FPLC analysis of recombinantly expressed α, ß and ϒ GS activities. IEC FPLC fractionation and GSt activity measurements of soluble extracts from ET8894 expressing pcGS-Eα (■), pcGS-Eß (●) and pcGS-Eϒ (◆) plasmids. Elution positions for the homologous GS isoenzymes are indicated.

that if recombinantly expressed gene products are too acidic or basic, relative to the *E.coli* intracellular pH (6.8 to 7.2), this could lead to product insolubility. However, from pI estimations of the α (5.8), ß (6.2) and ϒ (6.6) GS subunits (Lara *et al*, 1984), their is no such apparent correlation between pI and product insolubility. Kane and Hartley (1988) have proposed a model for recombinant product aggregation formation, postulating that during protein synthesis, the elongating polypeptide begins to assume secondary, tertiary and quarternary structure. The polypeptide could either assume a normal conformation, forming a stable soluble product, or alternatively, fold abnormally, resulting in a protease sensitive soluble product or an insoluble aggregate. Most proteins would be expected to behave between these extremes, and the recombinant α, ß and ϒ GS polypeptides could provide soluble, intermediately soluble and insoluble examples. Alternatively, Schein and Noteborn (1988) have demonstrated a role for host growth temperature in the solubility of recombinantly expressed human interferon-α2, interferon-ϒ and a murine protein termed Mx, postulating that these three structurally divergent molecules undergo sulfhydryl group shuffling at high expression temperatures (approximately 37°C and above), enabling inter-molecular bond formation and product aggregation. However, the ability of three structurally similar GS polypeptides to behave, in terms of solubility, in such a divergent manner when recombinantly expressed (at 42°C), cannot at present be explained.

Examination of the native M_r of the individual recombinant GS activities has identified that all three GS subunits can assemble in *E.coli* into an oligomeric form. However, the values obtained for the recombinant α, ß and ϒ GS isoforms expressed in the *E.coli* strain ET8894 (FIG 6.4F) are not in agreement with previously determined values (Cullimore *et al*, 1983), which questions the authenticity of the results obtained. In addition, both recombinant α and ß GS activities expressed in the *E.coli* host streain N5151 have native M_r's comparable with native α and ß *P. vulgaris* GS isoforms (FIG 6.3F). Further gel filtration analysis of the ET8894 expressed recombinant α, ß and ϒ GS isoforms are thus required to explain this inconsistency before the previously published *P. vulgaris* root and nodule GS native M_r values are questioned. The assembly of the recombinantly expressed soluble *P. vulgaris* cytosolic GS subunits can apparently occur in the absence of any special plant assembly factors.

However, this does not rule out the possibility that the presence of plant assembly factors might not improve polypeptide solubility and/or the efficiency of GS polypeptide assembly, or that a bacterial assembly protein may play a role in the assembly process (see Hemmingsen *et al*, 1988). IEC FPLC analysis has identified that the recombinant α and ß GS activities elution characteristics do not correspond, and instead elute prior, to their homologous GS activities (FIG 6.3D). This behaviour is indicative of some positive charge modification to the recombinant GS subunits, such as the N-terminal addition of a pEV3 vector derived lysine residue during the cloning of all three GS cDNAs (see FIG 6.2B). N-terminal modifications to a recombinantly expressed large subunit RUBISCO gene resulted in the loss of enzyme activity (Kettleborough *et al*, 1987). However, similar changes to the three *P. vulgaris* GS coding regions have no apparent effect on recombinant enzyme GSt activity, suggesting the relative unimportance of this region, in agreement with the heterogeneity of other plant GS sequences within the N-terminal coding region (see FIG 3.6A).

Expression studies using the *E.coli glnA* mutated host strain ET8894 (MacNeil, 1981) have identified that the pcGS-Eα and pcGS-EΥ expression constructs (in addition to a bacterial GS gene) are able to complement the genetic lesion, enabling growth on minimal media in the absence of a glutamine supplement (FIG 6.4A). The ability of the pcGS-Eα and pcGS-EΥ expression vectors to complement the *glnA* mutation provides the definitive proof that the plant recombinant GS α and Υ polypeptide products can assemble into active enzymes that are able to effectively integrate and function in the bacterial *E.coli* nitrogen assimilatory pathway. In contrast, pEV3 recombinants can only grow on minimal media in the presence of a glutamine supplement, indicative of a non-complementing genotype. The inability to isolate any pcGS-Eß recombinants featuring the nonrepressed synthesis of the ß GS gene product in the presence or absence of glutamine is suggestive that the pcGS-Eß product is not only unable to complement the *glnA* mutation, but also encodes some lethal phenotype, possibly through the nonrepressed synthesis of the insoluble and inactive ß product. The ability to isolate pcGS-Eß recombinants under repressed conditions (in the presence of the cI857 repressor), confirmed that it was the recombinant ß GS product, and not another feature of the pcGS-Eß plasmid, that exhibited this phenotype.

