

**A Thesis Submitted for the Degree of PhD at the University of Warwick**

**Permanent WRAP URL:**

<http://wrap.warwick.ac.uk/80168>

**Copyright and reuse:**

This thesis is made available online and is protected by original copyright.

Please scroll down to view the document itself.

Please refer to the repository record for this item for information to help you to cite it.

Our policy information is available from the repository home page.

For more information, please contact the WRAP Team at: [wrap@warwick.ac.uk](mailto:wrap@warwick.ac.uk)

CHARACTERISATION AND EXPRESSION OF THE GLUTAMINE SYNTHETASE  $gln-\alpha$   
GENE OF FRENCH BEAN.

by Adam Terence Watson BSc (Hons) Biochemistry

Thesis submitted for the degree of Doctor of Philosophy.

Department of Biological Sciences,

University of Warwick.

May 1993.

to the dogs

declaration:

The work contained in this thesis is the result of original research conducted by myself under the supervision of Dr J.V. Cullimore. All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used in any previous application for a degree.

A.T. Watson.



<u>contents:</u>	<u>page no.</u>
list of figures	I
list of tables	VI
abbreviations	VII
abstract	X
<u>Chapter 1: Literature Review.</u>	
1.1 Introduction	2
1.2 Ammonia assimilation	6
1.2.a Asparagine metabolism	11
1.2.b Ureide metabolism	14
1.3 GS in higher plants	16
1.4 GS in <u>Phaseolus vulgaris</u>	17
1.5 GS in other dicotyledons	22
1.6 Regulation of GS genes by ammonium	25
1.7 Precis of project	29
<u>Chapter 2: Materials and Methods.</u>	
2.1 Chemicals	31
2.2 Growth and storage of bacterial strains	31
2.3 Construction of a <u>P. vulgaris</u> genomic library in bacteriophage lambda	34
2.3.a Plant material	34
2.3.b Isolation of plant DNA	34
2.3.c Determination of optimal conditions for the production of 15-20Kbp fragments	34
2.3.d Large scale preparation of 15-20Kbp genomic fragments for cloning	35

2.3.e Fractionation of the genomic fragments using sucrose density gradient centrifugation	35
2.3.f Ligation of the genomic fragments to lambda Dash arms	36
2.3.g <u>In-vitro</u> packaging of DNA and titration	37
2.4 Screening of the genomic library for <u>gln-a</u> sequences	37
2.4.a Immobilisation of phage DNA onto nylon membranes	38
2.4.b Hybridisation of bacteriophage plaques to radiolabelled $\alpha$ -cDNA probes	38
2.5 <u>In-vitro</u> synthesis of radiolabelled nucleic acid probes	42
2.5.a Transcription	42
2.5.b Oligolabelling	43
2.6 Purification of phage DNA from positive clones	44
2.7 Sub-cloning of promoter sequences into plasmid vectors	45
2.8 Southern blotting and hybridisation of genomic and phage DNA	46
2.8.a Restriction endonuclease digestion	46
2.8.b Agarose gel electrophoresis	47
2.8.c Transfer of DNA to nylon membranes	47
2.8.d Hybridisation of radiolabelled probe to filter immobilised DNA	47
2.9 Preparation of plasmid DNA	48
2.9.a Small-scale preparations of plasmid DNA	48
2.9.b Large-scale preparations of plasmid DNA	48

2.10 Mapping of the <u>gln-<math>\alpha</math></u> transcriptional start	49
2.10.a using an RNase protection technique	49
2.10.b using a primer extension technique	52
2.11 DNA sequencing	53
2.11.a sequencing of plasmid DNA	54
2.11.a direct sequencing of PCR products	55
2.12 Construction of the fusion of the <u>gln-<math>\alpha</math></u> promoter to the <u>E. coli uidA</u> reporter gene	55
2.13 Plant transformation	59
2.13.a <u>Nicotiana tabacum</u> (tobacco) cv. Samsun	59
2.13.b <u>Lotus corniculatus</u> cv. Leo	60
2.14 Pre-fixation of plant material	61
2.15 Histochemical staining	61
2.16 Tissue sectioning	62
2.17 Wounding of tobacco leaves	62
2.18 Fluorimetric assay of extractable GUS activity	63
<u>Chapter 3: Results - Cloning and characterisation of the gln-<math>\alpha</math> gene promoter and upstream region.</u>	
3.1 Introduction	66
3.2 Construction and screening of the genomic library	68
3.3 Southern blots of phage DNA and genomic DNA	69
3.4 Sub-cloning of promoter sequences into plasmid vectors	75
3.5 Direct sequencing of the PCR product	76
3.6 Sequencing of the <u>gln-<math>\alpha</math></u> promoter	81
3.7 To determine if the 5' 28bp of the $\alpha$ -cDNA are	

represented in the cultivar Tendergreen $\alpha$ -mRNA	84
3.8 Mapping the transcriptional start site	85
3.8.a primer extension	85
3.8.b RNase protection	88
3.9 Consensus sequences	91
3.10 Discussion	96
3.11 Conclusions	106
<u>Chapter 4: Results - Activity of the gln-<math>\alpha</math> promoter in transgenic <i>Nicotiana tabacum</i> (tobacco).</u>	
4.1 Introduction	108
4.2 Tobacco transformation	110
4.3 Measurement of the level of extractable GUS activity of the root-tips of transgenic plants	113
4.4 Histochemical analysis of roots of transgenic plants	114
4.5 Measurement of the level of extractable GUS activity of leaves of transgenic plants	129
4.6 Histochemical analysis of leaves of transgenic plants	141
4.7 Histochemical analysis of flowers of transgenic plants	146
4.8 Histochemical analysis of transgenic F1 seedlings	167
4.9 The effect of mechanical wounding on the level of extractable GUS activity of the leaves of transgenic plants	176
4.10 Histochemical analysis of wounded leaves of transgenic plants	177
4.11 Discussion	183

4.11.a between-line variation in GUS activity	183
4.11.b possible physiological functions relating to <u>gln-<math>\alpha</math></u> expression	186
4.11.c the effect of mechanical wounding on <u>gln-<math>\alpha</math></u> promoter activity	192
4.12 Conclusion	194
<u>Chapter 5: Results - Activity of the gln-<math>\alpha</math> promoter in transgenic</u>	
<u>Lotus corniculatus.</u>	
5.1 Introduction	196
5.2 Plant transformation	198
5.3 Histochemical analysis of nodulated transgenic roots	198
5.4 Chimeric gene activity during nodule development and senescence	201
5.5 Discussion	209
5.6 Conclusions	215
<u>Chapter 6: Concluding discussion.</u>	217
<u>Chapter 7: References.</u>	227

list of figures:

<u>fig. no.</u>	<u>title</u>	<u>page no.</u>
fig. 1.1.	The glutamate synthase cycle.	8
fig. 1.2.	Proposed route for asparagine synthesis.	13
fig. 2.1.	Restriction map of the $\alpha$ -cDNA clone pcPvGS- $\alpha$ 1.	41
fig. 2.2.	Construction of plasmid clones for transcription probe synthesis for use in RNase protection assays.	51
fig. 2.3.	Production of the <u>gln-<math>\alpha</math>:uidA</u> construct and the subcloning of the construct into the plant transformation vector Bin19.	57
fig. 3.1.	Hybridisation of the $\alpha$ -cDNA: <u>HindIII</u> transcription probe to the Southern blot of phage "a" and "i" DNA and <u>P. vulgaris</u> genomic DNA.	71
fig. 3.2.	Hybridisation of the $\alpha$ -cDNA: <u>EcoRI</u> oligolabelled probe to the Southern blot of phage "a" and "i" DNA and <u>P. vulgaris</u> genomic DNA.	74
fig. 3.3.	Hybridisation of the <u>gln-<math>\alpha</math></u> oligolabelled probe to the Southern blot of phage "a" DNA.	78
fig. 3.4.	Hybridisation of the <u>gln-<math>\alpha</math></u> oligolabelled probe to the Southern blot of phage "a" DNA restricted using <u>EcoRI</u> and <u>HindIII</u> and the <u>gln-<math>\alpha</math></u> promoter fragment restricted from <u>pgln-<math>\alpha</math></u> using <u>EcoRI</u> and <u>HindIII</u> .	80

fig. 3.5. The DNA sequence of the <u>gln-<math>\alpha</math></u> upstream region isolated from phage clone "a" using the PCR technique.	83
fig. 3.6. RNase protection assay to determine whether the 5' 28bp of the $\alpha$ -cDNA are represented in the $\alpha$ -mRNA of the <u>P. vulgaris</u> cultivar Tendergreen.	87
fig. 3.7. Primer extension analysis to determine the <u>gln-<math>\alpha</math></u> transcriptional start site.	90
fig. 3.8. RNase protection assay to determine the <u>gln-<math>\alpha</math></u> transcriptional start.	93
fig. 3.9. Possible restriction enzyme maps for the phage clone "a" insert.	100
fig. 4.1. DNA sequence of the fusion site between the <u>gln-<math>\alpha</math></u> gene promoter and the coding sequence of the <u>E. coli uidA</u> gene.	112
fig. 4.2. Extractable GUS activity from the root-tips of 20 individual transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant lines.	116
fig. 4.3. Extractable GUS activity from the root-tips of 25 individual transgenic 35S:: <u>uidA</u> tobacco plant lines.	118
fig. 4.4. Extractable GUS activity from the root-tips of 12 individual transgenic promoterless:: <u>uidA</u> tobacco plant lines.	120

- fig. 4.5. Histochemical localisation of GUS activity in the root tip tissues of transgenic gln- $\alpha$ ::uidA tobacco plants. 123
- fig. 4.6. Histochemical localisation of GUS activity in lateral root initiation sites and emerging lateral roots of transgenic gln- $\alpha$ ::uidA tobacco plants. 126
- fig. 4.7. Histochemical localisation of GUS activity in those root epidermal cells damaged during lateral root emergence in a transgenic gln- $\alpha$ ::uidA tobacco plant. 128
- fig. 4.8. Histochemical localisation of GUS activity in the root hairs of a transgenic gln- $\alpha$ ::uidA tobacco plant. 131
- fig. 4.9. Histochemical localisation of GUS activity in the roots of a transgenic 35S::uidA tobacco plant. 133
- fig. 4.10. Extractable GUS activity from the leaves of 20 individual transgenic gln- $\alpha$ ::uidA tobacco plant lines. 136
- fig. 4.11. Extractable GUS activity from the leaves of 25 individual transgenic 35S::uidA tobacco plant lines. 138
- fig. 4.12. Extractable GUS activity from the leaves of 12 individual transgenic promoterless::uidA tobacco plant lines. 140
- fig. 4.13. Histochemical localisation of GUS activity in a leaf of a transgenic gln- $\alpha$ ::uidA tobacco plant. 145



fig. 4.14. Histochemical localisation of GUS activity in a leaf of a transgenic 35S:: <u>uidA</u> tobacco plant.	148
fig. 4.15. Histochemical localisation of GUS activity in the anthers of a transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant.	151
fig. 4.16. Histochemical localisation of GUS activity in the ovary of a transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant flower.	154
fig. 4.17. Histochemical localisation of GUS activity in the stigma and style of a transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant flower.	156
fig. 4.18. Histochemical localisation of GUS activity in the anthers and stigma and style of a transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant flower.	158
fig. 4.19. Histochemical localisation of GUS activity in transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant pollen.	160
fig. 4.20. Histochemical localisation of GUS activity in the 11 day-old fruits of transgenic (a) <u>gln-<math>\alpha</math>::uidA</u> and (b) 35S:: <u>uidA</u> tobacco plants.	163
fig. 4.21. Histochemical localisation of GUS activity in the 21 day-old fruits of transgenic (a) <u>gln-<math>\alpha</math>::uidA</u> and (b) 35S:: <u>uidA</u> tobacco plants.	165
fig. 4.22. Histochemical localisation of GUS activity in the root-tip tissues of 2 week-old transgenic <u>gln-<math>\alpha</math>::uidA</u> seedlings.	169

fig. 4.23. Histochemical localisation of GUS activity in emerging lateral root tissues of 2 week-old transgenic <u>gln-<math>\alpha</math>::uidA</u> seedlings.	171
fig. 4.24. Histochemical localisation of GUS activity in the vascular tissue of a cotyledon of a 2 week-old transgenic <u>gln-<math>\alpha</math>::uidA</u> seedling.	173
fig. 4.25. Histochemical localisation of GUS activity in the 2 week-old transgenic 35S:: <u>uidA</u> seedling.	175
fig. 4.26. Fold induction in extractable GUS activity 24 hours after wounding of (a) <u>gln-<math>\alpha</math>::uidA</u> leaves and (b) 35S:: <u>uidA</u> leaves.	179
fig. 4.27. Histochemical localisation of GUS activity in the vascular tissue of a transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant leaf 24 hours after mechanical wounding.	181
fig. 5.1. Histochemical localisation of GUS activity in a transgenic <u>gln-<math>\alpha</math>::uidA Lotus corniculatus</u> root.	200
fig. 5.2. Histochemical localisation of GUS activity in emerging lateral root tissues of a transgenic <u>gln-<math>\alpha</math>::uidA Lotus corniculatus</u> root.	203
fig. 5.3. Histochemical localisation of GUS activity in nodule primordia/lateral root initiation site tissues of transgenic <u>gln-<math>\alpha</math>::uidA Lotus corniculatus</u> root.	206
fig. 5.4. Histochemical localisation of GUS activity in senescing transgenic <u>gln-<math>\alpha</math>::uidA Lotus corniculatus</u> nodules.	208

list of tables:

<u>table no.</u>	<u>title</u>	<u>page no.</u>
table 1.	<u>E. coli</u> strains used and their genotype.	33
table 2.	variability in transgenic <u>gln-<math>\alpha</math>::uidA</u> tobacco plant leaf extractable GUS activity within the same transgenic plant line with repeated sampling.	143

abbreviations:

A = adenine

AAT = aspartate aminotransferase

ADP = adenosine-5'-diphosphate

ALA = allantoin

ALN = allantoic acid

AS = asparagine synthetase

ATP = adenosine-5'-triphosphate

BAP = benzaminopurine

bp = base pair

C = cytosine

CA = cinnamic acid

CaMV = cauliflower mosaic virus

cDNA = complementary DNA

CGA = ribonucleotide mix; deoxyribocytidide-5'-triphosphate, deoxyriboguanosine-5'-triphosphate and deoxyriboadenosine-5'-triphosphate

C/N = carbon/nitrogen ratio

cpm = counts per minute

dATP = deoxyadenosine-5'-triphosphate

dCTP = deoxycytidine-5'-triphosphate

dGTP = deoxyguanosine-5'-triphosphate

dTTP = deoxythymidine-5'-triphosphate

E. coli = Escherichia coli

EDTA = disodium ethylene diamine tetraacetate  
EtBr = ethidium bromide  
G = guanine  
GDH = glutamate dehydrogenase  
GOGAT = glutamate synthase  
GS = glutamine synthetase  
GS<sub>1</sub> = cytosolic GS  
GS<sub>2</sub> = chloroplastic GS  
GUS =  $\beta$ -glucuronidase  
hr = hour  
IPTG = iso-propyl- $\beta$ -D-thio-galactopyranoside  
Kbp = kilo base pair  
min = minute  
M<sub>r</sub> = molecular weight  
MS = Murashige and Skoog  
MU = 4-methylumbelliferone  
MUG = 4-methylumbelliferyl glucuronide  
NAA = indolacetic acid  
NAD<sup>+</sup>/NADH = nicotinamide adenine dinucleotide  
NADP<sup>+</sup>/NADPH = nicotinamide adenine dinucleotide phosphate  
NH<sub>4</sub><sup>+</sup> = ammonium  
OLB = oligolabelling buffer  
O/N = overnight  
PAL = phenylalanine ammonia-lyase  
PCR = polymerase chain reaction  
PEG = polyethylene glycol

pfu = plaque forming units

P<sub>i</sub> = phosphate

P. vulgaris = Phaseolus vulgaris L.

<sup>32</sup>P-GTP = <sup>32</sup>P-deoxyguanosine-5'-triphosphate

<sup>32</sup>P-UTP = <sup>32</sup>P-deoxyribouridine-5'-triphosphate

RUBISCO = ribulose biphosphate carboxylase/oxygenase

RT = room temperature

<sup>35</sup>S-dATP = <sup>35</sup>S-deoxyadenosine-5'-triphosphate

SDS = sodium dodecyl sulphate

SDW = sterile distilled water

SSC = saline sodium citrate buffer

T = thymidine

TAE = Tris-acetate EDTA buffer

TBE = Tris-borate EDTA buffer

TE = Tris-EDTA buffer

Tris = 2-amino-2(hydroxymethyl)-1,3-propanediol

UV = ultraviolet

V = volts

v/v = volume per volume

w/v = weight per volume

w/w = weight per weight

X-gal = 5-bromo-4-chloro-3-indolyl-β-galactoside

X-gluc = 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

abstract:

Approximately 900bp of the Phaseolus vulgaris cytosolic glutamine synthetase gln- $\alpha$  gene promoter has been cloned and the DNA sequence determined. The transcriptional start site was mapped using primer extension and RNase protection techniques. The promoter fragment was fused to the E. coli uidA reporter gene encoding the  $\beta$ -glucuronidase (GUS) enzyme and introduced into transgenic Nicotiana tabacum (tobacco) and Lotus corniculatus. In transgenic tobacco, the gln- $\alpha$  promoter directed uidA expression in the root-tip, in incipient lateral roots and in emerging lateral roots of all the transgenic plant lines studied. The promoter also directed uidA expression in the root vascular tissue and in the root hairs of a proportion of the lines studied. With the exception of root hairs, the promoter conferred a similar pattern of expression in transgenic L. corniculatus roots and expression of uidA was also observed in senescing mature nodules and possibly in incipient nodules. Expression of uidA was absent from young and mature transgenic nodules. Activity of the gln- $\alpha$  promoter was also associated with several tobacco flower structures including anthers, ovary placenta and vascular tissue, ova, pollen and the stigma and style. Several developing tobacco fruit structures including the placenta, vascular tissue, the developing seed cotyledon and the developing seed coat were also associated with gln- $\alpha$  promoter activity. The level of extractable leaf GUS activity was observed to increase between 2- and 5-fold 24 hours after

mechanical wounding, relative to non-wounded transgenic leaf tissue. Although twenty four hours after wounding, uidA expression was not associated with those leaf tissues adjacent to the wound site, a higher proportion of wounded leaves showed uidA expression associated with the vascular tissue than in non-wounded leaves. The extractable leaf GUS activity of control cauliflower mosaic virus 35S promoter::uidA transgenic plants were seen to decrease up to 50% 24 hours after mechanical wounding relative to non-wounded leaf tissue. Possible physiological functions for gln- $\alpha$  gene expression are discussed.



acknowledgements:

I would sincerely like to thank my supervisor, Dr. J.V. Cullimore for her help and encouragement over the past three years, and to the Science and Engineering and Research Council for financing this research.

## Chapter 1: Literature Review.

## 1.1 Introduction:

Nitrogen is an essential plant nutrient and its availability is normally the limiting factor in plant growth in the Western world. Various forms of inorganic nitrogen are accessible to assimilation by plants and include the direct uptake of soil ammonium, the reduction of soil nitrate and, in legumes the reduction of atmospheric dinitrogen by rhizobial symbionts. These are collectively known as "primary" sources of nitrogen and are assimilated mainly in different organs; roots, roots and leaves, and nodules respectively.

The reduction of soil nitrate to ammonium is catalysed sequentially by nitrate reductase and a series of further reductions to lower oxidation states by nitrite reductase. Irrespective of whether the process of nitrate reduction is located in photosynthetic or non-photosynthetic tissues, it is generally accepted that the reduction of nitrate into nitrite occurs in the cytosol whereas the subsequent steps of nitrite reduction take place in the plastid (Kleinhofs and Warner, 1990).

The soil bacteria of genera Rhizobium and Bradyrhizobium elicit hypertrophic growth on the roots of legume plants to form a highly specialised new organ; the nodule. Ultimately, the two organisms establish metabolic co-ordination. The bacteria reduce (fix) atmospheric dinitrogen into ammonium which is secreted into the plant-host cytosol and assimilated. The plant supplies carbon compounds, derived from photosynthesis in the shoot, to

the bacteria for generation of ATP and reducing electrons. The symbiotic association makes the legume plant autotrophic for external nitrogen. In the initial stages of nodule development, the bacterium induces three processes; root hair curling and deformation, infection thread formation and cortical cell division. The induction of mitotic activity leads to the formation of a globular nodule primordia in the root cortex. Infection threads ramify and penetrate target cells within the nodule and the bacteria are released into the plant cytosol, enveloped in plant plasma membrane and form structures referred to as "bacteroids". The bacteria and plant cells differentiate further and begin symbiotic nitrogen fixation and metabolite exchange. A cascade event controlling the expression of plant nodule specific or "nodulin" genes allows for the development of the infection by Rhizobia through to the establishment of the symbiotic state.

Certain plant metabolic pathways such as photorespiration, phenylpropanoid metabolism and amino acid and ureide catabolism release ammonium (Joy, 1988).

Photorespiration is the process that results in the significant loss of photosynthetic capacity in  $C_3$  plants. Photorespiration occurs under a high ratio of  $O_2:CO_2$  in the atmosphere and is therefore a consequence of photosynthesis. Photorespiration involves the consumption of  $O_2$  and the release of  $CO_2$ . Ribulose biphosphate carboxylase/oxygenase (RUBISCO) has two catalytic activities allowing either carboxylation or

oxygenation of ribulose biphosphate (RuBP) depending on the relative concentrations of CO<sub>2</sub> and O<sub>2</sub>. During photorespiration, RuBP is oxidised to produce the two carbon compound phosphoglycolate as well as phosphoglycerate. Phosphoglycolate is converted within the chloroplast to glycolate which is subsequently metabolised within the peroxisome to glycine. Finally, within the mitochondrion, two molecules of glycine are converted to one molecule each of serine, CO<sub>2</sub> and ammonium. The importance of the release of ammonium from the photorespiratory cycle was first realised by Keys and co-workers (1978) who calculated the cycle to release 10-fold more ammonium than that obtained from the uptake and reduction of the plant's primary, inorganic nitrogen. The reassimilation of this ammonium is essential for the maintenance of the nitrogen economy of the plant and to avoid the potentially toxic accumulation of ammonium. Despite the photorespiratory deamination reaction occurring in the mitochondrion, there is good evidence to suggest that the ammonium produced is reassimilated in the chloroplast (Wallsgrave et al., 1987; Blackwell et al., 1987).

The phenylpropanoid pathway is responsible for the production of various secondary phenolic plant metabolites including flavonoid pigments (Brouillard, 1988), lignin (Rubery and Northcote, 1968), antimicrobial phenolics (phytoalexins; Lamb et al., 1989), UV protectants (Schmeltzer et al., 1989) and polar auxin transport regulators (Jacobs and Rubery, 1988). Moreover, phenylpropanoid products function as signals in the rhizosphere

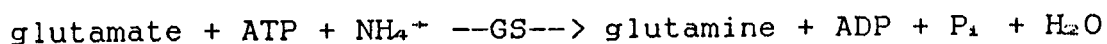
and act as transcriptional activators of the tumour-inducing bacterium Agrobacterium tumefaciens (Bolton et al., 1986) and the symbiotic dinitrogen-fixing Rhizobium (Long, 1989). Ammonium is produced during the deamination of L-phenylalanine to yield trans-cinnamic acid. The reaction is the first committed step in the pathway and is catalysed by the enzyme phenylalanine ammonia-lyase (PAL) and fluctuations in PAL levels are a key element in the control of phenylpropanoid biosynthesis (Jones, 1984).

In P. vulgaris there is a multigene family of three PAL genes; PAL1, PAL2 and PAL3 (Cramer et al., 1989) that encode distinct polypeptides (Liang et al., 1989a). Studies have shown the PAL genes in P. vulgaris to be differentially regulated during plant development and by environmental cues (Liang et al., 1989a).

## 1.2 Ammonia Assimilation:

Irrespective of source, in most plants and microorganisms studied to date the initial steps of ammonium assimilation are completed by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyses the initial, ATP-dependent incorporation of ammonium into glutamate to form glutamine (reaction 1), and GOGAT catalyses the transamidation of the glutamine amido-nitrogen to the  $\alpha$ -amino position of 2-oxoglutarate to form two moles of glutamate (reaction 2). As the enzymatic reactions are dependent on the provision of substrate by the other, the two reactions constitute a cycle. The cycle is known as the "glutamate synthase cycle" (see fig. 1.1) and results in the net biosynthesis of glutamate from ammonium and 2 oxo-glutarate (Mifflin and Lea, 1980). This is considered the major route for ammonium assimilation in higher plants and the glutamate derived from the glutamate synthase cycle is the nitrogen donor in the biosynthesis of essentially all nitrogenous compounds in higher plants including amino acids, nucleic acids and polyamines.

### reaction 1:



### reaction 2:

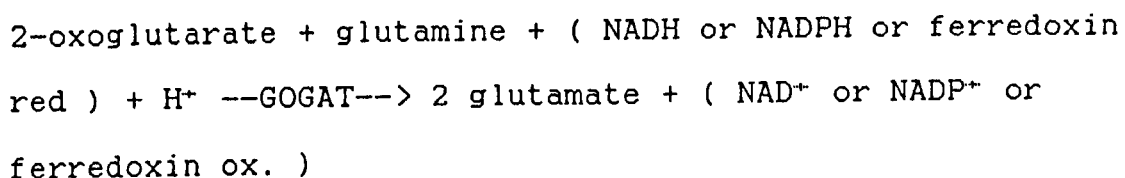
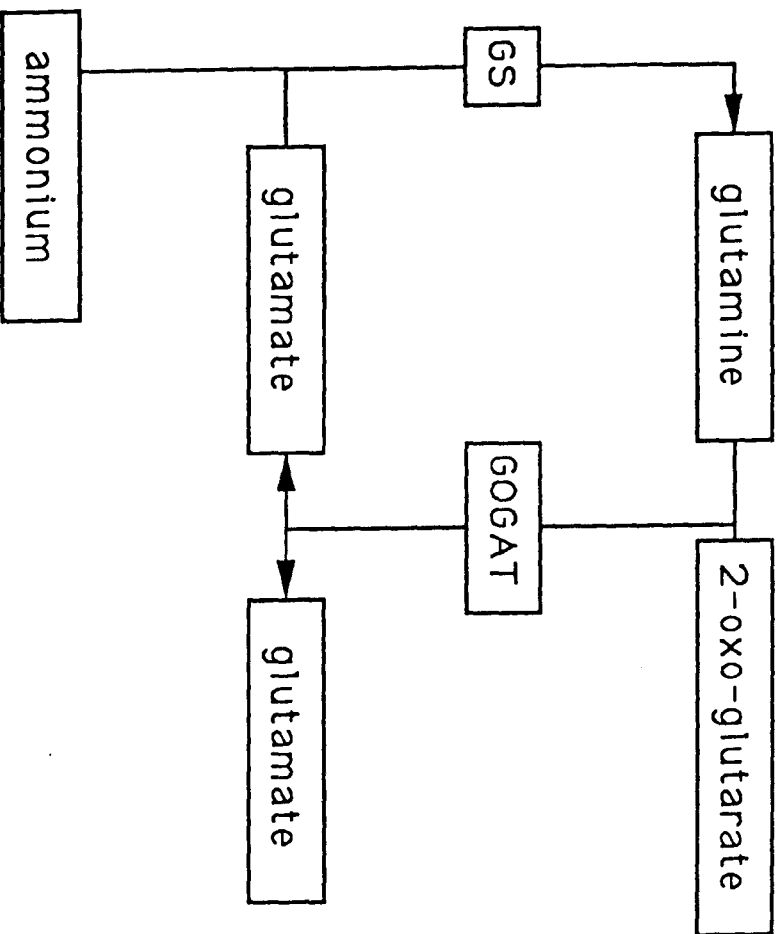


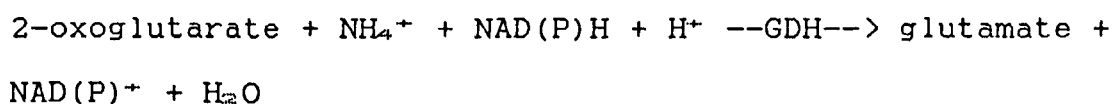
fig. 1.1. The glutamate synthase cycle (Miflin and Lea 1980);





For many years the assimilation of ammonium was thought to occur via the reductive amination of 2-oxoglutarate to give glutamate as catalysed by glutamate dehydrogenase (GDH) (reaction 3).

reaction 3:



Despite GDH activity having been found in many plant species and been shown to be abundant in most plant tissues, overwhelming evidence supports the glutamate synthase cycle to be the major route for ammonium assimilation in plants. Studies by Meeks et al. (1978) demonstrated that ammonium was first incorporated into the amide position of glutamine in the reaction catalysed by GS. The incorporation was blocked by methionine sulfoximine, an inhibitor of GS (Meeks et al., 1978). The subsequent transfer of the amide group to oxoglutarate as catalysed by GOGAT is blocked by azaserine, an inhibitor of GOGAT activity. In addition, GS has a much lower  $K_m$  for ammonium than GDH and allows the glutamate synthase cycle to function at very low ammonium concentrations. This is important in maintaining a favourable gradient in the export of ammonium from bacteroids (Rawsthorne et al., 1980). Further evidence for the glutamate synthase cycle to be the primary assimilatory route for ammonium came from developmental studies of nodule enzyme activities. When alfalfa nodulated roots were deprived of their shoot systems, both GS and GOGAT

declined in parallel with nitrogenase activity. During nodule rebuilding, GS and GOGAT activities increased with the increase in nitrogenase activity (Groat and Vance, 1981).

Studies regarding the reassimilation of ammonium released through photorespiration have shown that the route of reassimilation is via the glutamate synthase cycle (Wallsgrave et al., 1987; Blackwell et al., 1987). The studies involved the analysis of mutant barley plant lines lacking the GS chloroplastic isoenzyme. Under photorespiratory conditions the ammonium concentration in leaves of mutant plant lines were shown to rise significantly despite the cytosolic GS isoenzyme being at levels comparable to those of wild-type plants (Wallsgrave et al., 1987; Blackwell et al., 1987). Studies regarding the reassimilation of ammonium resulting from the catabolism of nitrogenous storage and transport compounds have yielded results consistent with the major pathway of ammonium reassimilation being via the glutamate synthase cycle (Lea et al., 1990). There is however little information concerning the reassimilation of ammonium produced through PAL activity which must reach significant levels in some plant tissues (Joy, 1988).

The subsequent metabolism of the glutamate derived from primary nitrogen sources differs between species and in certain cases, within species, depending on whether dinitrogen or nitrate is the overriding primary nitrogen source being assimilated (Pate and Atkins, 1983). Plant species synthesise one or only a few nitrogenous solute(s) at the site(s) of primary nitrogen

assimilation which are then transported through the xylem and phloem to other parts of the plant where they may be stored or subsequently used as sources of nitrogen for synthesis of nitrogenous compounds. Examples of nitrogenous transport and storage solutes include the amides asparagine and glutamine, the ureides allantoin and allantoic acid, and certain non-protein amino compounds such as citrulline, homoserine and methylene glutamate (Pate and Layzell, 1990).

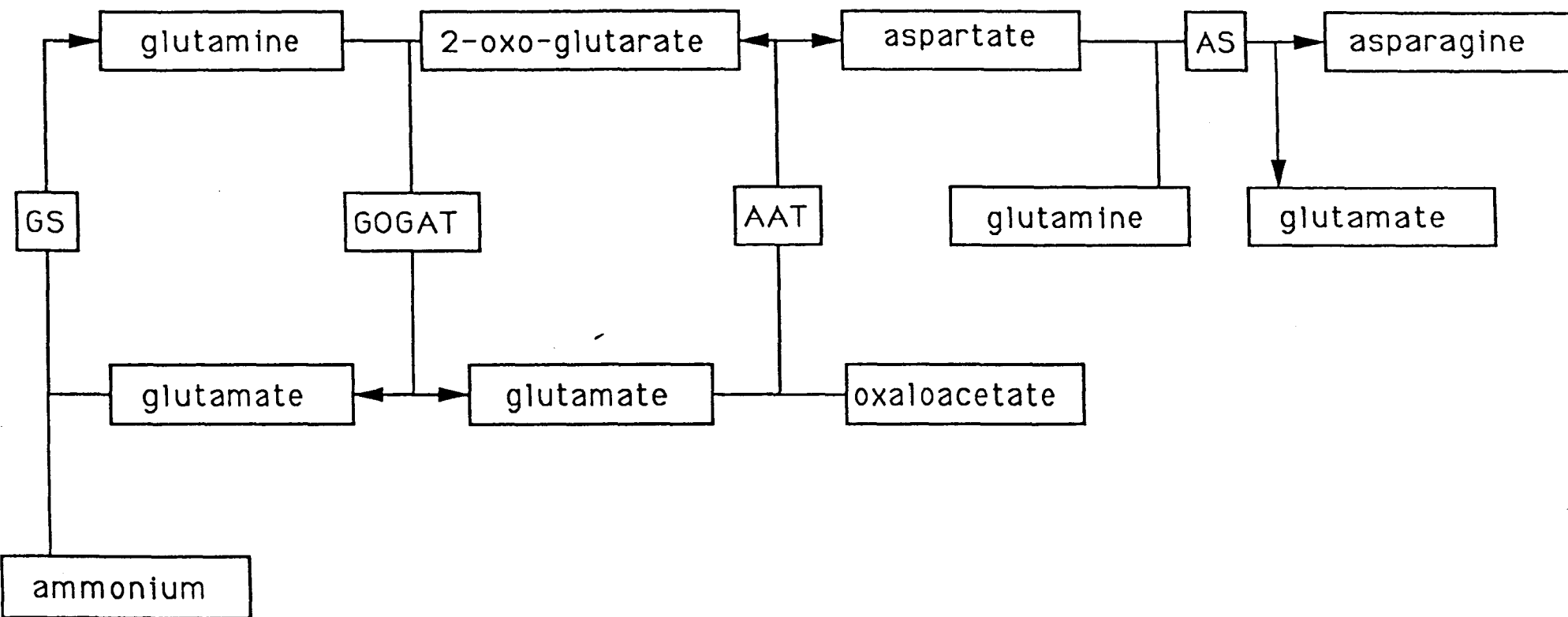
#### 1.2.a Asparagine metabolism:

The amides asparagine and, to a lesser extent, glutamine, are the main nitrogenous transport compounds of many plants including nodulated or non-nodulated temperate legumes (e.g. Pisum, Lupinus, Vicia, Trifolium, Medicago, and Lotus), non-nodulated tropical legumes (e.g. Glycine, Vigna and Phaseolus) and several actinorhizal plants (e.g. Casuarina and Myrica).

Asparagine synthesis is mediated by aspartate aminotransferase (AAT) and asparagine synthetase (AS). AAT catalyses the formation of aspartate and 2-oxoglutarate from glutamate and oxaloacetate and AS catalyses the ATP-dependent transfer of the amide group of glutamine to aspartate producing asparagine and glutamate (Robertson and Farnden, 1980) (see fig. 1.2). Studies have shown active GS and GOGAT are essential for asparagine synthesis (Sieciechowicz et.al. 1988).

Asparaginase hydrolyses the amide group of asparagine to produce aspartate and ammonium and provides a route for the

fig. 1.2.. Proposed route for asparagine synthesis (Robertson and Farnden 1980);



utilisation of asparagine for the synthesis of amino acids, nucleic acids and polyamines (Sieciechowicz et.al. 1988). A second route for asparagine degradation has been proposed and involves the transamination of the amino nitrogen by asparagine:2-oxoacid transaminase (Sieciechowicz et.al. 1988). The ammonium produced is reassimilated most probably via the glutamate synthase cycle (Lea et al., 1990).

#### 1.2.b Ureide metabolism;

The ureides allantoin (ALN) and allantoic acid (ALA) are important nitrogen transport compounds exported from the nodulated roots of tropical legumes (e.g. Glycine, Vigna and Phaseolus). The results of several studies have shown uriede production to be intrinsically associated with dinitrogen fixation in legumes (Schubert and Boland, 1990). The growth of nodulated cowpea and soybean plants in the presence of nitrate resulted in the increase of amino acid content of the xylem sap and the decreases in xylem sap ureide content, nodule mass and nitrogenase activity (Schubert and Boland, 1990). Furthermore, ALN and ALA have been shown to be synthesised from the products of recent dinitrogen fixation in the nodules of tropical legumes (Schubert and Boland, 1990).

Studies have firmly established ALA and ALN to be synthesised through the oxidation of purine bases and nucleotides derived from a pathway of de novo purine synthesis which utilises the products of recent dinitrogen fixation. During root nodule development, the activities of several uriede biosynthetic

enzymes have been shown to increase several-fold over a time course similar to the increase of nitrogenase in the bacteroids (Schubert and Boland, 1990).

The breakdown of allantoic acid can occur via two major pathways; the allantoicase pathway and the allantoate (ALA) amidohydrolase pathway and provide possible routes for the utilisation of ALA for the synthesis of organic nitrogen compounds. Available evidence suggests that in soybean, ALA and ALN metabolism occurs via the ALA amidohydrolase pathway (Schubert and Boland, 1990). The pathway produces ammonium, carbon dioxide and glyoxylate, the ammonium most probably being reassimilated via the glutamate synthase cycle (Lea et al., 1990). Results from studies on ureide-metabolising tissues strongly suggests that ALA degradation in plants is via the ALA amidohydrolase pathway (Winkler et al., 1987).



### 1.3 GS in higher plants:

In higher plants, GS is an octameric protein with subunits of  $M_r$  38,000–45,000 and a native  $M_r$  of approximately 350,000 (Stewart et al., 1980). Plants possess multiple isoenzymes of GS which are targeted to different subcellular compartments; chloroplastic or cytosolic. Chloroplastic GS (GS<sub>2</sub>) and cytosolic GS (generally GS<sub>1</sub>) are distinct and show organ-specific distribution. The ratio of the leaf chloroplastic and cytosolic GS isoenzymes in mature leaves vary with the higher plant species examined (McNally et al., 1983). The multiplicity of GS isoenzymes function to meet the varying ammonium-assimilatory demands of different plant organs (McNally and Hirel, 1983).

The molecular basis of these isoenzymes is the synthesis of different subunits encoded by different genes (Forde and Cullimore, 1989). The genetic basis of the multiple subunits is now established and in many dicotyledons it has been shown that there is a small multigene family encoding three or four distinct polypeptides. There is a single gene encoding the chloroplastic GS polypeptide and two or three genes encoding polypeptides which form the cytosolic GS isoenzymes of roots, leaves and, for legumes, nodules.

A similar picture of multiple GS gene families is now emerging for monocotyledons. Three genomic GS clones have been isolated from Oryza sativa (rice), two encode cytosolic GS polypeptides and one encodes a plastidic polypeptide (Kozaki et al., 1991)

#### 1.4 GS in Phaseolus vulgaris:

GS in P. vulgaris is encoded by a small multigene family of four nuclear genes. The genes gln- $\alpha$ , gln- $\beta$  and gln- $\gamma$  encode the cytosolic GS subunits  $\alpha$ ,  $\beta$  and  $\gamma$ , respectively, previously identified in plant tissue (Lara et al., 1984; Gebhardt et al., 1986; Bennett et al., 1989). The cytosolic subunits assemble into a variety of homologous and heterologous cytosolic isoenzymes (Bennett and Cullimore, 1989). The fourth gene, gln- $\delta$ , encodes the plastidic  $\delta$  GS subunit (Lightfoot et al., 1988). The  $\delta$  subunit is initially synthesised as a higher molecular weight precursor (pre- $\delta$ ) containing an N-terminal presequence responsible for targeting the polypeptide to the chloroplast. The imported polypeptide is cleaved to produce the mature  $\delta$  subunit (Lightfoot et al., 1988). The presequence shows similarities to the conserved sequences in chloroplast transit peptides (Karlin-Neumann and Tobin, 1986). The plastidic GS isoenzyme is composed only of  $\delta$  subunits. The sequence of clones coding for the  $\alpha$ ,  $\beta$  and  $\gamma$  polypeptides show greater than 85% nucleotide sequence homology (Cullimore and Bennett, 1988). The clone corresponding to the  $\delta$  polypeptide shows only 70% nucleotide sequence homology.

Studies show that the different GS genes are differentially expressed during development in an organ-specific manner (Cullimore et al., 1984; Gebhardt et al., 1986). The gln- $\beta$  gene is expressed in all tissues so far studied (Lara et al., 1984; Gebhardt et al., 1986). The studies of Forde et al. (1989)

demonstrated the gln- $\beta$  gene expression to be regulated during nodule development. The gln- $\beta$  promoter directed uidA reporter gene expression in both the cortical and infected regions of young nodules of transgenic Lotus corniculatus plants. As the nodule matured however, gln- $\beta$  expression became restricted to the vascular tissue.

The gln- $\gamma$  gene is strongly induced during nodule development (Lara et al., 1984; Gebhardt et al., 1986; Bennett et al., 1989) and prior to the onset of nitrogen fixation with initial gln- $\gamma$  expression being independent of nitrogenase activity (Padilla et al., 1987). Although initially thought to be nodule specific, gln- $\gamma$  mRNA has recently been detected in stems, petioles, and green cotyledons although at lower levels (Bennett et al., 1989).

Analysis of the gln- $\gamma$  promoter has identified sequences common to the promoter regions of late nodulins from other legume species. Nuclear factors from P. vulgaris have been shown to bind specific multiple A/T-rich gln- $\gamma$  promoter sequences (Forde et al., 1990) and, moreover, have a high affinity for a protein binding site from a soybean leghaemoglobin gene. The factors are also closely related to the soybean nodule factor which binds to A/T-rich sequences in the lbc<sub>3</sub> and nodulin 23 genes (Jacobsen et al., 1990). It appears therefore that these P. vulgaris factors belong to a conserved set of factors which interact with A/T-rich gene sequences from a number of plant species.

The gln- $\beta$  and gln- $\gamma$  genes display contrasting developmental and spatial-patterns of expression in nodules (Forde et al.,

1989). Localisation of reporter gene activity in transgenic Lotus corniculatus nodules showed that the gln- $\gamma$ :gusA gene construct was expressed specifically in the rhizobially infected cells but, as described earlier, the gln- $\beta$  gene construct was expressed in both cortical and infected regions of young nodules, and, as the nodule matured, expression became restricted to the vascular system. These results indicate that the  $\gamma$ -isoenzyme may be responsible for the assimilation of ammonium from nitrogen fixation in nodules and that the  $\beta$ -isoenzyme functions to generate nitrogenous solutes for intercellular transport.

The plastid-located GS of P. vulgaris is encoded by the gln- $\delta$  gene. The gene is highly expressed in photosynthetic tissues and the  $\delta$ -polypeptide forms the major GS isoenzyme in mature leaves (Lightfoot et al., 1988; Cock et al., 1991; Swarup et al., 1990). In leaves, gln- $\delta$  is regulated by both light and possibly by the photorespiratory status of the leaf. It is therefore probable that one of the main functions of the isoenzyme in leaves is the reassimilation of ammonium from photorespiration (Wallsgrave et al., 1987; Blackwell et al., 1987). As discussed earlier, the role of chloroplastic GS in the reassimilation of photorespiratory ammonium is crucial since the mass of ammonium generated by photorespiration exceeds by an order of magnitude, that acquired during primary ammonium assimilation (Keys et al., 1978).

The gln- $\delta$  gene is also expressed in nodules and dark grown leaves (Bennett et al., 1989; Cock et al., 1991). As these

organs are non-photorespiratory, the plastid-located GS may perform a different role such as the assimilation of ammonium produced from nitrogen fixation or from nitrate reduction.