The activity of the recombinant α and Υ GS products could be limiting, and hence would dictate the growth rate of the auxotrophic *glnA* host. The increased growth rates of the pcGS-Eα and pcGS-EΥ complemented ET8894 host strains at lower incubation temperatures, contrasting with the lower rate of pDC1 complementation, could be indicative of the recombinant α and Υ GS activities temperature optima. Alternatively, it could reflect the increased solubility and efficiency of assembly of the recombinant α and Υ GS subunits, roles that growth temperature have previously been associated with (O'Hare *et al*, 1987; Schein and Noteborn, 1988). DasSarma *et al* (1986) have demonstrated the synthesis, assembly and kinetic activity of the product of an alfalfa GS gene recombinantly expressed in *E.coli*, and furthermore, achieved the complementation of an *E.coli glnA* mutant with the plant GS gene. However, the authors proposed, following the failure of the plant GS gene to complement the *glnA* mutant at low media concentrations of NH_4^+ (approximately 0.1 mM) or using an alternative nitrogen source such as arginine, that the plant GS enzyme was not efficiently integrated into the *E.coli* host NH_4^+ assimilatory pathway.

In contrast, the *P. vulgaris* pcGS-Eα and pcGS-EΥ allowed the growth of the *glnA* auxotrophic ET8894 strain even at low ammonium concentrations (0.1 mM), or in the presence of the alternative nitrogen source arginine. However, this might not be due to differences between the recombinantly expressed *P. vulgaris* and alfalfa GS gene products, but alternatively reflect the temperature of incubation for the alfalfa GS mediated complementation, which was unspecified by DasSarma *et al* (1986). If DasSarma *et al* (1986) performed alfalfa GS mediated complementation at 37°C, then both *P. vulgaris* and alfalfa GS gene products would appear to behave in a similar manner at lower concentrations of media NH_4Cl or using the alternative nitrogen source, arginine (see Table 6.4A).

No kinetic characterisation of the recombinant pcGS-Eα, pcGS-Eß or pcGS-EΥ GS products has been performed. However, the results of this chapter suggest that a recombinant source of each cytosolic GS isoform is available to perform a comparative study of their kinetic similarities and differences.

CHAPTER 7

CONCLUSION

CONCLUSION

The data presented in this thesis can be summarised under three separate headings, and the major points arising from the data are as follows:-

A) The isolation and characterisation of an essentially full length nodule GS cDNA from *P. vulgaris*.

i) Three GS cDNAs (F14, F23 and F27) were isolated from a cDNA library prepared to *P. vulgaris* root nodule poly (A) RNA, and characterised by restriction analysis (FIG 3.2D). The F14 cDNA was further characterised by DNA sequencing (FIG 3.5C), appearing identical at its 3' end to the partial GS cDNA, pcPvNGS-01 (Cullimore *et al*, 1984). Furthermore, the F14 5' sequence appeared identical with a nodule expressed GS genomic clone (B.G.Forde, pers. commun.), with which first base of the F14 sequence corresponded to the major transcriptional start site. F14 therefore represents an essentially full length GS cDNA.

ii) The polypeptide encoded by the F14 cDNA and two previously identified GS cDNA clones, pR-1 and pR-2 (see Gebhardt *et al*, 1986) have been produced *in vitro* by transcription/translation and shown to co-migrate on two-dimensional gels with the cytosolic ϒ, β and α GS polypeptides respectively (FIG 3.3). From this work the cDNA clones were renamed accordingly pcGS-α1, pcGS-β1 and pcGS-ϒ1, and their genomic equivalents *gln-α*, *gln-β* and *gln-ϒ* respectively (Bennett *et al*, submitted).