The gln- $\alpha$  gene is highly expressed in developing roots (Ortega et al., 1986), developing leaves (Cock et al., 1991), developing nodules (Bennett et al., 1989) and in the cotyledons of germinating seeds (Swarup et al., 1990). The gln- $\alpha$  polypeptide and/or mRNA is detectable in dry-seed radicles, plumules and cotyledons and in young nodules. Levels then decrease within several days of further growth with other GS subunits becoming predominant. In the cotyledons of germinating seeds, all four GS genes are differentially expressed and may function in the re-assimilation of the ammonium liberated during proteolysis of seed storage protein and metabolism of the resulting amino acids.

The gln- $\alpha$  gene is also expressed during nodule senescence and in response to pathogen attack and mechanical wounding (Cock et al., 1990; Daniell, 1992). In experiments where the root systems of nodulated P. vulgaris plants were grown under an atmosphere of Ar/O<sub>2</sub> as opposed to an atmosphere of N<sub>2</sub>/O<sub>2</sub>, the nodules senesced early. Nodule senescence induced the expression of the gln- $\alpha$  and phenylalanine ammonia lyase (PAL1) genes but lead to a decrease in the level of expression of the  $\gamma$ ,  $\beta$  and leghaemoglobin genes but (Cock et al., 1990). As the co-induction of PAL1 and gln- $\alpha$  gene expression was also observed in response to mechanical wounding and Pseudomonas phaseolicola infection of

P. vulgaris plants (Daniell, 1992), it was therefore suggested that under such conditions, a GS isoenzyme formed predominantly from  $\alpha$ -subunits was responsible in the reassimilation the ammonium produced by PAL as it catalyses the deamination of L-phenylalanine to form trans-cinnamic acid.

Studies by Kawakami and Watanabe (1988) demonstrated a senescence-specific increase in cytosolic GS and its mRNA in radish cotyledons. The relative content of the cytosolic isoenzyme (GS<sub>1</sub>) increased two-fold in senescing cotyledons, whereas the chloroplastic isoenzyme (GS<sub>2</sub>) declined to half its initial level. Senescence also induced the increase in the relative level of translatable GS<sub>1</sub> mRNA seven-fold after 72 hr and decreased rapidly that for the GS<sub>2</sub> and for other nuclear encoded chloroplastic proteins. Cotyledons also accumulated GS<sub>1</sub> mRNA when they senesced after growth under continuous light. Senescing leaves become a source of nitrogen for the plant, the leaf's constituent protein is hydrolysed and the protein moiety is redistributed throughout the plant (Thomas, 1978). Thomas (1978) demonstrated that several enzymes responsible for the conversion of proteins into nitrogenous transport molecules increase during leaf senescence. GS may function to reassimilate the ammonium produced during such conversion in cotyledons. During P. vulgaris cotyledon senescence, gln- $\alpha$  mRNA levels were also shown to increase (Swarup et al., 1990), possibly to serve a similar function.

### 1.5 GS in other dicotyledons:

Molecular analysis has uncovered nuclear GS multigene families in other species of dicotyledons including Pisum sativum (pea) (Tingey et al., 1987; Tingey et al., 1988), Medicago sativa (alfalfa) (Tisher et al., 1986), Glycine max. (soybean) (Hirel et al., 1987), Nicotiana plumbaginifolia (Tingey and Coruzzi, 1987), and Arabidopsis thaliana (Peterman and Goodman, 1991)

Both the pea and Arabidopsis thaliana chloroplastic GS isoenzyme are encoded by a single nuclear gene which are expressed predominantly in leaves and in a light dependent manner (Tingey et al., 1987; Peterman and Goodman, 1991). Similarly to P. vulgaris chloroplastic ( $\delta$ ) mRNA, the pea chloroplastic GS mRNA preferentially accumulates in the leaves of plants grown under photorespiratory conditions (Edwards et al., 1989).

As with the chloroplastic polypeptide of P. vulgaris, the primary translation products of the chloroplastic GS mRNA of both pea (Tingey et al., 1987) and N. plumbaginifolia (Tingey and Coruzzi, 1987) possess an N-terminal extension necessary for targeting the polypeptide into the chloroplast where it is removed to give the mature chloroplastic polypeptide size.

Cytosolic GS isoenzymes in pea are encoded by two distinct classes of genes. The first class contains two nearly identical or "twin" GS genes (GS341 and GS132), while the second contains a single GS gene (GS299) distinct in both coding and non-coding regions from the "twin" genes (Tingey et al., 1987; Walker and

Coruzzi, 1989). Studies indicate that the GS gene family of pea, like P. vulgaris, does not contain a GS gene that is expressed exclusively in nodules and instead, all four GS genes are recruited during nodule development. This situation is similar to that reported for soybean where at least two of the genes for cytosolic GS are expressed at high levels in nodules and low but detectable levels in roots (Hirel et al., 1987). However, such findings contradicted the earlier results of Sengupta-Gopalan and Pitas (1986) which indicated the existence of a nodule-specific GS in soybean.

The pea "twin" cytosolic GS genes are coordinately expressed during nodule development and in germinating cotyledons (Walker and Coruzzi, 1989). In contrast to this parallel "twin" gene expression, the GS299 gene shows differences in developmental expression. The GS299 mRNA accumulates coordinately with the "twin" GS mRNAs during nodule development but GS299 mRNA however, shows a distinct pattern of accumulation in certain mutant nodules and GS299 mRNA fails to accumulate in germinating cotyledons (Walker and Coruzzi, 1989).

The promoters of the chloroplastic and cytosolic GS isoenzymes of pea show non-overlapping patterns of expression in transgenic tobacco plants (Edwards et al., 1990). The promoter for the chloroplastic GS gene directed  $\beta$ -glucuronidase reporter gene expression within transgenic photosynthetic tissues and conferred light regulation in a manner as seen in pea. These expression patterns reflect the probable physiological role of



the chloroplastic GS isoenzyme in the assimilation of ammonium from photorespiration and nitrite reduction. In contrast the cytosolic GS341 promoter directed expression specifically within the phloem elements in all organs of the mature plant studied, revealing a possible function of the cytosolic GS isoenzyme in the generation of glutamine for intercellular nitrogen transport. In germinating seedlings, the cytosolic GS341 promoter directed intense expression in the vascular tissue, suggesting the cytosolic GS isoenzyme may be involved in the mobilisation of seed-storage reserves.

### 1.6 Regulation of GS genes by ammonium:

The GS genes expressed in roots of P. vulgaris are not apparently induced, directly or indirectly, by ammonium, the enzyme's substrate (Cock et al., 1990). Neither is expression changed under conditions of nitrogen starvation. When the nitrogen fixation of P. vulgaris nodules is prevented by either growth under an Ar/O<sub>2</sub> atmosphere or when formed by certain Fix<sup>-</sup> rhizobial mutants, gln- $\delta$  gene expression is still strongly induced compared to roots. These results, and those of Padilla et al. (1987) suggest that the ammonium from dinitrogen fixation is not the primary signal for increasing the expression of gln- $\delta$  in nodules and instead, gln- $\delta$  gene expression is primarily under developmental control. However dinitrogen fixation appears to have a quantitative effect on expression of gln- $\delta$  as the abundance of the  $\delta$ -mRNA is about 2 to 4-fold higher under nitrogen fixing condition than under non-fixing conditions. However this is not specific to the gln- $\delta$  gene as mRNA from a nodulin gene encoding leghaemoglobin is similarly affected. Work by Chen et al. (1990) has shown the isoenzyme activity of NADH-dependent GOGAT II of P. vulgaris is regulated in a manner similar to the regulation of gln- $\delta$ . The NADH-dependent GOGAT isoenzyme is the most active isoenzyme in root nodules and its expression appears to be confined to this organ (Chen et al., 1990).

An increase in dinitrogen fixation in nodules of P. vulgaris by growth under high CO<sub>2</sub> (0.1%) correlated with a reduction of

GS- $\delta$  isoenzyme (Ortega et al. 1992). A higher carbon/nitrogen (C/N) ratio is expected in nodules grown either under an excess of CO<sub>2</sub> or an absence of N<sub>2</sub>, suggesting that the C/N balance within the nodule and not the ammonium generated through dinitrogen fixation is the primary modulating factor of the P. vulgaris GS- $\delta$  isoenzyme.

These results on P. vulgaris GS differ from those reported by Hirel et al. (1987) for soybean who observed an increase in GS mRNA with the addition of ammonium to soybean roots. Furthermore, when nitrogen fixation in nodules was prevented by growth under an Ar/O<sub>2</sub> atmosphere or when nodules were formed with Fix<sup>-</sup> mutants, the amounts of GS mRNA did not increase over that of roots. Miao et al. (1991) cloned the cytosolic GS gene induced in roots by the availability of ammonium and fused the promoter region to the  $\beta$ -glucuronidase reporter gene. The promoter directed root-specific expression in both transgenic tobacco and Lotus corniculatus plants, the expression being restricted to the growing root tips and the vascular bundles of mature roots. However, ammonium treatment only induced chimeric gene expression in the roots of transgenic L. corniculatus; transgenic tobacco roots showed no induction. It was therefore proposed that the factor responsible for ammonium induction is present only in legumes. Histochemical localisation of chimeric gene expression in ammonium-treated roots of transgenic L. corniculatus plants showed a uniform distribution across all cell types. The chimeric gene was also shown to be expressed in the infection zone,

including the uninfected cells, and in the inner cortex of transgenic L. corniculatus nodules, presumably where the flux of ammonium from dinitrogen fixation is assimilated.

Finally, ammonium has also been reported to induce the activity of GS and the activity of NADH-dependent GOGAT in the roots of alfalfa (Groat and Vance, 1982) and in the seedlings of maize (Handa et al., 1985).

Species differences in the relative proportions of chloroplastic and cytosolic GS isoenzyme activities may reflect differences in regulation of the respective genes. As described earlier for P. vulgaris and P. sativum, a probable function of the chloroplastic GS is the reassimilation of photorespiratory ammonium and studies indicate that some of the differences in chloroplastic GS activities between different species may relate to the magnitude of photorespiration. Tobin et al. (1988) found that the activities of GS and glycollate oxidase, a key enzyme in the photorespiratory pathway, increased in apparent synchrony with photorespiration during chloroplast biogenesis in wheat leaves. A comparative study of Panicum plants differing in their photosynthetic pathways showed chloroplastic GS activity to be highest in the  $C_3$  species and lowest in the  $C_4$  species (Chandler et al., 1985).  $C_4$  plants are a lot less susceptible to photorespiration than  $C_3$  plants as  $CO_2$  is concentrated at the site of assimilation in  $C_4$  plants through the  $C_4$  pathway. Studies on pea leaves have shown that under conditions of elevated levels of  $CO_2$ , which effectively inhibit photorespiration, the level of

chloroplastic GS mRNA decrease (Edwards and Coruzzi, 1989).

It is worth noting that studies on photorespiratory mutants of Arabidopsis (Somerville and Ogren, 1980) and barley (Kendall et al., 1986) have demonstrated that the chloroplastic ferredoxin-dependent GOGAT is also essential in the reassimilation of photorespiratory ammonium in leaves.

### 1.7 Precis of project:

In the absence of gln- $\alpha$  promoter sequences, initial studies concerning the cell-specific expression of the gln- $\alpha$  gene involved in-situ hybridisation techniques (Daniell, 1992). Studies involving the hybridisation of probes synthesised from the  $\alpha$ -cDNA to sections of P. vulgaris nodules were however unsuccessful due possibly to the relatively low abundance of  $\alpha$ -mRNA in these tissues (Daniell, 1992). The present study made use of transgenic plant systems. A P. vulgaris gln- $\alpha$  genomic clone was isolated, characterised and the promoter region fused to the E. coli uidA reporter gene which encodes the enzyme  $\beta$ -glucuronidase (GUS). The chimeric gene gln- $\alpha$ ::uidA gene was introduced into non-legume (tobacco) and legume (Lotus corniculatus) plants. The resulting transgenic plants were studied with respect to gln- $\alpha$  promoter activity in response to both developmental and environmental cues.

## Chapter 2: Materials and Methods.

## 2.1 Chemicals;

The chemicals used were normally of the highest analytical grade available.

## 2.2 Growth and storage of bacterial stocks;

The E. coli bacterial strains used for this study are listed in Table. 1.

For long term storage, bacteria were maintained as frozen glycerol stocks. 0.7 ml of overnight (O/N) culture grown in L-broth (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.2M NaCl) was mixed with 0.7 ml of "freezer mix" (Maniatis et al., 1982), frozen in liquid nitrogen and stored at -80°C.

E. coli strains were maintained in the short term on L-agar plates (L-broth solidified with 1.5% (w/v) bacto-agar) containing the appropriate antibiotic(s). A loopful of a fresh overnight culture or frozen glycerol stock was streaked onto a plate and incubated at 37°C O/N and then stored for up to 4 weeks at 4°C.

Agrobacterium and Rhizobium strains were maintained on TY-agar plates (1% (w/v) bacto-tryptone, 1% (w/v) yeast extract, 0.2M NaCl solidified with 1.5% (w/v) bacto-agar) supplemented with 6mM CaCl and appropriate antibiotic(s). A loopful of 3 day culture (grown at 30°C) or glycerol stock was streaked onto a plate and incubated for a further 3 days at 28°C and stored at 4°C for up to 4 weeks.

Bacteriophage lambda stocks were stored at 4°C in 5ml of SM buffer (20mM Tris-Cl pH 7.5, 20mM NaCl, 10mM MgSO<sub>4</sub>) and 25μl of chloroform.



table 1. E. coli strains used and their genotype;

<u>strain</u>	<u>genotype</u>
NM554	recA1, araD139, del (ara-leu)7969, del(lac)17A, galU, galK, hsdR, strA, mcrA, mcrB
LE392	e14- (mcrA), hsdR514, supE44, supF58, lacY1 or del (lacZY)6, galT22, metB1, trpR55
P2392	LE392 (P2 lysogen)
TG2	recA1, endA1, gyrA96, thi-1, hsdR17 (rk-, mk+), supE44, relA1

### 2.3 Construction of a *P. vulgaris* genomic library in bacteriophage lambda:

The genomic library was constructed in the bacteriophage lambda Dash vector (Sorge, 1988; Stratagene Cloning Systems) from a partial SauIIIA digest of *P. vulgaris* genomic DNA. The library was constructed as follows;

#### 2.3.a Plant material:

*P. vulgaris* L. cv. Tendergreen seeds were surface sterilised using 10-14% Na hypochlorite and washed several times in sterile distilled water (SDW). The seeds were germinated in the dark at 30°C on TY plates containing 6mM CaCl for 3 days and then transplanted to trays containing sterile perlite. The plants were watered using sterile nitrate-free nutrient solution (Bennett and Cullimore, 1989) and at day 10, root material was harvested, frozen in liquid nitrogen and stored at -80°C.

#### 2.3.b Isolation of plant DNA:

The isolation of high molecular weight plant genomic DNA was as essentially described by Dellaporta et al. (1983). From an initial 6.2g of root tissue, a total of 665µg of genomic DNA was extracted. The degree of shearing was determined using agarose gel electrophoresis and the DNA was sized by the co-electrophoresis of uncut lambda DNA.

#### 2.3.c Determination of optimal conditions for the production of 15-20 Kbp genomic fragments:

Initially, *P. vulgaris* root genomic DNA was partially restricted with SauIIIA in small-scale, pilot reactions to

determine the number of enzyme units/ $\mu$ g genomic DNA ratio which produces the greatest yield of 15-20Kbp DNA fragments. A 2-fold dilution series of enzyme units/ $\mu$ g genomic DNA were set up between 2 units/ $\mu$ g DNA and 1/128 units/ $\mu$ g DNA. The digest reactions were incubated at 37°C for 1 hr and then transferred to ice and mixed with 0.75 $\mu$ l of 0.4M EDTA to stop the reaction. 5 $\mu$ l of 6 x loading buffer was added to each sample and electrophoresed O/N on a 0.5 (w/v) agarose, 0.4 $\mu$ g/ml ethidium bromide (EtBr), 1 x Tris-Borate EDTA (TBE) gel with digested lambda DNA size markers and an uncut genomic DNA marker. The gel was visualised and the DNA sample with the greatest fluorescence in the size range 15-20Kbp noted.

#### 2.3.d Large scale preparation of 15-20Kbp genomic fragments for cloning;

The optimal conditions previously noted were used in the large scale preparation of genomic fragments for cloning. To increase the chances of cloning any given sequence of interest, a large scale digest was also carried out using the enzyme:DNA ratio immediately below the optimum. This in effect enables the production of a more random population of cloned DNA fragments. Large scale digests were carried out using 1/64 units/ $\mu$ g DNA (DNA digested; 75 $\mu$ g) and 1/128 units/ $\mu$ g DNA (DNA digested; 64 $\mu$ g) with reaction conditions identical to those used before. The size distribution of the digested DNA was checked by gel electrophoresis as before.

#### 2.3.e Fractionation of the genomic fragments using sucrose

#### density gradient centrifugation:

For optimal ligation conditions it was necessary to isolate clonable genomic fragments (i.e. 15-20Kbp) from the combined pool of fragments generated by the partial digests. This was achieved using sucrose density gradient centrifugation. The DNA sample was loaded without preheating onto a 38ml, 10-40% (w/v) sucrose density gradient in a Beckman SW 28 Ultraclear tube. The sucrose solution contained 1M NaCl, 20mM Tris-Cl pH 8 and 5mM EDTA, in addition to the sucrose. The gradient was centrifuged at 26,000 rpm for 24 hrs at 20°C in a Beckmann SW28 rotor and brought to rest without using the brake. 0.5ml fractions were collected from the top of the gradient using a fraction collector.

Aliquots of every third sample were analysed by gel electrophoresis on a 0.4% (w/v) agarose, 0.4µg/ml EtBr, 1 x TBE gel along with digested lambda DNA size markers. The sucrose and NaCl concentrations of the size marker samples were adjusted to correspond with the genomic samples in the middle of the gel. Six fractions containing the DNA within the desired range were pooled and dialysed O/N against TE pH 8 buffer (10mM Tris-Cl pH8, 1mM EDTA pH 8) and the volume of the DNA solution was then reduced to approximately 1 ml by butan-1-ol extraction. After ethanol precipitation and centrifugation, the DNA pellet was vacuum dried and redissolved in TE pH 8 to an approximate concentration of 0.3 µg/µl. The exact DNA concentration was determined by spectrophotometry.

#### 2.3.f Ligation of the genomic fragments to lambda Dash arms:

By considering the average molecular weight of the purified genomic fragments and those of the respective lambda arms, the expected optimum ratio (w/w) of arms:insert for ligation would be 2:1 (the lambda arms were supplied pre-linearised with BamHI by Stratagene Cloning Systems). However, two different ligation reactions were set up (1:1 and 2:1 (w/w) arms:insert) as some molecules may have lacked cohesive termini and thereby altered the effective concentration of ends available for ligation. The ligation components and volumes (10 $\mu$ l) were as recommended in the Stratagene Lambda Dash booklet (Stratagene Cloning Systems).

#### 2.3.g In-vitro packaging of DNA and titration:

The Gigapack Gold II packaging kit (Stratagene Cloning Systems) was used to package the DNA in accordance to the manufacturer's recommendations. The yield of bacteriophage was assayed by preparing 10<sup>-1</sup> to 10<sup>-4</sup> dilutions of the packaged phage and plating out 100 $\mu$ l of each of these. The E. coli host strains used for this purpose were LE392 and P2392 and the preparation, adsorption and plating procedures were carried out as previously described. The size of the library and the percentage of non-recombinants was thus determined.

#### 2.4 Screening of the genomic library for gln- $\alpha$ sequences:

In order to maximise the chances of detecting any given cloned sequence of interest, the entire in-vitro packaged bacteriophage library was plated out and the resulting plaques screened directly. This direct screening approach kept to a minimum the problems associated with unequal growth rates of

recombinant clones. Plaques were grown at a density of 250 pfu/cm<sup>2</sup> on the E. coli host strain NM554 (see table 1). Plates were grown overnight at 37°C and chilled at 4°C for a minimum of 1 hr prior to plaque lifting.

#### 2.4.a Immobilisation of phage DNA onto nylon membranes;

Bacteriophage DNA was transferred onto nylon filters using a method modified from that of Benton and Davies (1977) as described by Maniatis et al. (1982). Duplicate nylon filters (Hybond N, Amersham International PLC.) were cut to fit the plates and numbered using a pencil. Each nylon filter was placed carefully on top of its corresponding plate for 1 min. During this minute the orientation of the filter was fixed by puncturing through both the filter and agar with a large gauge hypodermic needle. After 1 min, the filter was removed by means of forceps and placed DNA side up on Whatman 3MM paper soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 min. The filter was then transferred to Whatman 3MM paper soaked in neutralising solution (1.5M NaCl, 0.5M Tris-Cl pH 8) for 5 min. Finally the filters were rinsed by transfer to 3MM paper soaked in 2 x SSC and allowed to dry on dry 3MM paper. The DNA was fixed to the filters by exposure to UV light, DNA side up, for 2 min followed by baking under vacuum for 2 hr at 80°C.

#### 2.4.b Hybridisation of bacteriophage plaques to radiolabelled $\alpha$ -cDNA probes.

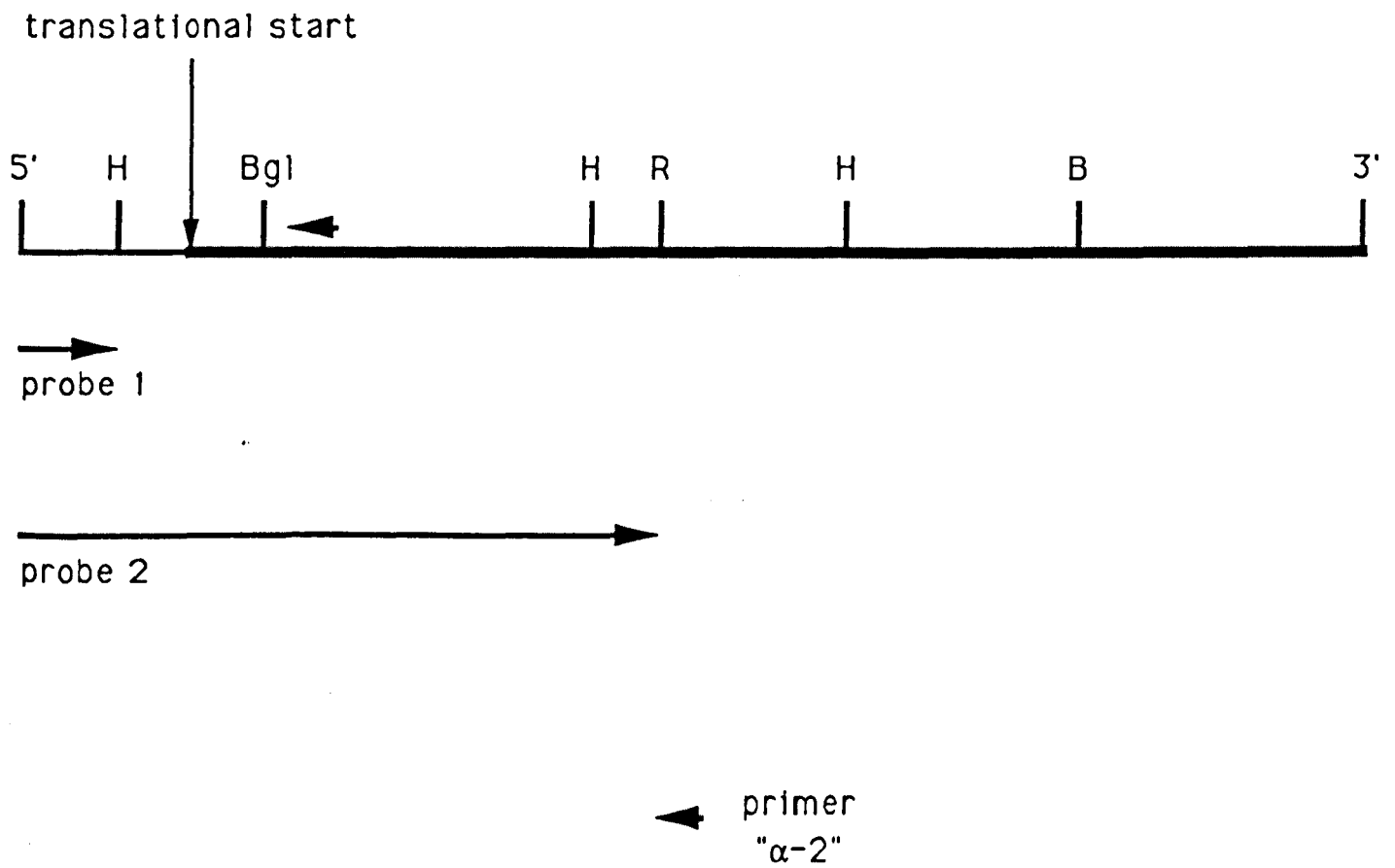
Prehybridisation and hybridisation of the filters was carried out as described by the Amersham International PLC

booklet for use with Hybond-N filters. Prehybridisation was carried out in a sealed bag for 4-5 hr at 65°C in 10ml of prehybridisation buffer (6 x SSC, 5 x Denhardt's solution, 0.5% (w/v) SDS and 100µg/ml sonicated salmon sperm DNA). DNA probes were denatured by heating in a boiling water bath for 5 min prior to addition to the 6ml of hybridisation solution (identical to the prehybridisation solution). RNA probes were added directly to the hybridisation solution without prior boiling. Hybridisation was carried out at 65°C overnight. Two <sup>32</sup>P-radiolabelled probes were used for screening. One was a 99bp RNA probe (49bp of which were derived from the vector) (Kreig and Melton, 1987) transcribed to the 5' HindIII site of the α-cDNA (see fig. 2.1; "probe 1"). The second probe was a 710bp EcoRI α-cDNA probe prepared by oligolabelling (Feinberg and Vogelstein, 1984) (see fig. 2.1; "probe 2"). Following hybridisation the filters were washed at low stringency (2 x SSC, 0.1% (w/v) SDS, 65°C, for 1 hr) and high stringency (0.1 x SSC, 0.1% SDS, 65°C, for 1 hr). After each wash, the filters were sealed in hybridisation bags and autoradiographed overnight at -80°C using Fuji X-ray film in the presence of a single intensifying screen. The gln-α positive clones were identified as black spots on the autoradiographs. By means of the alignment marks, cores of top agar corresponding to positive signals were removed using the bulb-end of pasture pipettes. These agar cores were stored in 1ml of SM buffer and 1 drop of chloroform and, after vortexing, the bacteriophages were left to elute overnight at 4°C. The tubes



fig. 2.1. Restriction map of the  $\alpha$ -cDNA clone pcPvGS- $\alpha$ 1 (Gebhardt et al., 1986) (H = HindIII, R= EcoRI, B = BamHI, Bgl = BglII, probe 1 =  $\alpha$ -cDNA:HindIII transcription probe, probe 2 =  $\alpha$ -cDNA:EcoRI oligolabelled probe);

total length approximately 1.4Kbp



were again vortexed and a dilution series from  $10^{-2}$  to  $10^{-4}$  was made from each bacteriophage eluate. 100 $\mu$ l of each dilution was plated and the number of resulting plaques counted and the titre of the original eluate calculated. The same procedure was followed for primary positives "a" and "i" for each round of screening using a progressively lower plaque density until it became possible to identify individual positive plaques. This was achieved within 3 rounds of screening. After individual positives plaques had been picked, one further round of screening was performed to check plaque purity had been attained. An amplified stock of each plaque pure clone was obtained and stored as previously described.

## 2.5 In-vitro synthesis of radiolabelled nucleic acid probes;

### 2.5.a Transcription;

In order to transcribe gene-specific probes, specific DNA sequences were subcloned into the plasmid vectors pGEM-3Z and pGEM-4Z (Promega) which contain both the bacteriophage SP6 and T7 polymerase initiation sequences which flank a multiple cloning site. DNA manipulations were as previously described (Maniatis et al. 1982) and the recombinant plasmids were linearised at an appropriate restriction site within the insert. The restriction digests were extracted once with TE-equilibrated 1:1 (v/v) phenol/chloroform mixture, the DNA ethanol precipitated and redissolved in TE pH 8 at a concentration of approximately 0.5  $\mu$ g/ $\mu$ l. Transcripts were then generated using either T7 or SP6 RNA polymerase (Kreig and Melton, 1987). Transcription reactions

were assembled at RT by mixing the following; 1.5 $\mu$ l of SDW (RNase protection probe), 1 $\mu$ l of 0.5 $\mu$ g/ $\mu$ l linearised DNA, 1 $\mu$ l of 200mM DDT, 0.5 $\mu$ l of human placental RNase inhibitor, 1 $\mu$ l of CGA ribonucleotide mix (5mM CTP, 5mM GTP, 5mM ATP), 1 $\mu$ l of 100 $\mu$ M UTP (RNase protection probe), 1 $\mu$ l (RNase protection probe) or 3.5 $\mu$ l (library screening probe) of  $^{32}$ P-UTP (20 $\mu$ Ci/ $\mu$ l, >800 Ci/mmol), 1 $\mu$ l of 10 x transcription buffer (0.4M Tris-Cl pH 7.5, 60mM MgCl<sub>2</sub>, 20mM spermidine-Cl) and 1 $\mu$ l of either T7 or SP6 RNA polymerase. After the mixture had been incubated at 37°C for 40 min, 0.5 $\mu$ l of RNase-free DNase (23 units/ $\mu$ l) was added and the mixture incubated for a further 10 min at 37°C. SDW was added to a final volume of 50 $\mu$ l and the preparation extracted with an equal volume of 1:1 (v/v) phenol:chloroform mixture. After centrifugation, the aqueous phase was transferred to a tube containing 25 $\mu$ l of 7M NH<sub>4</sub> acetate, 1 $\mu$ l of 5 $\mu$ g/ $\mu$ l yeast tRNA and 200 $\mu$ l of ethanol. The preparation was incubated at -20°C for 20 min and the RNA pelleted by centrifugation for 15 mins. If the probe was to be hybridised to genomic library screening filters or Southern blot filters, the resulting pellet was washed with 500 $\mu$ l of 70% ethanol, vacuum dried and redissolved in 50 $\mu$ l of SDW. If the probe was to be used in an RNase protection assay, the pellet was vacuum dried and redissolved in 5 $\mu$ l of formamide loading buffer. The probe was then purified by acrylamide gel electrophoresis as described previously by Kreig and Melton (1987).

#### 2.5.b Oligolabelling:

A modification of the oligolabelling method of Feinberg and Vogelstein (1984) was used to prepare  $^{32}\text{P}$ -radiolabelled DNA probes. The probes were used for hybridisation to both Southern blots and genomic library screening filters. Initially, the DNA fragment to be radiolabelled was isolated from a suitable clone and dissolved to a final concentration of approximately 50 ng/ $\mu\text{l}$  in SDW. The DNA fragment was then denatured by incubation in a boiling water bath for 5 min. The preparation was then transferred to a 37°C water bath for 5-10 min while the remainder of the reaction mixture was assembled in an Eppendorf tube. This consisted of 3 $\mu\text{l}$  of 5 x oligolabelling buffer (OLB) (0.24M Tris-Cl pH 8, 25mM  $\text{MgCl}_2$ , 50mM  $\beta$ -mercaptoethanol, 0.1mM dATP, 0.1mM TTP, 0.1mM dCTP, 1M HEPES-NaOH buffer pH 6.6, 54  $\text{A}_{260}$  units/ml hexadeoxyribonucleotides), 0.6 $\mu\text{l}$  of 10mg/ml bovine serum albumin, 2-5 $\mu\text{l}$  of  $\alpha$ - $^{32}\text{P}$  dGTP (>3,000 Ci/mmol) and 0.6 $\mu\text{l}$  of 5 units/ $\mu\text{l}$  DNA polymerase I Klenow fragment. A 50ng aliquot of denatured DNA was added to this mixture and the final volume adjusted to 15 $\mu\text{l}$  with SDW. The preparation was incubated at RT for 4-5 hr and the unincorporated nucleotides removed by ethanol precipitation as described in section 2.4.a.

## 2.6 Purification of phage DNA from positive clones:

Phage DNA was purified using the polyethylene glycol (PEG)/NaCl method as described in the Amersham International p.l.c cDNA cloning system booklet.

## 2.7 sub-cloning of promoter sequences into plasmid vectors;

Promoter sequences were amplified directly from the phage clones, using a polymerase chain reaction (PCR) method as modified from the method of Saiki et al. (1988), and subcloned into pGEM-4Z.

The following "working solution" was sufficient for 3 PCR reactions; 18 $\mu$ l PCR stock (3M KCl, 1M Tris-Cl pH 8.3, 1M MgCl<sub>2</sub>), 1 $\mu$ l DDT (0.1M stock), 1.6 $\mu$ l deoxyribonucleotide mix (100mM stock) (Pharmacia), 1 $\mu$ l gelatine (100 mg/ml stock), 237 $\mu$ l H<sub>2</sub>O, 3 $\mu$ l of each oligonucleotide primer (100 pg/ $\mu$ l stock), and 1 $\mu$ l (5 units) AmpliTaq Taq DNA polymerase (Perkin Elmer Cetus). 80 $\mu$ l of "working solution" and 20 $\mu$ l of DNA template solution were combined to give the final PCR reaction mix of 100 $\mu$ l. The reaction mix was overlaid with 50 $\mu$ l of liquid paraffin and run for 30 cycles on a Techne PHC-2 thermal cycler. The cycle temperatures used were 92°C for denaturation, 40°C for hybridisation and 72°C for extension, each for 90 secs. To prevent the possible introduction and amplification of non-specific aerosolic DNA fragments, Gilson positive displacement pipettes were used to add all solutions and buffers and a new tip used for each new addition. 1 $\mu$ l of phage stock was directly amplified using the 23mer " $\alpha$ -2" antisense-strand primer (5' CTG TGG ATT CTG AGA GGT TGA GG 3') (hybridises 26bp 3' to the  $\alpha$ -cDNA translational start) and either the 20mer "T7" sense-strand primer (5' CTC TAA TAC GAC TCA CTA TA 3') or the 17mer "T3" primer (5' ATT AAC CCT CAC TAA AG 3') (hybridises to the T7 and

T3 RNA polymerase recognition sites of the lambda Dash arms, respectively). The first denaturing step destroys the protein coat of the phage allowing hybridisation of the primers to the DNA template and sequence amplification. PCR products were sized by electrophoresis of 10  $\mu$ l of the final reaction mix on 0.8% (w/v) agarose, 1 x Tris-Acetate EDTA (TAE) gels using digested lambda DNA markers.

To clone the PCR product, the remainder of the final reaction mix was agarose gel isolated using the Geneclean kit (Bio 101 Inc.). The DNA fragments were then restricted with EcoRI (site resulting from the lambda Dash polylinker) and BglII (site resulting from the gln- $\alpha$  sequence) and agarose gel isolated again using Geneclean and ligated into pGEM-4Z digested with EcoRI and BamHI. The resulting plasmid was termed pgln- $\alpha$ . The DNA manipulations were as described by Maniatis et al. (1982) and the resulting ligations transformed into competent cells of E. coli strain TG2 as prepared by the method of Hanahan (1983). The transformed cells were plated out onto L-agar plates containing 50  $\mu$ g/ml ampicillin and supplemented with 0.2 mg/ml IPTG and 0.17 mg/ml X-gal. In the presence of IPTG and X-gal, non-recombinant transformations give rise to blue colonies, whereas colonies from recombinant transformations are white. Thus any non-recombinant clones were discarded at this stage.

## 2.8 Southern blotting and hybridisation of genomic and phage DNA:

### 2.8.a Restriction endonuclease digestion:

10 $\mu$ g of P. vulgaris genomic DNA was restricted in a reaction

volume of 100 $\mu$ l with a 10-fold excess of restriction enzyme (100 units) and in the presence of 4mM spermidine-Cl. 3-5 $\mu$ g of phage DNA was restricted under similar conditions but the spermidine-Cl was omitted. Digestion was carried out at 37°C for 2 hr. Following digestion the DNA was ethanol precipitated, pelleted by centrifugation, washed in 70% ethanol, vacuum dried and redissolved in 20 $\mu$ l TE pH 8.

#### 2.8.b Agarose gel electrophoresis;

For high resolution of genomic and phae fragments, 0.6% (w/v) agarose gels, 0.4 $\mu$ g/ml EtBr were made and run in 1 x TAE buffer as described by Maniatis et al. (1982). Gels were run with either HindIII-digested lambda DNA size markers or with 1Kbp DNA ladder (BRL) size markers. Gels were run at 30 V for between 16 and 18 hr. Gels were then visualised on a UV trans-illuminator and photographed using a Polaroid instant camera with Polaroid 665 and 667 films. The gels were photographed aligned with a ruler.

#### 2.8.c Transfer of DNA to nylon membranes;

The method used was that modified from the method of Southern (1975) as described in Maniatis et al. (1982). The DNA was transferred to Hybond-N (Amersham International p.l.c) nylon membrane and the DNA fixed to the membrane as described in section 2.4.c.

#### 2.8.d Hybridisation of radiolabelled probe to filter immobilised DNA;

The procedure used was as recommended by the Amersham booklet as described in section 2.4.c. Following hybridisation, membranes were washed and autoradiographed as in section 2.4.c.



## 2.9 Preparation of plasmid DNA;

### 2.9.a Small-scale preparations of plasmid DNA;

Minipreparations of plasmid DNA for preliminary restriction analysis were obtained using an alkaline lysis method modified from the method of Birnboim and Doly (1979) as described by Maniatis et al. (1982) except that the RNase treatment was performed prior to the phenol:chloroform extraction.

### 2.9.b Large-scale purification of plasmid DNA;

Large-scale preparations of plasmid DNA were obtained using the alkaline lysis method as modified from the method of Birnboim and Doly (1979) as essentially described by Maniatis et al. (1982). An extraction with an equal volume of 1:1 (v/v) phenol:chloroform mixture was however included prior to isopropanol DNA precipitation when the plasmid DNA was to be subsequently purified by polyethylene glycol precipitation. The purification of plasmid DNA by polyethylene glycol precipitation differed considerably from that described by Maniatis et al. (1982) and was as follows; the ethanol precipitated DNA pellet was redissolved in 1.6ml of SDW and 0.4ml of 4M NaCl and 1.0ml of 13% (w/v) PEG 8000 were added and mixed well. After incubation on ice for 60 mins, the precipitated plasmid DNA was recovered by centrifugation at 10,000g for 10 mins, washed in 70% ethanol, vacuum dried and redissolved in 500µl of TE pH 8.

Purification of plasmid DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients was as previously described (Maniatis et al., 1982)

The plasmid DNA concentration was calculated by measuring the OD<sub>260</sub> of a 2:500 dilution of the DNA solution and using the conversion factor; 1 OD<sub>260</sub> unit = 50µg of plasmid DNA/ml.

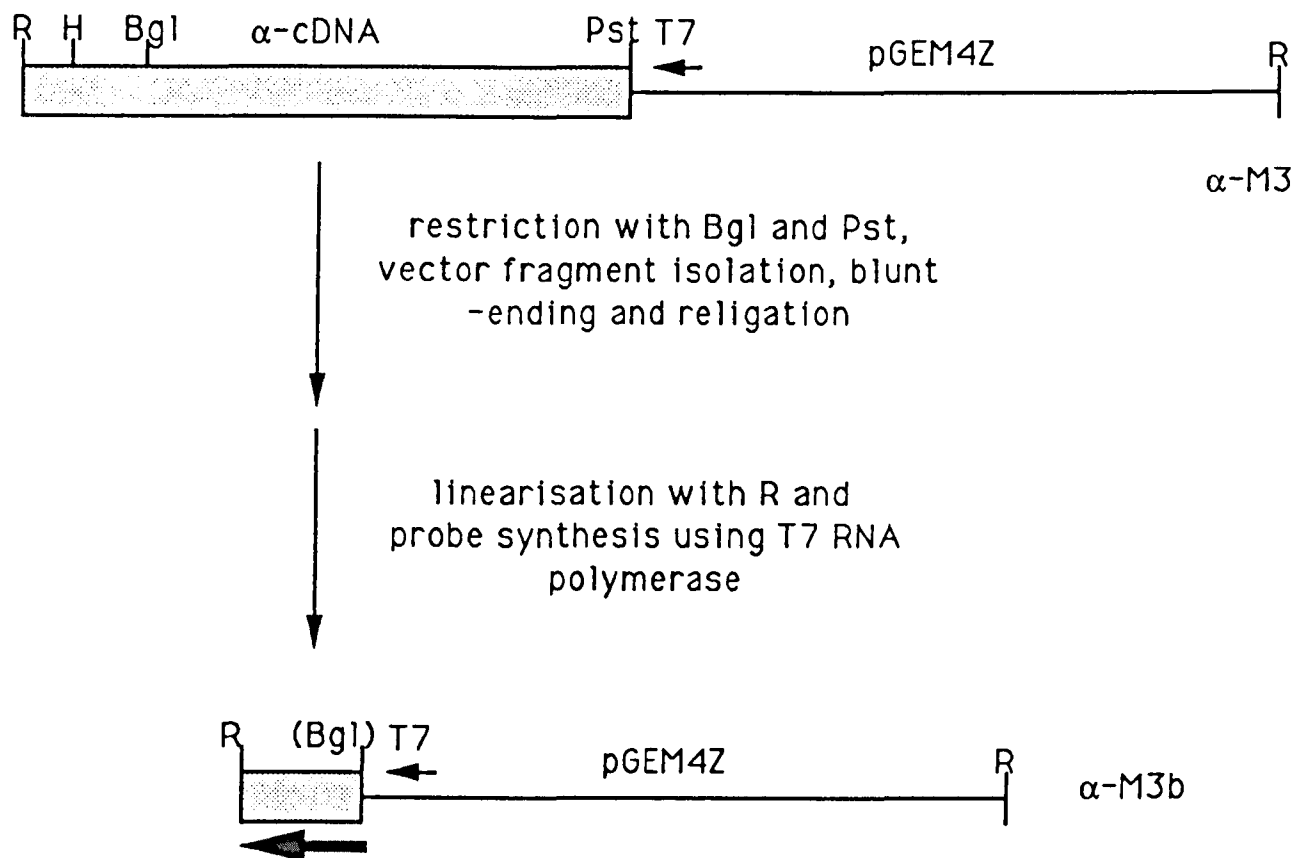
## 2.10 Mapping of the gln-α transcriptional start;

### 2.10.a using an RNase protection technique;

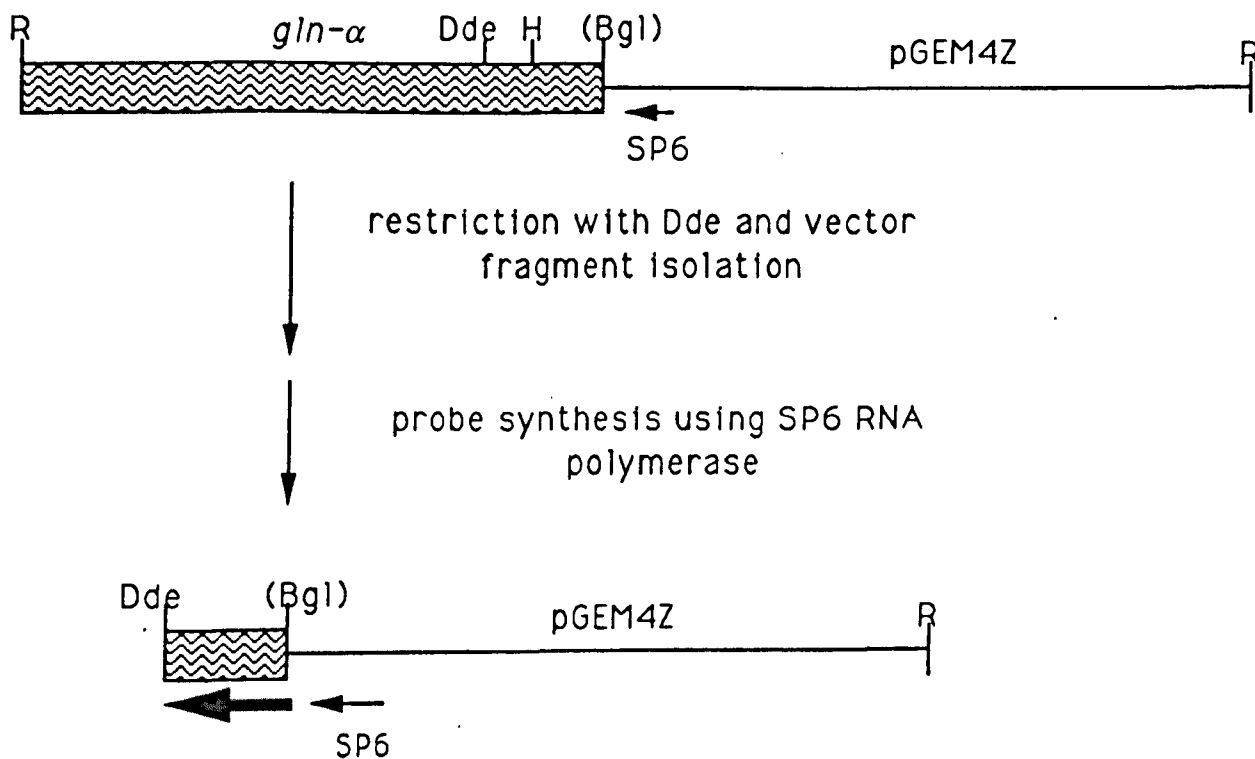
The RNase protection method (Krieg and Melton, 1987) was used to map the transcriptional start site of the gln-α clone. Total RNA was extracted from the leaves of overnight-imbibed P. vulgaris seeds using a mini-preparation method (Logemann et al., 1987) with the modification that the RNA was further purified by LiCl precipitation before the sodium acetate washes. Duplicate or triplicate 10µg total RNA samples were analysed to map the transcriptional start. The probes used were prepared by synthesis of single stranded <sup>32</sup>P-labelled anti-sense RNA of a defined size and were purified by elution from polyacrylamide gels (Krieg and Melton, 1987). The cloning of the DNA fragments to produce these probes is described as follows. The α-cDNA was restricted with PstI and BglII and the vector fragment gel isolated and blunt-ended using T4 DNA polymerase. Following vector re-ligation, the recombinant plasmid (termed α-M3b) was linearised with EcoRI and an anti-sense RNA probe of 112bp (38bp of which were derived from the vector) was synthesised by in-vitro transcription using T7 RNA polymerase (see fig. 2.2.a and section 2.5.a). A second probe of 171bp (46bp of which were derived from the vector) was synthesised from gln-α clone linearised with DdeI using SP6 RNA polymerase (see fig. 2.2.b).

fig. 2.2. Construction of plasmid clones for transcription probe synthesis for use in RNase protection assays (H = HindIII, R = EcoRI, Bgl = BglII, Pst = PstI, Dde = DdeI, T7 and SP6 = T7 and SP6 RNA polymerase recognition sites (arrows indicate direction of transcription);

(a)



(b)



Hybridisations, RNase digestions and analysis of the probe fragment protected from the RNase digestion by the complementary RNA were performed as previously described by Kreig and Melton (1987) except that hybridisation was carried out at 37°C overnight (instead of at 45°C overnight) and the RNase digestion was performed at RT (approx. 25°C) (instead of at 37°C). Protected fragments were sized by the co-electrophoresis of sequencing reactions.