B) The analysis of the expression of the *gln-ϒ* mRNA and the ϒ polypeptide in *P. vulgaris*.

i) An RNase protection technique has been used to specifically and quantitatively determine the abundance of the *gln-β*, *gln-ϒ* and *Lhb* mRNAs during nodule development. The *gln-β* mRNA is constitutively expressed during nodulation, whereas the *gln-ϒ* and *Lhb* mRNAs are coordinately expressed, initially detectable at least one day before the onset of dinitrogen fixation (FIG 5.2F).

ii) A study of the abundance of the *gln-ϒ* and *Lhb* mRNAs from a variety of *P. vulgaris* organs has identified that in contrast with *Lhb* the

expression of the *gln*-Y gene is not nodule specific as previously suggested (Cullimore *et al*, 1984; Gebhardt *et al*, 1986), since *gln*-Y mRNA is also detectable, albeit at a lower abundance, in stems, petioles, and green cotyledons (FIG 3.4C).

iii) From studies which show that the relative abundance of the *gln*-Y mRNA and the Y polypeptide in different organs (FIG 3.4C and D) and during nodulation (FIG 5.2F and E) is approximately constant, it is suggested that the appearance of the Y polypeptide is largely under transcriptional control with little regulation at the translational level.

iv) It is concluded, following the detection of *gln*-Y mRNA in nodules prior to the onset of dinitrogen fixation, and the identification of the Y polypeptide in nodules grown in the absence of dinitrogen (under $Ar:O_2$, see FIG 5.3E), that a product of dinitrogen fixation does not provide the primary signal for *gln*-Y nodule expression. However, the reduced abundance of the Y polypeptide in nodules grown under $Ar:O_2$ relative to $N_2:O_2$ samples, and the enhanced level of the Y polypeptide in nodules initially grown under $Ar:O_2$ but later switched to $N_2:O_2$ (FIG 5.3E), suggests that a product of dinitrogen fixation may have a role in the level of *gln*-Y nodule expression or on the accumulation of the Y polypeptide.

C) The structure of *P. vulgaris* GS has been studied using both recombinantly expressed and native plumule and nodule sources of the enzyme.

i) The three cytosolic GS cDNAs (pcGS-α1, pcGS-β1 and pcGS-Y1) have been expressed individually in *Escherichia coli*, synthesising GS subunits of the correct M , which appear for α, Y and β to be mainly soluble, intermediate in solubility and insoluble respectively (FIG 6.4E). The soluble α, β and Y GS subunits are able to assemble into kinetically active oligameric isoforms, in the absence of any specific plant assembly factors (FIG 6.4F). In addition, the recombinant α, β and Y GS isoforms retain similar ion exchange properties to their homologous native GS isoforms (FIG 6.4E). The α and Y cDNAs have been shown to complement an *E.coli glnA* mutation, suggesting that the recombinant α and Y higher plant GS enzymes can effectively function in the *E.coli* nitrogen assimilatory pathway

(FIG 6.4A). Furthermore, the ability of the plant α and ϒ GS cDNAs to complement the *E.coli glnA* mutant is strongly temperature dependent, achieving faster growth rates at temperatures of 25°C and 30°C, compared to 37°C (FIG 6.4B).

ii) The structure of *P. vulgaris* nodule and plumule GS has been studied through the resolution of the component GS isoforms, and the determination of their respective GS polypeptide compositions by IEC FPLC and two-dimensional western blotting respectively (see sections 4.2 and 4.4). These studies have identified that both α + ß, and ß + ϒ GS subunits may assemble, perhaps randomly, to produce a heterogeneous mixture of GS isoforms. However, a study of the changes in GS isoforms during nodulation (FIG 5.3C) has shown that the ß isoform is present at higher activity than would be expected for random subunit mixing, which could reflect differences in the temporal and/or spatial expression of the ϒ and ß GS subunits within the nodule. Similar conclusions have been drawn to interpret the changes in GS isoforms during plumule development (FIG 4.4A and C).

iii) The increase in GS activity during nodulation was correlated to the initial detection of *gln-ϒ* mRNA and the appearance of the ϒ polypeptide and isoform (FIG 5.3F, E and C). During later stages of nodule development the appearance and increased importance of nodule GS isoforms composed of both ϒ and ß GS subunits reflected the decrease in the abundance of the *gln-ϒ*, relative to the *gln-ß*, mRNAs.

iv) A minor nodule plastid GS isoform has been identified (FIG 4.2A), detectable during later stages of nodule development following the onset of dinitrogen fixation (FIG 5.2C).