#### 2.10.b using a primer extension technique:

Prior to 5'-end-labelling of the oligo "α-2" by phosphorylation using bacteriophage T4 polynucleotide kinase, the oligo was purified on a 16% (w/v) acrylamide gel to remove possible contaminating sequences. Following electrophoresis, the gel was UV shadowed and the gel section containing the oligo was cut from the rest of the gel and the oligo eluted by immersion O/N in elution buffer (300mM NaAc (pH 6.5), 10mM MgAc, 1mM EDTA, 0.1% SDS). 2ng of purified oligo was end-labelled using T4 polynucleotide kinase (10 units), 2μl 10 x P.N.K buffer (0.5M Tris-Cl (pH 7.6), 0.1M MgCl<sub>2</sub>, 50mM DTT, 1mM spermidine, 1mM EDTA), 3μl <sup>32</sup>P ATP and the reaction volume adjusted to 20μl using SDW. Following incubation at 37°C for 1 hr, the reaction mix was extracted once with a 1:1 (v/v) mixture of phenol:chloroform and ethanol precipitated. The nucleic acids were pelleted by centrifugation and redissolved in 3 x P.E.B buffer (5 x P.E.B; 2M NaCl, 50mM Pipes pH 6.4, 2.5mM EDTA). To approximately 3 x 10<sup>4</sup> c.p.m of end-labelled oligo was added 7.5μl 5 x P.E.B, RNA

adjusted to a final volume of 40 $\mu$ l using SDW, 1 $\mu$ l placental RNase inhibitor and the overall volume made up to 52.5 $\mu$ l using SDW. The mix was incubated O/N at 45°C, ethanol precipitated and the nucleic acids pelleted by centrifugation. The pellet was resuspended in 20 $\mu$ l reverse transcriptase buffer (R.T.B) (10 x R.T.B; 50mM Tris-Cl pH 8.3, 6mM MgCl<sub>2</sub>, 10mM DTT, 1mM dNTPs). and 0.5 $\mu$ l of reverse transcriptase added. Following incubation at 37°C for 30 mins, 2 $\mu$ l of RNase (20 mg/ml) was added to the mix and incubation continued for a further 10 mins at 37°C. Following phenol extraction, isopropanol precipitation and centrifugation, the resulting nucleic acid pellet was resuspended in 5 $\mu$ l of formamide sequencing dyes (Maniatis et al., 1982) and the extension products analysed on an 8% sequencing gel.

## 2.11 DNA sequencing:

### 2.11.a sequencing of plasmid DNA:

The method of DNA sequencing was carried out using the dideoxynucleotide chain termination method of Sanger et al. (1977) but using double-stranded plasmid template DNA.

The volume of approximately 10 $\mu$ g of plasmid DNA preparation (small or large scale) was adjusted to 20 $\mu$ l with SDW. The DNA strands were denatured by the addition of 1 $\mu$ l of 0.5N NaOH for exactly 5 min. The solution was then passed through a desalting Sepharose 6LB (Pharmacia) spin column and the resulting DNA solution was then immediately used for sequencing exactly as described in the Sequenase II (USB) instruction booklet and using the kit components and <sup>35</sup>S-dATP radiolabeled nucleotide.

#### 2.11.b direct sequencing of PCR products;

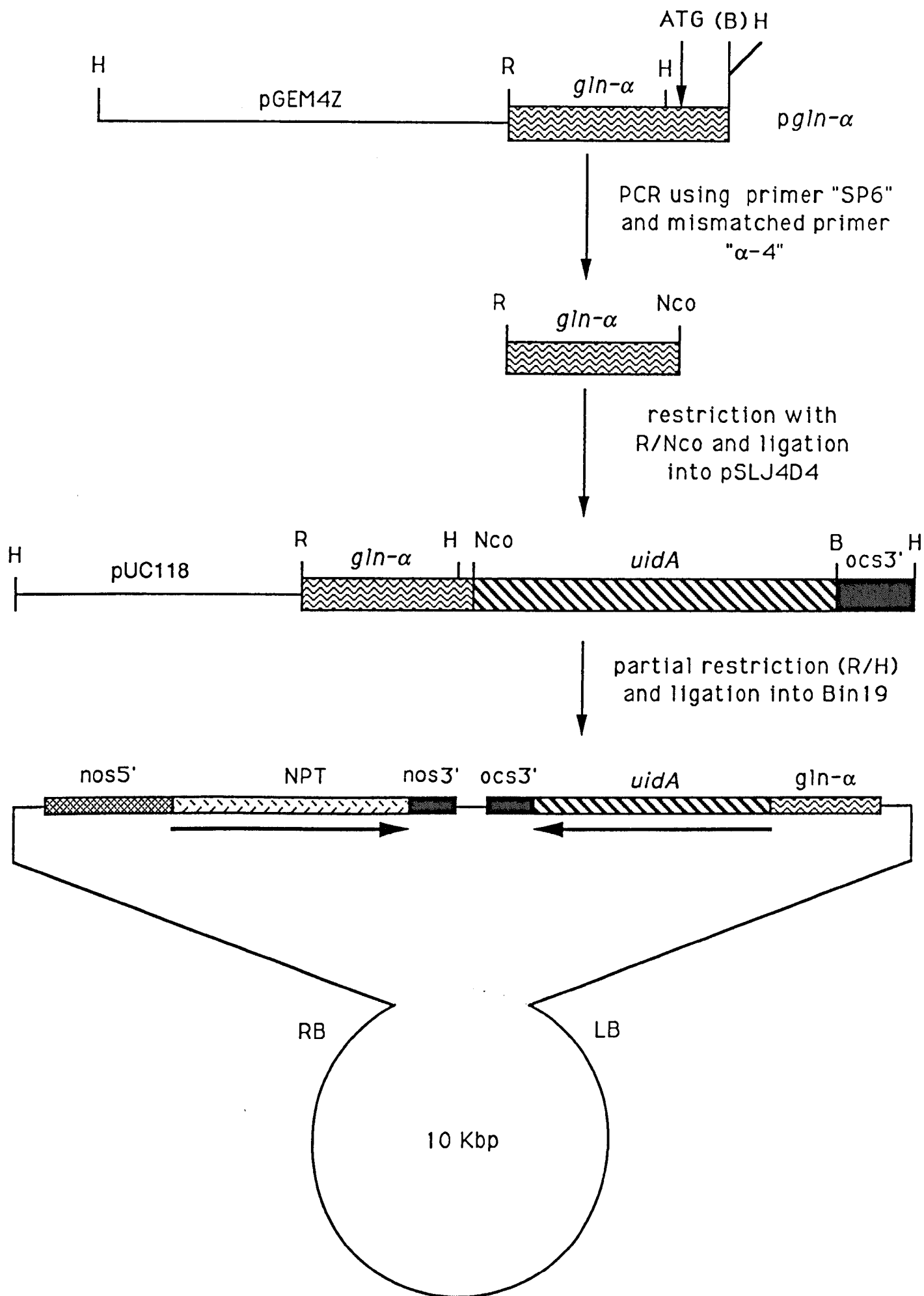
Double-stranded PCR products were directly sequenced using the method of Casanova et al. (1990) and the Sequenase II kit. The annealing temperature used was  $-70^{\circ}\text{C}$ , the extension time used was 1 min, and the molar ratio of primer:template was 1:20.

## 2.12 construction of the fusion of the gln- $\alpha$ promoter to the *E. coli uidA* reporter gene:

The gln- $\alpha$  transcriptional start site (5' CCATGT 3') was mutated to the NcoI restriction enzyme recognition site (5' CCATGG 3') using PCR. The PCR reaction was performed as described in section 2.7 and used the mismatched 23mer " $\alpha$ -4" antisense-strand primer (5' GAT CTG AAA GCA AAG CCA TGG TG 3'), the 19mer "SP6" sense-strand primer (5' GGA TTT AGG TGA CAC TAT A 3') and 20ng of plasmid DNA template. The PCR product was separated by electrophoresis on a 0.8% (w/v) agarose, 1 x TAE gel and isolated using GeneClean. Following gel isolation, the DNA fragments were restricted with EcoRI and NcoI and gel isolated using GeneClean for a second time. The resulting DNA fragments were subcloned into the plasmid pSLJ4D4, a pUC18 derivative containing the cauliflower mosaic virus (CaMV) 35S promoter fused to the *E. coli uidA* gene encoding  $\beta$ -glucuronidase (GUS) and the osc (octapine synthase) terminator, so that it replaced the 35S promoter (see fig. 2.3). The resulting plasmid was termed pgln- $\alpha$ D4. The 35S promoter fragment was restricted from the plasmid prior to subcloning using EcoRI and NcoI and the plasmid fragment agarose gel isolated. Sequence analysis by the dideoxy method (Sanger et al., 1977) verified the gln- $\alpha$ ::uidA fusion to be in-frame. The sequencing primer was a 17mer sense strand primer " $\alpha$ -5" (5' CAC TTC ACC CTA TCA AG 3') which hybridised 65bp 5' to the gln- $\alpha$  transcriptional start site. The construct (termed gln- $\alpha$ ::uidA) was subcloned into the plant transformation vector Bin19 (Bevan,



fig. 2.3. Production of the gln- $\alpha$ ::uidA construct and the subcloning of the construct into the plant transformation vector Bin19 (Bevan, 1984) (H = HindIII, R = EcoRI, Nco = NcoI, ATG = gln- $\alpha$  translational start, nos3' = nopaline synthase terminator sequence, nos5' = nopaline synthase promoter sequence, NPT = neomycin phosphotransferase coding sequence, ocs3' = octapine synthase terminator sequence);



1984) linearised using EcoRI and HindIII and agarose gel isolated (see fig. 2.3). Because of the presence of a HindIII restriction enzyme site in the gln- $\alpha$  promoter sequence, the entire gln- $\alpha$ ::uidA construct was isolated from a partial digest of p<sub>gln- $\alpha$</sub> D4. The products of the partial digest were separated by electrophoresis on an agarose gel and the band corresponding to the intact construct was gel isolated and subcloned into the Bin19 vector. Contained within the Bin19 T-DNA border repeats is not only the multiple cloning site into which the gln- $\alpha$ ::uidA construct was subcloned but also a chimeric nopaline synthase promoter::neomycin phosphotransferase gene (nos::nptII) which confers plant kanamycin resistance. The orientation of construct subcloning was such that the two chimeric genes were transcribed in opposite directions (see fig. 2.3). Studies have shown the influence of surrounding DNA on the transcriptional activity of an introduced chimeric gene acts over at least several kilobases (Odell et al., 1987) and therefore the nos promoter may have a marked effect on the transcriptional activity of the gln- $\alpha$ ::gusA gene if the orientation of the two chimerc genes was the same. The gln- $\alpha$ ::uidA construct and two control constructs; pBI121.1 (a 35S::uidA fusion) and pBI101.1 (a promoterless uidA) (Jefferson et al., 1987) were directly introduced into Agrobacterium rhizogenes (strain AR10) and Agrobacterium tumefaciens (strain LBA4404) using the freeze-thaw method as essentially described by An et al. (1988). The constructs were checked for rearrangements by restriction mapping. Plasmid DNA was isolated from

Agrobacterial cultures using an alkaline lysis procedure and the resulting plasmid used to transform competent E. coli TG2 cells. Plasmid DNA was re-isolated from the resulting E. coli strains and restriction mapped.

## 2.13 plant transformation:

### 2.13.a Nicotiana tabacum (tobacco) cv. Samsun;

The constructs were individually transformed into tobacco using the leaf disc method of Horsch et al. (1985). 4 to 5 inch leaves from 5 to 7 week old chamber grown plants were excised and sterilised by submergence in 10% chlorox, 0.1% SDS for 30 minutes. After rinsing the leaves in SDW, leaf discs were punched out into a diluted A. tumefaciens culture and incubated for 5 mins. Discs were then transferred, bottom surface up, to shoot induction plates (4.6g Murashige and Skoog (MS) salts (Murashige and Shoog, 1962), 30g sucrose, 1ml BAP (1mg/ml), 0.1ml NAA (1mg/ml) and 8g Bacto agar per litre (pH adjusted to 5.8)). After 3 days incubation at 25-28°C and in the light, the discs were transferred to selective shoot induction medium containing 200 µg/ml carbenicillin (to remove contamination by Agrobacteria) and 100 µg/ml kanamycin (to select for transgenic lines). After 3 weeks incubation the discs were transferred to fresh selective shoot induction media and with two further weeks incubation, shoots were excised from the discs and transplanted to selective root induction media (4.6g MS salts, 30g sucrose and 8g Bacto agar per litre (pH adjusted to 5.5)) containing 100µg/ml kanamycin and 200µg/ml carbenicillin for 3 to 4 weeks.

Transformed plants were propagated vegetatively in magenta pots on selective root induction media (referred to as "in-vitro maintained" plants) and also grown in greenhouses in compost and watered using tap water.

#### 2.13.b Lotus corniculatus cv. Leo;

Transgenic hairy root lines of L. corniculatus were generated essentially as described by Hansen et al. (1989). L. corniculatus seeds were surface sterilised by immersion in 10% chlorox for 15 mins followed by several washes in SDW. Seeds were then grown in the dark at 25-28°C for 7 to 10 days on 1/2 MS media (2.3g MS salts, 30g sucrose, 8g bacto-agar per litre). Seedlings were infected with A. rhizogenes at several sites on each hypocotyl by wound inoculation. The respective A. rhizogenes strains were grown on plates containing 100 µg/ml kanamycin for two days at 28°C and a portion of culture scraped from the plate using a sterile scalpel blade. The blade was then used to make several small incisions on the hypocotyl surface. After 2 to 4 weeks, when the hairy roots had grown to 3 to 4cm, the original roots were excised and the plants were immersed in Fahraeus medium (Fahraeus, 1957) containing 0.5mM KNO<sub>3</sub> and 250 µg/ml carbenicillin to remove agrobacterial contamination. After several rinses in SDW, the plants were transferred to sterile growth pouches containing paper wicks soaked in Fahraeus medium containing 0.5mM KNO<sub>3</sub>. The plants were grown for a further 2 weeks and then inoculated with a suspension of Rhizobium loti, nodules appearing 3 to 4 weeks later. A number of plants were

then treated with 20mM KNO<sub>3</sub> in order to cause senescence of the nodules, which were then harvested 3 days later.

#### 2.14 pre-fixation of plant material:

Tissue was fixed prior to histochemical staining by the vacuum infiltration of 50mM Na-phosphate buffer pH7 (Maniatis et al., 1982) containing 0.3% (v/v) formaldehyde for 10 min. The tissue was then incubated on ice for 30 min and then vacuum infiltrated for a further 10 min with Na-phosphate buffer alone. After a 30 min incubation on ice, the tissue was ready for staining (see section 2.15).

#### 2.15 histochemical staining:

Intact or handcut sections of transformed plant material were immersed, with or without pre-fixing, in 50mM Na-phosphate buffer pH7 ,0.01% triton X-100, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc), and subjected to vacuum infiltration for between 10 and 15 minutes and incubated at 37°C O/N (Jefferson et al.,1987). An oxidation catalyst (2.5mM K ferricyanide and 2.5mM K ferrocyanide) was included to avoid artefacts that could result from peroxidase activity or microaerobic conditions (Pearse, 1972).

For the localisation of GUS activity in tobacco flowers and pollen, methanol was added to the phosphate buffer to a final concentration of 20% (v/v) to suppress endogenous GUS activity (Kosugi et al., 1990 ). Mannitol was also added to a final concentration of 0.3M.

Tissues were cleared of photosynthetic pigments by boiling

in either 100% ethanol or a methanol:acetone (3:1) mixture.

#### 2.16 tissue sectioning:

Following staining, the tissues were washed in 50mM Na-phosphate buffer pH7 and incubated at RT in 50mM Na-phosphate buffer containing 1% (v/v) glutaraldehyde. This solution was then replaced by 70% ethanol and the tissue stored at 4°C until sectioned. Fixed tissue was embedded in 3% agar and 100 µm sections taken using a Bio-Rad micro-cut H1200. Sections were slide mounted and brightfield photographed using a Wild Leitz auto-exposure binocular camera using Fuji 'reala' film at between 10x and 40x magnification.

#### 2.17 wounding of tobacco leaves:

Leaves were excised from in-vitro maintained transformed tobacco plants and approximately 1cm of the leaf tip was cut away, frozen in liquid nitrogen and stored at -80°C. This sample was subsequently assayed as the time 0 extractable GUS activity. The remainder of the leaf was lacerated using a sterile scalpel. Cuts were perpendicular to, and away from the leaf midrib and down to the leaf edge and were approximately 1 to 2mm apart. The leaf was cut several times this way on both sides of the midrib. The leaf was floated on sterile MS media supplemented with 1mM final concentration reduced glutathione ensuring the cut leaf petiole was fully immersed in the media. Flotation on MS media was necessary in order to avoid leaf stress and therefore limit any induction of GUS activity to be a result of the wounding. 6 hrs after wounding, half of the remaining leaf was cut away,

frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  and subsequently assayed for extractable GUS activity. 24 hrs after wounding the remainder of the leaf was frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  and subsequently assayed. To avoid either bacterial or fungal infection of the wounded leaves, the experiment was conducted under sterile conditions in a lamina-flow hood using sterile solutions and implements.

#### 2.18 fluorimetric assay of extractable uidA activity:

Plant material was harvested, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The tissue was then ground in liquid nitrogen in either a 1.5ml centrifuge tube using a disposable plastic pestle or in a mortar and pestle. Extraction buffer was added (50mM  $\text{Na}^{+}$  phosphate pH7.0, 10mM  $\beta$ -mercaptoethanol, 10mM  $\text{Na}^{+}$  EDTA, 0.1% sarcosyl and 0.1% triton X-100) and grinding continued. The samples were microcentrifuged for 10 minutes twice, the supernatant being transferred each time to a new tube. Extracts were assayed for GUS activity using 4-methylumbelliferyl glucuronide (MUG) as the substrate and the extraction buffer as the reaction buffer (Jefferson et al. 1987). The total reaction volume was 2ml with a final MUG concentration of 1mM. The reaction mix was prewarmed to  $37^{\circ}\text{C}$  and an aliquot of extract added. The reaction proceeded at  $37^{\circ}\text{C}$  and 400 $\mu\text{l}$  aliquots of the reaction mix were taken at 0 min, 30 min and 2 hr and added to 2ml of stop solution (0.2M  $\text{Na}_2\text{CO}_3$ ). Fluorescence was measured using a Fluoroskan II (excitation 355 nm; emission 460 nm). The fluorimeter was calibrated using 100nM 4-methylumbelliferone



(MU). Crude extract protein concentrations were determined using the method of Bradford (1976) using the Bio-Rad protein dye-binding reagent and bovine  $\gamma$ -globulin as standard.

Chapter 3: Results - Cloning and characterisation of the gln- $\alpha$   
gene promoter and upstream region.

### 3.1 Introduction:

Previous attempts to clone the gln- $\alpha$  gene were unsuccessful. Only gln- $\gamma$  sequences were cloned from a P. vulgaris genomic library screened at low stringency using a probe synthesised from the  $\gamma$ -cDNA (Cullimore, unpublished data) and demonstrated the need for specific gln- $\alpha$  probes. Probes subsequently synthesised from the  $\alpha$ -cDNA were however unsuccessful in isolating gln- $\alpha$  sequences from a P. vulgaris genomic library (Daniell, 1992) from which gln- $\delta$  clones had been successfully isolated (Cock et al., 1991).

It was possible that gln- $\alpha$  sequences may undergo modification during the cloning. E. coli contains at least three types of restriction-modification system (RMS). The systems involve an endonuclease (ENase) which digests unmodified DNA, and a methylase (MTase), which protects endogenous DNA from restriction through methylation. The functions of RMSs are thought to include both defence (restriction of bacteriophage DNA) and recombination (the conversion of introduced bacterial DNA into recombinogenic fragments). The hsd genes of E. coli are responsible for encoding type I RMSs such as EcoK and EcoB and cleave specific DNA sequences unprotected by methylation at N-6 of specific adenines (Bickle, 1982). In order to prevent the potential restriction of cloned sequences, hsd mutants of E. coli are used as cloning hosts. The cloning host used previously to screen P. vulgaris genomic libraries for gln- $\alpha$  sequences was an hsd mutant (strain P2392) (Cullimore, unpublished results;

Daniell, 1992) and it is worth noting that the cloning host used in the present study was also an hsd mutant (strain NM554).

In contrast to the hsd ENases, a second type of RMS ENases encoded by the genes mcrA and mcrB specifically recognise DNA sequences only when they are methylated. The mcrA and mcrB genes encode ENase which specifically recognise and restrict DNA containing 5-methylcytosine in certain sequence contexts (Raleigh and Wilson, 1986). As in plants up to 40 to 50% of all cytosine residues may be methylated, including over 80% of CG and C<sup>A</sup>/T<sup>G</sup>, significant skewing of plant genomic libraries screened using mcr positive host strains may occur (see Blumenthal, 1989). Although host strain P2392 is an mcrA mutant, the host strain used in this study, NM554, is both an mcrA and an mcrB mutant.

The root tissue used in the construction of this and previous genomic libraries was extracted from the roots of approximately 10 day old P. vulgaris plants. The studies of Ortega et al. (1986) clearly demonstrated that the  $\alpha$ -polypeptide is the main component of the GS isoenzyme in the embryo and in up to 5 day old roots but by day 10, the  $\alpha$ -polypeptide is barely detectable. This down regulation may be through gene methylation. Therefore, either the pattern and extent of methylation of the gln- $\alpha$  gene per se or the possible methylation of the gln- $\alpha$  gene during regulation of root expression may render the gene restrictable by the mcr gene system and thus unclonable from genomic libraries screened in mcr positive host strains.

Recent evidence has also shown that mcr ENases can also

degrade genomic DNA during in-vitro packaging reactions (Graham et al., 1990). The packaging mix used previously in the construction of genomic libraries was Gigapack I and was prepared from lambda lysogens derived from an mcrA<sup>+</sup>, mcrB<sup>-</sup> strain (Stratagene Cloning Systems). However the packaging extract used in this study was Gigapack II and is derived from an mcrA<sup>-</sup>, mcrB<sup>-</sup> strain (Stratagene Cloning Systems).

### 3.2 Construction and screening of the genomic library;

Genomic DNA was extracted from the roots of 10 day-old P. vulgaris plants. The genomic DNA was then partially restricted using SauIIIA and 15-20Kbp DNA fragments were isolated by sucrose density centrifugation. A portion of these DNA fragments were ligated into the bacteriophage lambda Dash cloning vector and in-vitro packaged into bacteriophage lambda heads using Gigapack II Gold packaging extract (Stratagene Cloning Systems). The percentage of library non-recombinant clones was determined using the E. coli host strains P2392 and LE392; both recombinant and non-recombinant clones plate out on strain LE392 but only recombinants plate out on strain P2392. The percentage of non-recombinants was determined as 21% thus allowing the titre of the library to be more accurately calculated.

It was calculated that in order to achieve a 95% probability of a gln-α gene being cloned,  $8.5 \times 10^{10}$  pfu must be plated out and screened (size of P. vulgaris haploid genome =  $1.7 \times 10^9$  and the size of average lambda insert estimated as 12Kbp). Therefore, the remainder of the library ( $12.7 \times 10^{10}$  pfu) was plated out

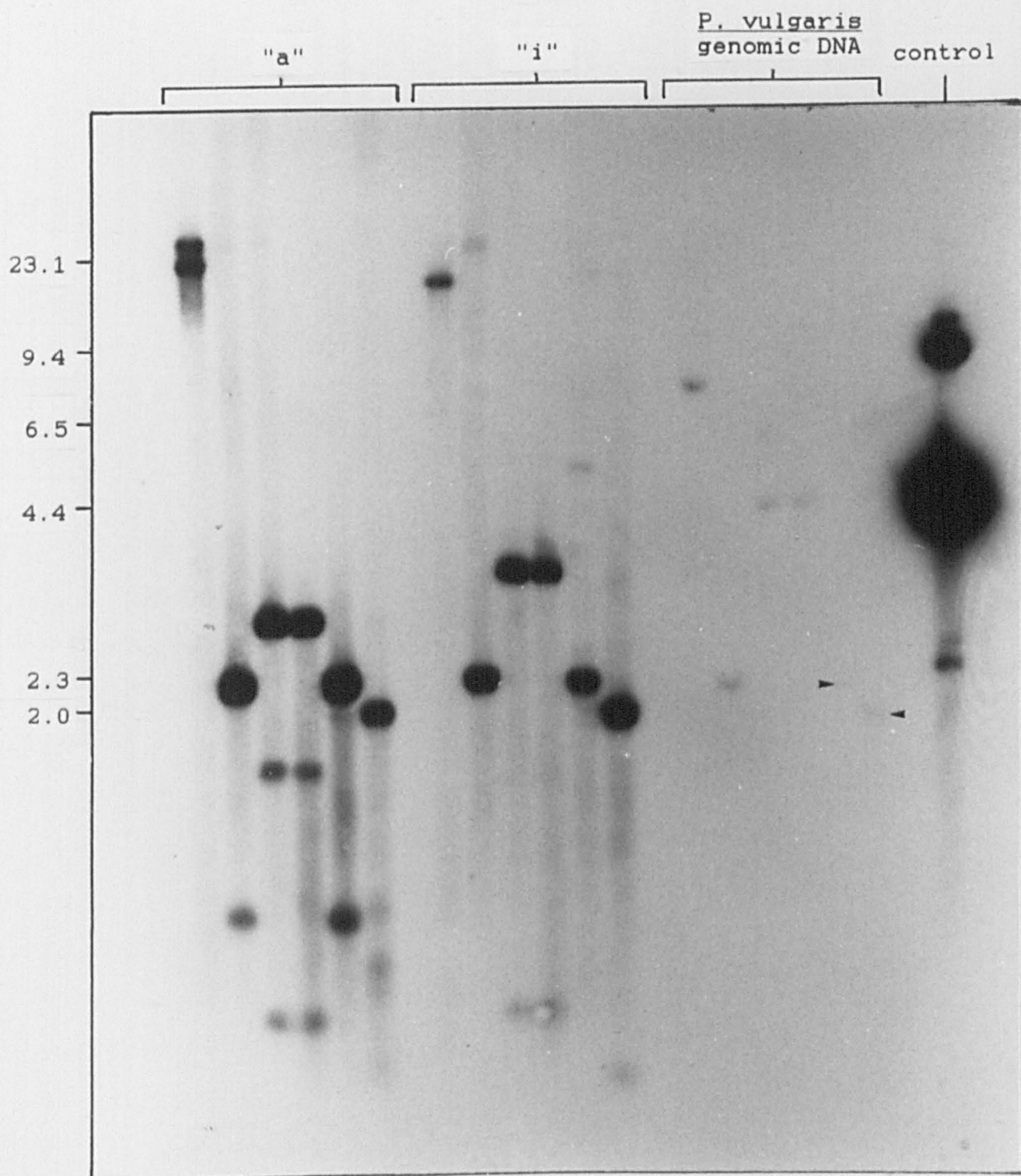
directly without amplification on host strain NM554 at a density of 250pfu/cm<sup>2</sup> and the phage DNA immobilised on nylon filters. Hybridisation to the  $\alpha$ -cDNA:HindIII probe (a 99bp RNA probe, 50bp derived from the 5' end of the  $\alpha$ -cDNA and 49bp derived from the vector, transcribed to the 5' HindIII site of the  $\alpha$ -cDNA; see fig. 2.1) revealed, at high stringency, 6 positives. The small 5' cDNA probe was used to screen the library to preferentially isolate promoter sequences. These 6 positives were then confirmed by re-hybridising the filters to the  $\alpha$ -cDNA:EcoRI probe (a DNA probe prepared by oligolabelling the 710bp  $\alpha$ -cDNA EcoRI fragment; see fig. 2.1). The latter probe however failed to hybridise to any further clones beyond those 6 hybridised to previously. The autoradiograph positives were aligned with the plates and one or a few cores of agar removed and stored prior to further screening. Two (clones "a" and "i") of the original 6 positives were plaque purified.

### 3.3 Southern blots of phage DNA and genomic DNA;

DNA was isolated from both phage clones "a" and "i" and genomic DNA was extracted from the roots of 10 day old P. vulgaris plants. Both sets of DNA were restricted with BamHI, HindIII, EcoRI and double restriction digests of these enzymes. Following 0.6% (w/v) agarose gel electrophoresis and Southern blotting, the filter was hybridised to the  $\alpha$ -cDNA:HindIII probe and then washed at high stringency. The autoradiograph resulting from exposure to the filter is shown in fig. 3.1. The patterns of hybridisation of both the phage clones and the genomic DNA

fig. 3.1. Southern blot of phage "a" and "i" DNA and P. vulgaris genomic DNA. Restriction order for each; BamHI, HindIII, EcoRI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI. Blot hybridised to the  $\alpha$ -cDNA:HindIII transcription probe and washed at high stringency (0.1 x SSC, 0.1% SDS, 65°C) (control: pcGS $\alpha$ M ( $\alpha$ -cDNA subcloned into pGEM3Z (Promega)) linearised with SacI);

show both similarities and differences. An identical sized

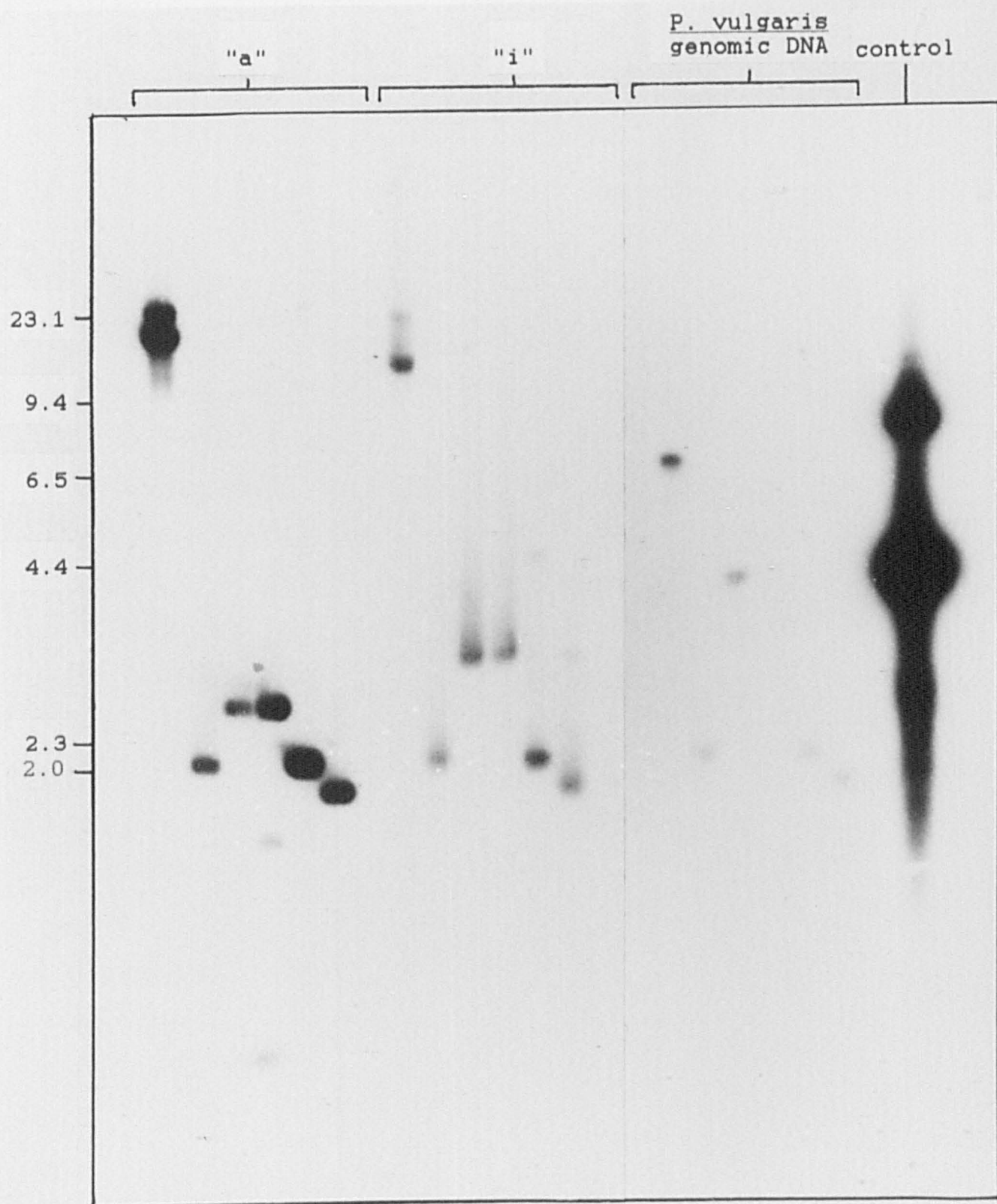




show both similarities and differences. An identically sized HindIII band of approximately 2.2Kbp is common to all three as is an EcoRI/HindIII band of approximately 2.0Kbp. Clones "a" and "i" show differing sizes of EcoRI band with both of these being smaller in size to the genomic EcoRI band of approximately 5.0Kbp. The genomic pattern of hybridisation is identical to that found previously by Gebhardt et al. (1986). Their studies involved the restriction of P.vulgaris genomic DNA with EcoRI, HindIII and BamHI. Southern blotting of the DNA fragments and the hybridisation to a probe synthesised from the entire  $\alpha$ -cDNA <sup>32</sup>P-labelled by nick translation. Filters washed at high stringency (5mM NaPO<sub>4</sub> pH7, 1mM EDTA, 0.2% SDS at 60-63°C) showed a hybridising BamHI fragment of approximately 8.0Kbp, a HindIII fragment of approximately 2.2 Kbp, and two EcoRI fragments of approximately 5.0Kbp and 6.5Kbp (Gebhardt et al., 1986). Southern blot data has also shown that a portion of the 5' end of the gln- $\alpha$  gene is contained within a 8Kbp BamHI fragment (Daniell, unpublished results). A blot identical to that hybridised to the  $\alpha$ -cDNA:HindIII probe was hybridised to the oligolabelled  $\alpha$ -cDNA:EcoRI probe and the pattern of hybridisation was similar to that described for the  $\alpha$ -cDNA:HindIII probe (see fig. 3.2). Increased periods of exposure of the X-ray film to the washed filter revealed that all of the lower molecular weight hybridising bands seen in fig. 3.1 were present. When considering the probe contained 3 HindIII fragments it was expected the probe would hybridise to several further HindIII restriction fragments.

fig. 3.2. Southern blot of phage "a" and "i" DNA and P. vulgaris genomic DNA. Restriction order for each; BamHI, HindIII, EcoRI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI. Blot hybridised to the  $\alpha$ -cDNA:EcoRI oligolabelled probe and washed at high stringency (0.1 x SSC, 0.1% SDS, 65°C) (control: pcGS $\alpha$ M ( $\alpha$ -cDNA subcloned into pGEM3Z (Promega)) linearised with SacI);

However, G. G. G. (1985) used the entire  $\alpha$ -DNA to probe



However, Gebhardt et al. (1986) used the entire  $\alpha$ -cDNA to probe genomic blots and only obtained hybridisation to the single HindIII fragment of approximately 2.2Kbp.

### 3.4 sub-cloning of promoter sequences into plasmid vectors;

Using the specific primer homologous to the  $\alpha$ -cDNA (" $\alpha$ -2"; hybridises 26bp 3' to the  $\alpha$ -cDNA translational start and 12bp 3' to the BglIII site) and the generic primers homologous to the T7 and T3 RNA polymerase initiation sites of the lambda vector arms ("T7" and "T3"), a PCR product of approximately 900bp was produced from the clone "a" DNA template. As the specific primer was complementary to sequences downstream of the  $\alpha$ -cDNA translational start site, the entire upstream fragment contained within clone "a" was amplified. Clone "i" however, failed to produce a PCR fragment, a surprising result when it was considered that the Southern blot data predicted a PCR fragment from clone "i" to be at least 500bp larger than that of the fragment produced by clone "a" (see fig. 3.9). The 900bp clone "a" fragment was subcloned as an EcoRI/BglIII fragment into pGEM-4Z and the resulting plasmid was termed pgln- $\alpha$ . The pgln- $\alpha$  plasmid was restricted with EcoRI and HindIII and the gln- $\alpha$  sequence oligo-labelled (termed "gln- $\alpha$  probe") and hybridised to P. vulgaris genomic DNA and phage "a" DNA. The pattern of hybridisation at high stringency to the P. vulgaris DNA was identical to that seen for both cDNA probes and the work of Gebhardt et al. (1986) (data not shown). However, the pattern of hybridisation of the gln- $\alpha$  probe to phage "a" DNA was different

to that seen for the  $\alpha$ -cDNA probes. The hybridising HindIII and HindIII/EcoRI fragments were approximately 10.0Kbp and 900bp respectively for the gln- $\alpha$  probe (see fig. 3.3) as opposed to 2.2Kbp and 2.0Kbp respectively for both the  $\alpha$ -cDNA probes (see figs 3.1 and 3.2). Further Southern blot analysis revealed a single hybridising BamHI/HindIII fragment of approximately 10.0Kbp indicating the BamHI/HindIII digest shown in fig. 3.3 to be partial. This BamHI/HindIII fragment is again different in size to the 2.2Kbp BamHI/HindIII fragment hybridised to by the  $\alpha$ -cDNA probes. The hybridising BamHI, EcoRI and BamHI/EcoRI fragments were however the same size for both the gln- $\alpha$  and  $\alpha$ -cDNA probes.

When an EcoRI/HindIII restriction digest of the pgln- $\alpha$  plasmid was co-electrophoresed with the digested phage "a" DNA to act as a positive control during probe hybridisation, it was observed that the clone "a" EcoRI/HindIII fragment and the gln- $\alpha$  EcoRI/HindIII fragment were both of similar size and both hybridised to the gln- $\alpha$  probe. This suggested that the gln- $\alpha$  EcoRI/HindIII fragment was the PCR product of the clone "a" EcoRI/HindIII fragment (see fig. 3.4).