A model is presented (FIG 7), based on the findings of this thesis and other data, for the synthesis and the assembly of the individual GS polypeptides in the root nodule of *P. vulgaris*. All four *P. vulgaris* GS genes (*gln-α*, *gln-ß*. *gln-ϒ* and *gln-δ*) are expressed during nodule development (Gebhardt *et al*, 1986; Padilla *et al*, 1987; Bennett *et al*, submitted), each featuring distinctive patterns and levels of expression. Their individual GS polypeptide products assemble into a variety of GS isoforms. The plastid GS subunits (δ), that are spatially separate from the cytosolic GS polypeptides following post-translational retargeting (Lightfoot *et al*, 1988), assemble into a plastid GS isoform

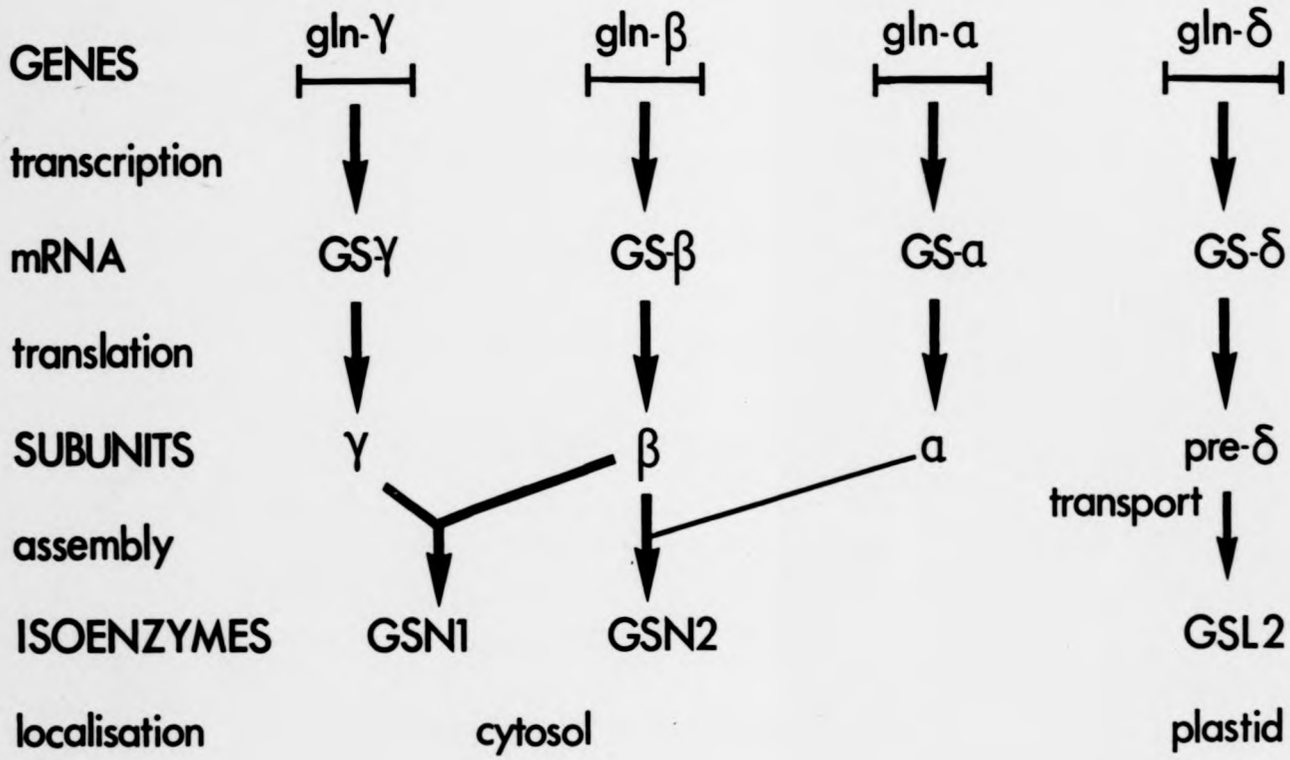

FIG 7 A model describing the synthesis of GS isoenzymes in root nodules of *P. vulgaris*. GSN1 and GSN2 are terms used to describe cytosolic isoenzymes that contain both γ + β and β (+ α) respectively, whereas the plastidic GSL2 isoenzyme is composed of δ polypeptide(s).

(GSL2). The cytosolic GS subunits (α, β and γ) assemble into a variety of GS isoforms, separable by IEC FPLC, composed of γ + β, and β (+ α) polypeptides, termed GSN1 and GSN2 respectively (FIG 4.2A-C). The increase in nodule GS activity, following the detection of dinitrogen fixation, is mainly attributable to the GSN1 isoform (Lara *et al*, 1983; FIG 5.2D) and, to a lesser extent, the plastid GS isoform.

CHAPTER 8

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TITLE

STRUCTURE AND EXPRESSION OF GLUTAMINE SYNTHETASE IN ROOT NODULES OF *PHASEOLUS VULGARIS* L.

AUTHOR

Malcolm John Bennett BSc (Hons) Manchester