### 3.5 direct sequencing of the PCR product:

Prior to subcloning, the double-stranded PCR product was directly sequenced from the 3' end using the 18mer " $\alpha$ -1" primer (5' GAA GAT ACC AAA AGG TGA 3') which primes 58bp upstream of the  $\alpha$ -cDNA translational start and 56bp downstream from the 5' end of the  $\alpha$ -cDNA. The sequencing method was as essentially described by the Sequenase II booklet and used the kit reagents. However, alterations were made to the protocol as recommended by Casanova et al. (1990) (see section 2.11.b). The sequence obtained was identical to that of the  $\alpha$ -cDNA but the sequences

fig. 3.3. Southern blot of phage "a" DNA. Restriction order; BamHI, HindIII, EcoRI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI. Blot hybridised to the gln- $\alpha$  oligolabelled probe and washed at low stringency (2.0 x SSC, 0.1% SDS, 65°C);

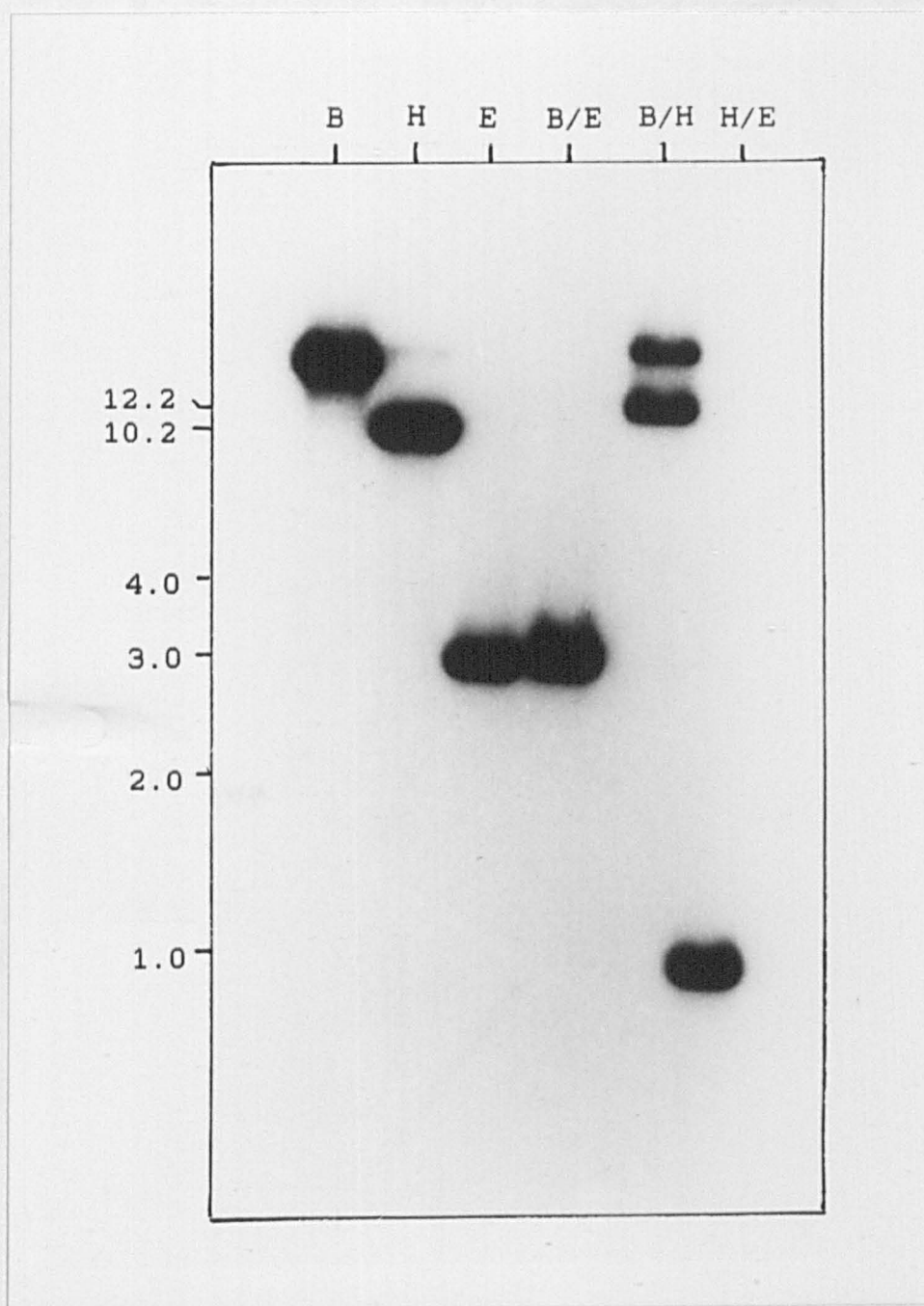
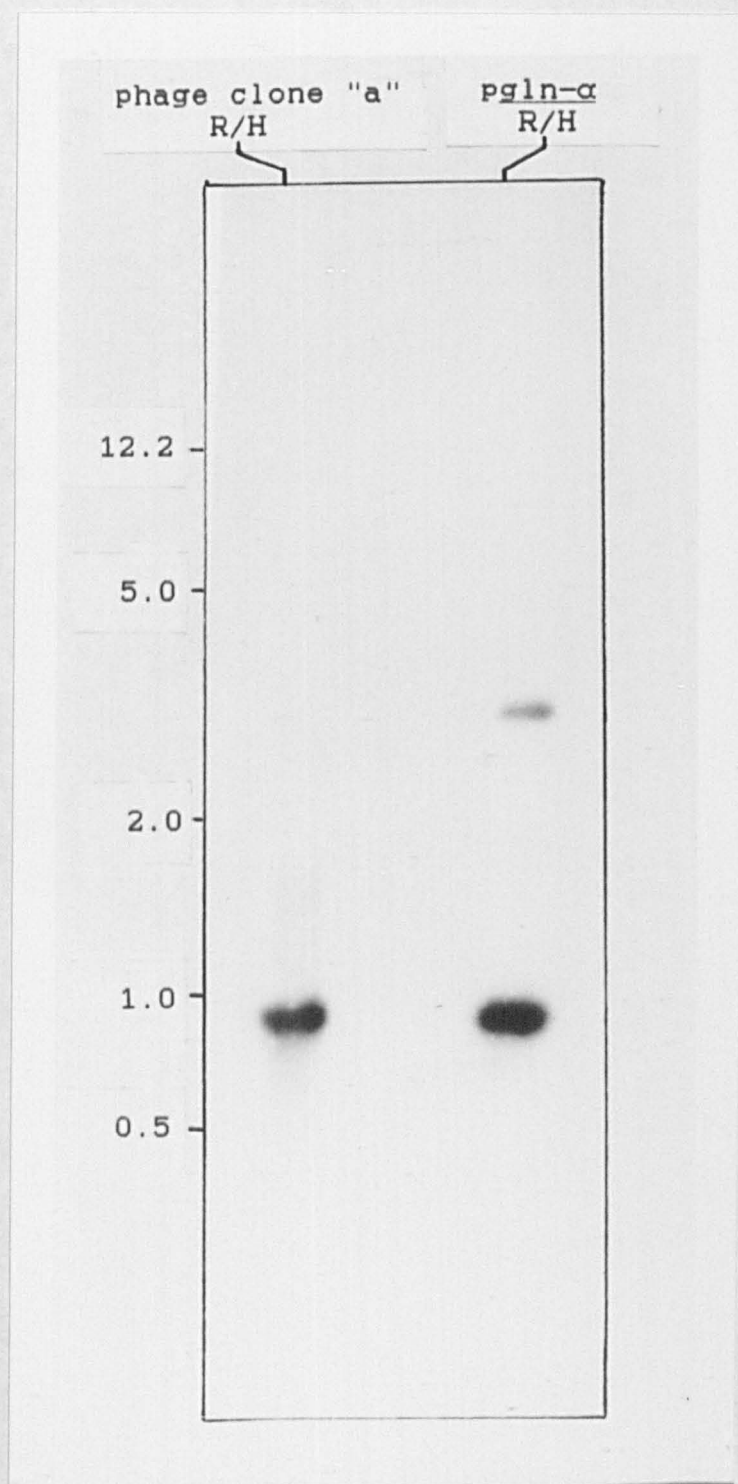


fig. 3.4. Southern blot of phage "a" DNA restricted using EcoRI and HindIII and the gln- $\alpha$  promoter fragment restricted from pgln- $\alpha$  using EcoRI and HindIII. Blot hybridised to the gln- $\alpha$  oligolabelled probe and washed at high stringency (0.1 x SSC, 0.1% SDS, 65°C);





diverged 28bp from the 5' end of the  $\alpha$ -cDNA sequence and those 5' cDNA 28bp were not present in the gln- $\alpha$  sequence obtained using the " $\alpha$ -1" primer. The implication of this sequence divergence are discussed in detail below.

### 3.6 sequencing of the gln- $\alpha$ promoter;

Sequencing of gln- $\alpha$  clone was as described in section 2.11a and involved the use of the Sequenase II kit and protocol. Using the "SP6" and "T7" primers, the clone was sequenced up to 300bp in each direction from within the plasmid vector. From this sequence information two further primers were synthesised and used to sequence further into the clone. The two primers synthesised were an 18mer " $\alpha$ -6" (5' GGT TGA ATT TTG AAA CCC 3') to prime downstream (see fig. 3.5) and an 18mer " $\alpha$ -8" (5' CAT ATA ATT TCT GTA TCC 3') to prime upstream (see fig. 3.5). The complete gln- $\alpha$  sequence is shown in figure 3.5. The sequencing confirmed earlier data indicating that the  $\alpha$ -cDNA sequence and gln- $\alpha$  sequences diverge 28bp from the 5' end of the  $\alpha$ -cDNA. Four possible reasons may account for the divergence in sequence; "1", an intron, "2", a cDNA cloning artefact, "3", a genomic cloning artefact, or "4" differences in plant variety. The  $\alpha$ -cDNA was isolated from cultivar Bush Blue Lake and the  $\alpha$ -genomic clone was isolated from cultivar Tendergreen. Possibility "1" was unlikely as the genomic sequence shows the pre-spliced  $\alpha$ -mRNA to lack, at the sequence divergence point, the 3' intron-exon consensus dinucleotide sequence AG, as found in all introns (Goodall and Filipowicz, 1989). Furthermore, all previously characterised

fig. 3.5. The DNA sequence of the gln- $\alpha$  upstream region isolated from phage clone "a" using the PCR technique (primers " $\alpha$ -2" and "T7") (A = point of divergence of the gln- $\alpha$  upstream region and  $\alpha$ -cDNA sequences, T = transcriptional start site as determined using primer extension analysis (template = total RNA), C = transcriptional start site as determined using primer extension analysis (template = poly A+ RNA), T = transcriptional start site as determined using an RNase protection assay, TATAAA = putative TATA box consensus sequence, ATG = gln- $\alpha$  translational start, CACGT and TGAC = sequences that possibly interact with certain basic/leucine zipper (bZIP) transcriptional activators, " $\alpha$ -6" and " $\alpha$ -8" = synthetic oligonucleotide primers used in promoter fragment sequencing (arrows indicate direction of priming).

-819  
 GAATTCGGAT CCGTTACATT TTGTTTCACT TACATTTTTC AGGGAAACAA ACTAATACGA  
 -759  
 GGTTTACAAC TATACTCTTT ACTATTGAAT AAAGTCACCA TCATTAAC TAATTAGTTAAT  
 -699  
 TAATAAGAT AAAATTAATA ACTCACTAGT TATTTTTGGT ACATAAAAGT ATATAAATTT  
 -639  
 ATTCAAAATA TATTTAAAGA CCATAATTAA AAATAGCAAA AAATCATGAA GTTTTGACGC  
 -579  
 GAAATGATAC AAAATATTAA TATGTACATA TAATTTCTGT ATCCACAAGA AGTAGTTGGT  
 —————  $\alpha$ -8 —————>  
 -519  
 GGAATAGTAA TATTTTATAT ACTAAAATAT CATAACTTAA TAATAACGAT TAGTGTTGTT  
 -459  
 AATAGTATGT AATCAAATCT AATGAAAGTA CTTAGTTAAT ACGCAACCTG CTTTTGGTTA  
 -399  
 AGTGCTTATA AATTTACAA TATAATAATC AAACCTTAGC CGCAAAACAT GCTCGTTTGT  
 -339  
 TTTTATTCAC AAAATCTTCA CCCATTTTTT TATTCATTTT TCATAAATTT TTTTCTCCC  
 -279  
 TCTTCTCAG CAAATGCAAC GCTAAGAATA TTTTTCAG TTTTGGTTA CACAGTAAAT  
 -219  
 ATTCTTTTAA CAATTGCTTA ATTATTAATT AGTTAATTAA TTAAAGIGAC ACTATTTTAT  
 -159  
 TCAGGGTTTC AAAATTCAAC CTTGTGGAAG AAAAAGTGTC TCCAAGCACA TTCAAACACG  
 ←————  $\alpha$ -6 —————→  
 -99  
 TCTATGCAAC AACAATCTTA GGTTTATTCA ATCAATATTG ACATCACTGA GTCATTGTTG  
 -39  
 TTATTTATTA AGGGACTCTG AGACCAAACA AAGTCCTTCA TTTCATTCACT TCACCCTATC  
 +21  
 AAAGCTTTTT CACCTTTTGG TATCTTCTAC AATATCCTCT CATACTCATC TTTTCTCT  
 +81  
 +109  
 TTATCACC ATG TCT TTG CTT TCA GAT CC  
 Met Ser Leu Leu Ser Asp Pro

plant cytosolic GS genes lack introns aligning to the 5' untranslated cDNA region (Tischer et al., 1986, Turton et al., 1986). Possibility "2" would have explained the irregular occurrence of an additional start codon 3bp from the 5' end of the  $\alpha$ -cDNA. Possibility "4" could not be dismissed and it was reasonable to consider that those divergent 28bp were simply not represented in the Tendergreen  $\alpha$ -mRNA but were in the Bush Blue Lake  $\alpha$ -mRNA.

### 3.7 to determine if the 5' 28bp of the $\alpha$ -cDNA are represented in the cultivar Tendergreen $\alpha$ -mRNA;

An RNase protection assay (Kreig and Melton, 1986) was used to determine if the 5' 28bp of the  $\alpha$ -cDNA are present in the mRNA extracted from cultivar Tendergreen. The protection assay involved the use of an anti-sense probe transcribed from the  $\alpha$ -M3b clone linearised with EcoRI using T7 RNA polymerase (see section 2.10 and fig. 2.2.a). The probe transversed the point of sequence divergence and the size of the protected fragment indicated whether the 5' 28bp of the  $\alpha$ -cDNA are represented in the Tendergreen  $\alpha$ -mRNA. The probe was isolated from an acrylamide gel and aliquots hybridised to duplicate 10 $\mu$ g samples of total RNA extracted from the leaves of overnight imbibed P. vulgaris seeds or, as a control, 10 $\mu$ g of yeast tRNA. Following hybridisation O/N at 37°C, the mix was treated with RNase A at RT (approximately 25°C). The acrylamide gel electrophoresis of the protected fragments and sequencing reactions (sequencing template:  $\alpha$ -M3b, sequencing primer: "T7") allowed the protected

fragments to be accurately sized. The resulting autoradiograph is shown in fig. 3.6. Track (p) shows the undigested probe, track (t), the yeast tRNA control and tracks (1) and (2), the total RNA samples. From the absence of protected fragments in track (t), it was assumed that those fragments present in tracks (1) and (2) were not the result of non-specific hybridisation of the probe to various RNA molecules. Although tracks (1) and (2) were identical, they each showed several protected fragments of differing sizes and intensities. This may be due in part to "breathing" of the termini of the RNA:RNA hybrid and cleavage from the hybrid of one or several ribonucleotide bases by RNase A. In previous studies where the RNase digestion was carried out at 37°C and not at RT (approximately 25°C) as in this study, the problem was more pronounced and no individual band had any greater intensity over any other band (unpublished data). From calculations using the upper, most intense band as the limit of the hybridisation of the probe to the  $\alpha$ -RNA (arrowed on fig 3.6), it was revealed that the size of this fragment aligned 3bp 5' to that point where the  $\alpha$ -cDNA and gln- $\alpha$  sequences diverged.

### 3.8 mapping the transcriptional start site;

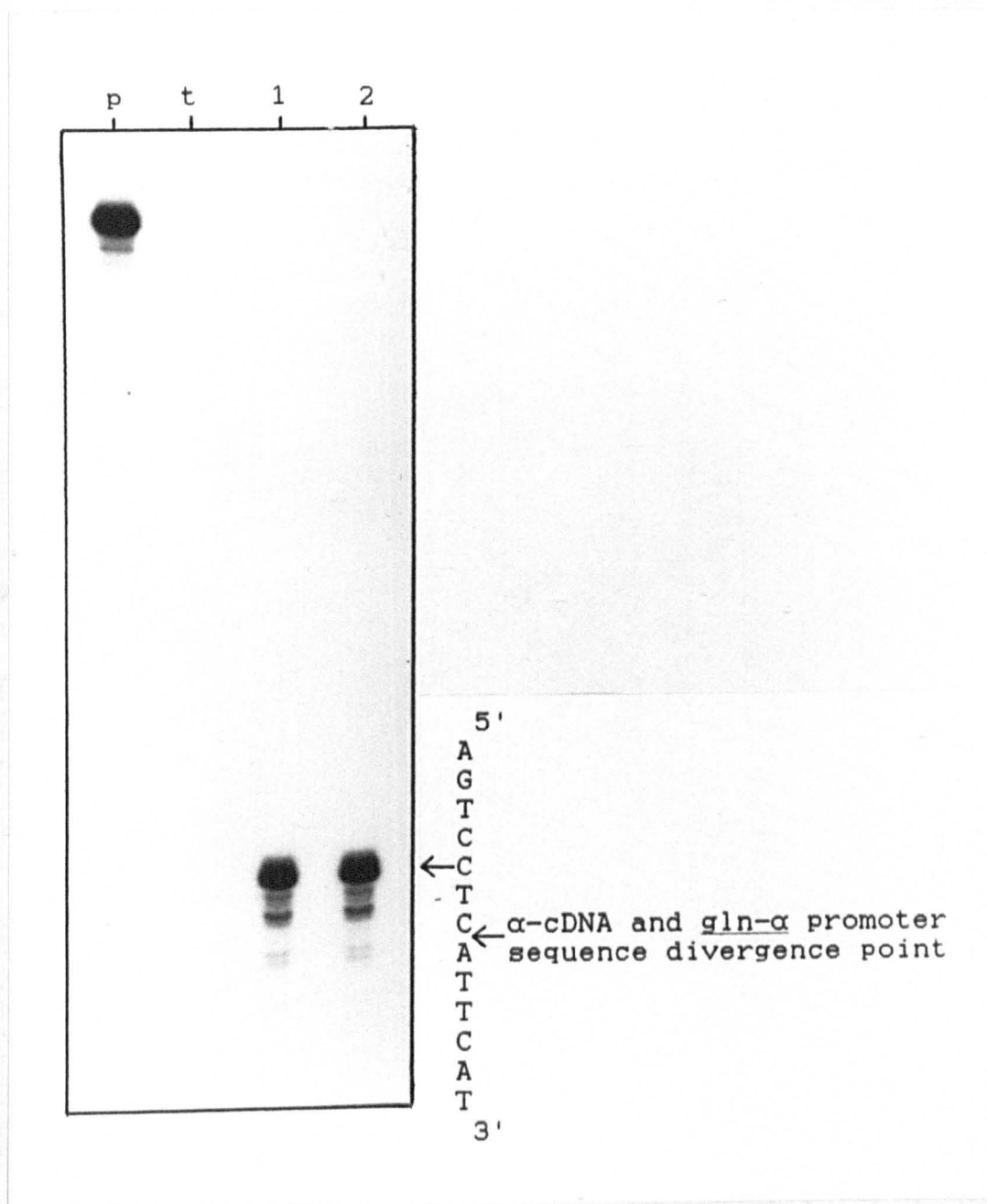
The gln- $\alpha$  transcriptional start site was determined by both primer extension and RNase protection.

#### 3.8.a primer extension;

Following acrylamide gel purification of 50 $\mu$ g of oligo " $\alpha$ -2" 2ng of purified oligo was radiolabelled by phosphorylation using bacteriophage T4 polynucleotide kinase. Approximately  $3 \times 10^4$  cpm

fig. 3.6. RNase protection assay to determine whether the 5' 28bp of the  $\alpha$ -cDNA are represented in the  $\alpha$ -mRNA of the P. vulgaris cultivar Tendergreen (track p = undigested probe, track t = yeast tRNA control, tracks 1 and 2 = total RNA samples extracted from the leaves of overnight imbibed P. vulgaris seeds, protected fragments sized by the co-electrophoresis of sequencing reactions (sequencing template;  $\alpha$ -M3b, sequencing primer; "T7") and aligned on the figure to the gln- $\alpha$  promoter sequence).





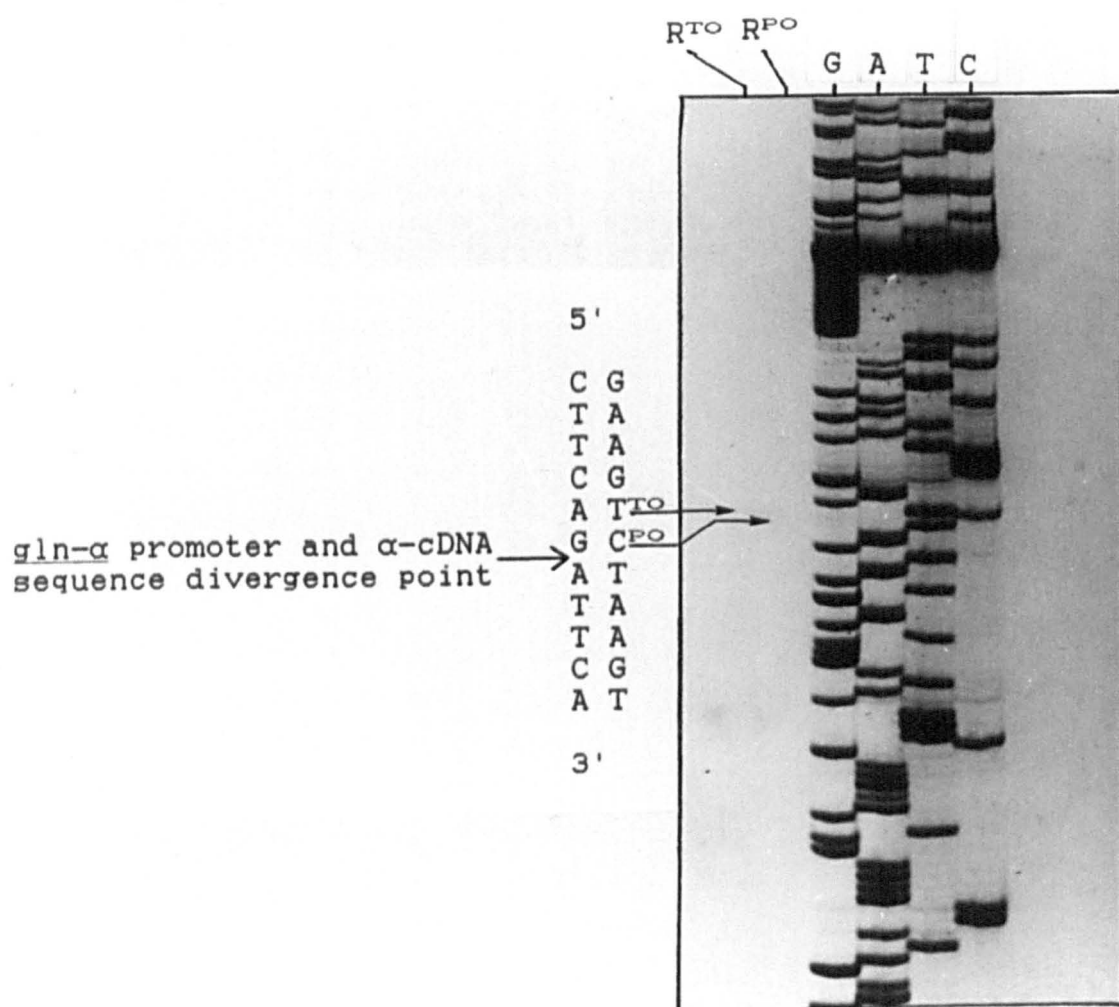


of labelled oligo was hybridised to both 5µg of total RNA extracted from leaves of P. vulgaris seeds germinated for 24hrs and 5µg of polyA<sup>+</sup> RNA extracted from P.vulgaris leaves. Following hybridisation, the DNA was pelleted and redissolved in a suitable buffer and reverse transcriptase was added (see section 2.10.b). Acrylamide gel electrophoresed of primer extension products and sequencing reactions (sequencing template: α-M3, sequencing primer: "α-2") allowed the accurate sizing of the extension products. As the same oligo was used in both the primer extension and sequencing reactions, the transcriptional start was directly read from the resulting autoradiograph (see fig. 3.7). The transcriptional start determined from hybridisation to poly A<sup>+</sup> RNA aligns 1bp 5' to the point of sequence divergence of the α-cDNA and gln-α sequences. The transcriptional start as determined from hybridisation to total leaf RNA aligns 2bp 5' to the point of sequence divergence. Despite both bands being faint, for both RNA samples, only a single band was observable.

3.8.b RNAse protection:

The RNAse protection assay involved an anti-sense probe transcribed from the pgln-α clone linearised with DdeI using T7 RNA polymerase (see section 2.10). The probe transversed the cDNA clone:genomic clone sequence divergence point and the size of the resulting protected fragment should correspond to the gln-α transcriptional start site. The probe was isolated from an acrylamide gel and probe aliquots were hybridised to triplicate 10µg aliquots of total RNA extracted from the leaves of overnight

fig. 3.7. Primer extension analysis to determine the gln- $\alpha$  transcriptional start site ( $R^{TQ}$  = total RNA template,  $R^{PQ}$  = poly A+ template,  $T^{TQ}$  = transcriptional start site using total RNA as the extension template,  $T^{PQ}$  = transcriptional start using poly A+ RNA as the extension template, DNA sequence =  $\alpha$ -cDNA (sequencing template;  $\alpha$ -M3, sequencing primer; " $\alpha$ -2") - note: protected fragments in figs. 3.6 and 3.8 are aligned to the gln- $\alpha$  promoter sequence).

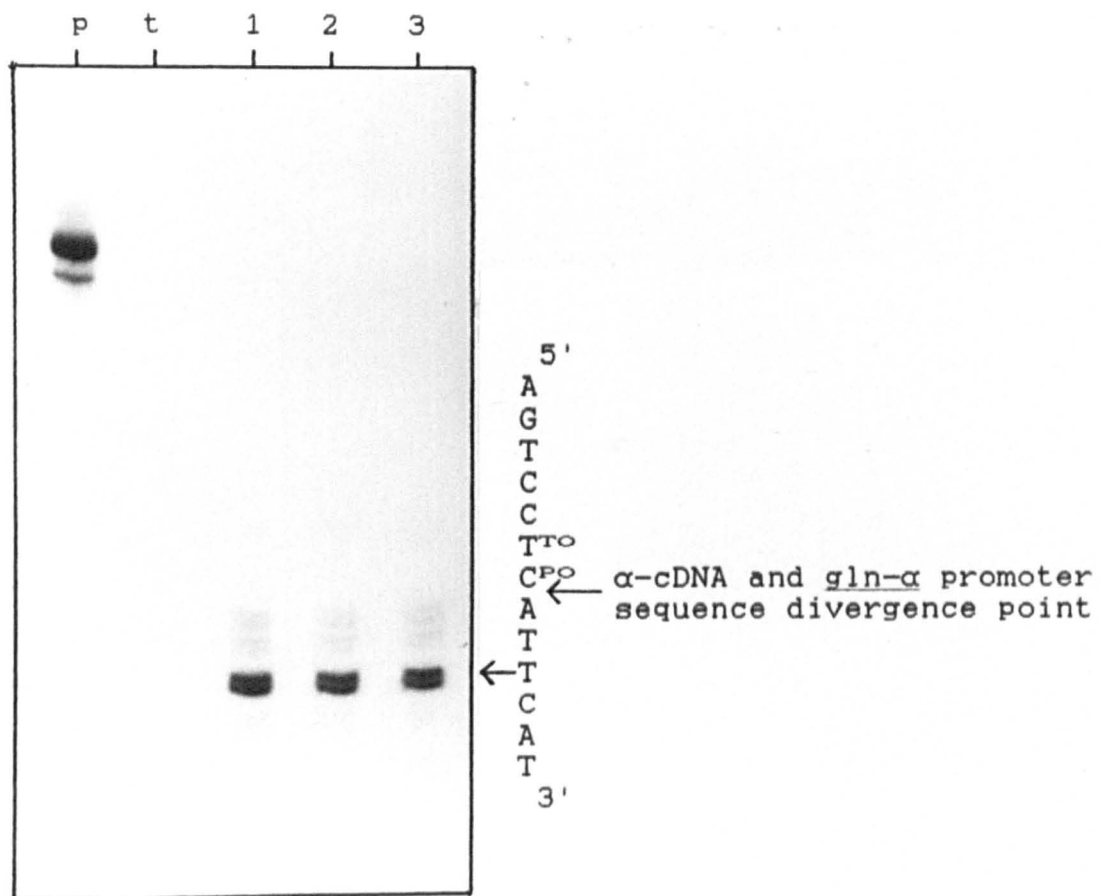


imbibed P. vulgaris seeds. Following hybridisation O/N at 37°C the mix was treated with RNaseA at RT (approximately 25°C). Acrylamide gel electrophoresis of the protected fragments and sequencing reactions (sequencing template: pgln- $\alpha$ , sequencing primer: " $\alpha$ -1") allowed the fragments to be sized. The resulting autoradiograph is shown in fig. 3.8. Track (p) contains the undigested probe, track (t), the yeast tRNA control, and tracks (1) to (3), the total RNA samples. As seen in section 3.7, multiple fragments were seen for each track although the tracks were seemingly identical. Again, the upper, most intense band (arrowed) was considered to be the extent to which the probe had hybridised to the  $\alpha$ -RNA and through calculations it was revealed that the size of this fragment aligned 3bp 3' to the transcriptional start calculated from the primer extension experiment (template: poly A<sup>+</sup> RNA) and 2bp 3' to the point of sequence divergence of the gln- $\alpha$  and  $\alpha$ -cDNA clones.

### 3.9 consensus sequences:

Further study of the gln- $\alpha$  sequence revealed a putative TATA box 28bp upstream from the transcriptional start site (designating the A residue as the start of transcription (see fig 3.5 and below)). The distance between the TATA box and the transcriptional start in the gln- $\alpha$  sequence is in agreement with the studies of Joshi (1987) which found that in the comparative study of 79 plant gene sequences, the majority of plant gene TATA boxes were between 32 +/- 7 nucleotides from the transcriptional start site. The gln- $\alpha$  TATA box and surrounding sequence (TAT TTA

fig. 3.8. RNase protection assay to determine the gln- $\alpha$  transcriptional start (track p = undigested probe, track t = yeast tRNA control, tracks 1, 2 and 3 = total RNA samples extracted from leaves of overnight imbibed P. vulgaris seeds, protected fragments sized by the co-electrophoresis of sequencing reactions (sequencing template; pgln- $\alpha$ , sequencing primer; " $\alpha$ -1") and aligned on the figure to the gln- $\alpha$  promoter sequence, T<sup>o</sup> = transcriptional start site determined using primer extension (template; total RNA), T<sup>o</sup> = transcriptional start determined using primer extension (template; poly A<sup>+</sup> RNA)).



TAA AGG G) however shows little homology (6 mismatches) to the consensus sequence used by Joshi (1987) (TCA CTA TAT ATA G).

The gln- $\alpha$  transcriptional initiation site consensus sequence (TTC ATT C) is significantly homologous (2 mismatches) to that deduced by Joshi (1987) ( $\text{C}^{\text{C}}/\text{TC}$  ATA A) during his comparative study of plant gene sequences. Joshi (1987) encountered varied consensus sequences for different groups of genes, the significance of which was unknown. Interestingly though, Joshi (1987) reported a remarkable conservation of A at the transcriptional initiation site in 85% of the genes studied and the gln- $\alpha$  consensus sequence agrees with this.

The translational initiation site consensus sequence deduced by Joshi (1987) (TAA ACA ATG GCT) showed poor homology to the gln- $\alpha$  translational initiation site (ATC ACC ATG TCT) (5 mismatches). The gln- $\alpha$  translational initiation site showed greater homology to the animal consensus sequence ( $\text{CC}^{\text{C}}/\text{C}$  CCA TG) (1 mismatch). The significance of this is unknown.

Assuming that the 5' 28bp of the  $\alpha$ -cDNA are a cloning artefact, the first AUG codon on the  $\alpha$ -mRNA would act as the translation initiation site. Joshi (1987) found that 92% of the genes he studied were consistent with the "first AUG" rule and that such findings were in agreement with previous studies (Kozak, 1986).

The motifs CACGT (position -103), TGAC (positions -61 and -173) and GACG (position -52) present within the gln- $\alpha$  promoter sequence (see fig. 3.5) are discussed in section 3.10 as possibly

interacting with certain basic/leucine zipper (bZIP) transcriptional activators and being responsible at least in part for the spatial expression pattern of the gln- $\alpha$  promoter.

The element CCTACC(N)<sub>7</sub>CT(N)<sub>4</sub>A (the H-box) has recently been shown to be important in the developmental regulation of a bean CHS gene and its induction by elicitors and other stress stimuli (Yu et al., 1993). The CCTACC motif of the H-box forms part of the consensus sequence present in the promoters of several genes involved in phenylpropanoid metabolism (Lois et al., 1989, Ohl et al., 1990). The CCTACC motif is however absent from the gln- $\alpha$  sequence.



### 3.10 Discussion:

P. vulgaris genomic libraries have previously been screened unsuccessfully for gln- $\alpha$  sequences (Cullimore, unpublished results; Daniell, 1992). However, in this study the cloning of gln- $\alpha$  sequences was successful and may be due to the use of mcr cloning host strains and in-vitro packaging mixes as described in section 3.1.

Two genomic library phage clones were plaque purified and named "a" and "i". PCR was used to amplify the sequences upstream of the translational start site. Although phage clone "a" produced a PCR fragment of approximately 900bp, clone "i" failed to produce a fragment. As discussed earlier, from the Southern blot data available, clone "i" was expected to have produced a PCR fragment approximately 500bp greater in length than that from clone "a". Possible reasons for the absence of PCR product from clone "i" include firstly, that the phage stock was contaminated resulting in the PCR reaction mechanism being significantly inhibited and secondly, a loss of priming sites either through rearrangement of the clone sequence or through mutation.

Phage "a" and "i" clone DNA and P. vulgaris genomic DNA were isolated, restricted and Southern blotted. Two radiolabelled nucleic acid probes synthesised from the  $\alpha$ -cDNA ( $\alpha$ -cDNA:HindIII and  $\alpha$ -cDNA:EcoRI (see fig. 2.1)) were hybridised to the blots and the resulting pattern of hybridisation for both probes was identical. This was surprising when considering that the  $\alpha$ -

cDNA:EcoRI probe traverses two further HindIII sites than does the  $\alpha$ -cDNA:HindIII. Furthermore, Gebhardt et al. (1986) probed P. vulgaris genomic DNA with a probe synthesised from the entire  $\alpha$ -cDNA and at high stringency, the hybridisation pattern was identical to that seen for both cDNA probes used in this study. The probe used by Gebhardt et al. (1986) transverses an EcoRI site, a BamHI site and an HindIII site above those traversed by the  $\alpha$ -cDNA:EcoRI probe and would therefore be expected to hybridise to further EcoRI, BamHI and HindIII fragments. A possible reason for this apparent identicality may be that the missing hybridising fragments were relatively small and were run off the bottom of the gel or diffused from the gel before transfer or were inefficiently transferred to the nylon membrane.

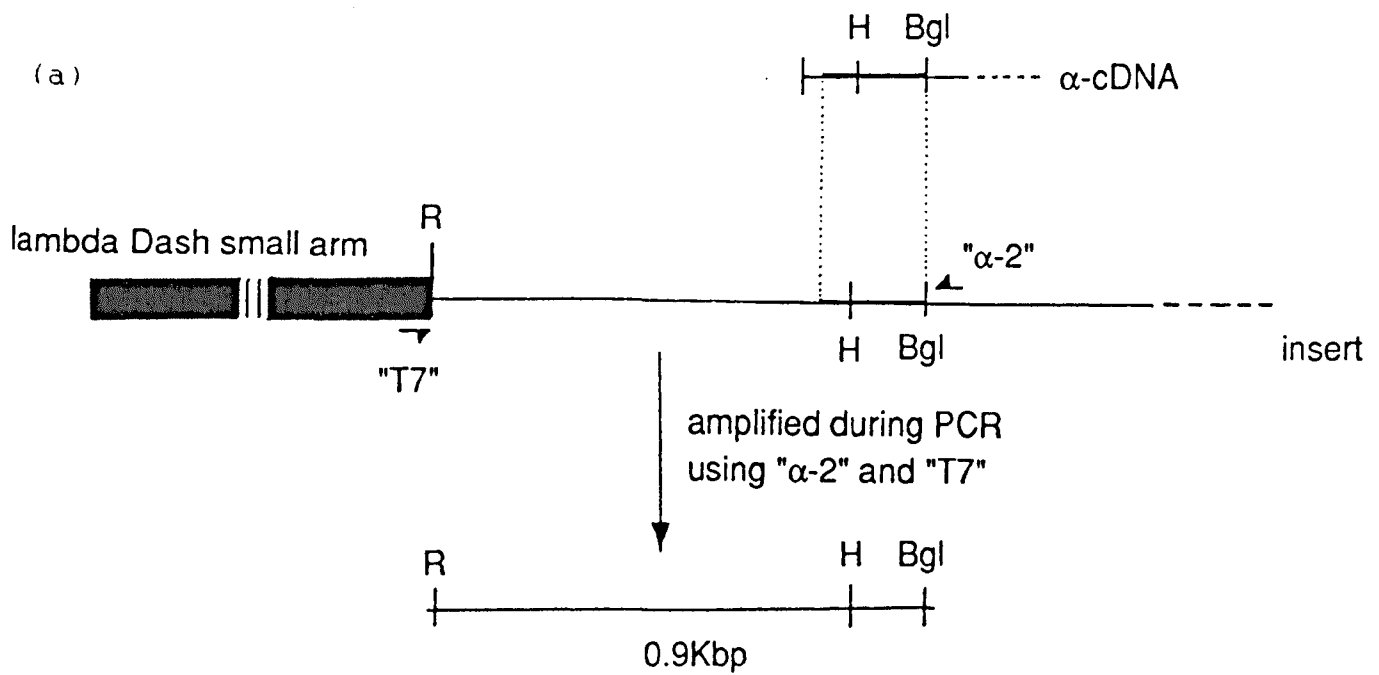
During the primary screen of the P. vulgaris genomic library, the  $\alpha$ -cDNA:EcoRI probe only hybridised to those six positives hybridised to by the  $\alpha$ -cDNA:HindIII probe and gives further indication that the hybridisation pattern of the two cDNA probes were seemingly independent of probe size.

The specific PCR primer used to isolate the upstream region from phage clone "a" primed 3' to the  $\alpha$ -cDNA BglIII site (see fig. 2.1) and the resulting PCR fragment was sub-cloned using this restriction enzyme site along with the EcoRI site derived from the lambda Dash multiple cloning site. The sequence of the 3' end of this gln- $\alpha$  clone was identical to that of the  $\alpha$ -cDNA upstream of the BglIII site and up to the point of  $\alpha$ -genomic clone: $\alpha$ -cDNA sequence divergence 28bp from the 5' end of the  $\alpha$ -cDNA.

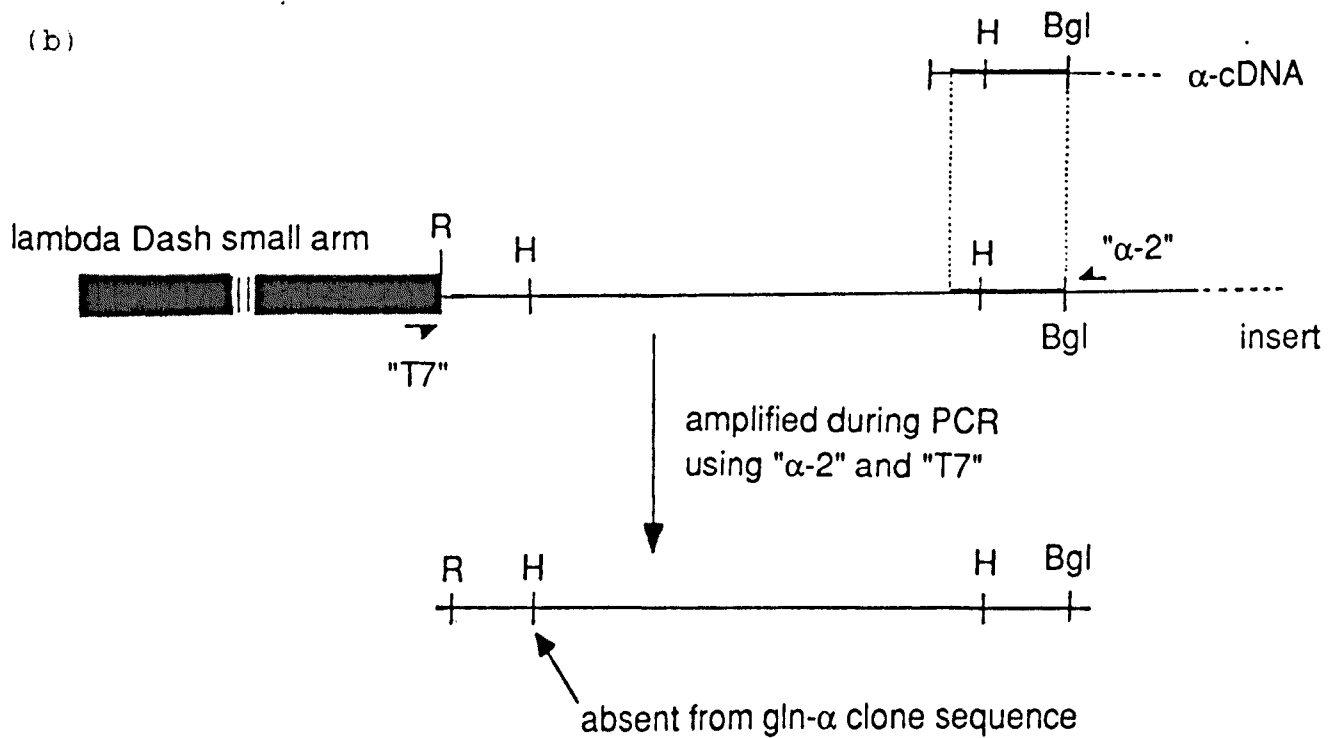
Sequencing of the gln- $\alpha$  clone revealed that the only HindIII site present within the upstream region aligned to the 5' HindIII site of the  $\alpha$ -cDNA (see fig. 3.9.a). Together, this information suggested that the hybridisation pattern of the gln- $\alpha$  and  $\alpha$ -cDNA:HindIII probes to the HindIII digests of clone "a" DNA and P. vulgaris genomic DNA would be identical. Although the hybridisation patterns of both probes to P. vulgaris genomic DNA were identical, the hybridisation patterns to phage clone "a" DNA were different. The gln- $\alpha$  probe hybridised to a HindIII fragment approximately 10.0Kbp in size and the  $\alpha$ -cDNA:HindIII probe hybridised to a fragment approximately 2.2Kbp in size (see figs 3.1 and 3.3). When considering possible restriction maps for the phage clone "a" insert, the hybridisation pattern of the gln- $\alpha$  probe is in agreement with subsequent sequencing data while the hybridisation pattern of the  $\alpha$ -cDNA:HindIII probe is not. The PCR primers used were designed to amplify the phage clone sequences upstream of the translational start site up to lambda Dash arm (see fig 3.9.a). As the HindIII site within the lambda Dash multiple cloning site was lost during linearisation of the lambda arms, probing of HindIII restricted clone "a" DNA would hybridise to a gln- $\alpha$  gene fragment connected to a lambda arm and this was indeed seen when using the gln- $\alpha$  probe. The size of the hybridising HindIII fragment was approximately 10Kbp and limited the gln- $\alpha$  fragment to be connected to the small 9.0Kbp lambda arm (the other vector arm is 20Kbp in length). Indeed, the generic primer "T7" used to amplify the gln- $\alpha$  fragment is

fig. 3.9. Possible restriction enzyme maps for the phage clone "a" insert based on data from (a) the hybridisation pattern of the gln- $\alpha$  probe to clone "a" DNA and (b) the hybridisation pattern of the  $\alpha$ -cDNA:HindIII probe to clone "a" DNA. R = EcoRI, H= HindIII, Bgl = BglII, " $\alpha$ -2" and "T7" = synthetic oligonucleotides, maps are not to scale.

(a)



(b)



homologous to the T7 RNA polymerase initiation site which was situated on the small 9.0Kbp arm. A restriction map based on this data is shown in fig. 3.9.a.

The 2.2Kbp clone "a" HindIII fragment hybridised to by the  $\alpha$ -cDNA:HindIII probe implied that this fragment is restricted from an internal region of the phage insert. The amplified gln- $\alpha$  fragment would have therefore have been expected to have contained at least a second HindIII site upstream of that HindIII site aligning to the 5'  $\alpha$ -cDNA HindIII site (see fig. 3.9.b). Sequencing of the resulting gln- $\alpha$  clone however showed no second HindIII restriction enzyme site to be present and the data therefore seemingly contradicts itself.

The hybridisation pattern of the  $\alpha$ -cDNA:HindIII probe to phage clone "a" DNA seemingly contradicts itself in another way. The gln- $\alpha$  probe was produced by oligolabelling the the gln- $\alpha$  clone. The sequence at the 3' end of the gln- $\alpha$  clone and the sequence of the 5' end the  $\alpha$ -cDNA were shown to be identical with the exception of the terminal  $\alpha$ -cDNA 28bp (see section 3.6). Thus considering that the gln- $\alpha$  clone was approximately 900bp in length, both the gln- $\alpha$  probe and the  $\alpha$ -cDNA:HindIII probe (transcribed from the 5' end of the  $\alpha$ -cDNA) were expected to hybridise to a HindIII/EcoRI clone "a" fragment of approximately 900bp. Although this was the case for the gln- $\alpha$  probe, it was not the case for  $\alpha$ -cDNA:HindIII probe which hybridised to HindIII/EcoRI of approximately 2.0Kbp in size (see fig. 3.1).

Overall therefore, the hybridisation pattern of the  $\alpha$ -

cDNA:HindIII to clone "a" DNA does not agree with data from subsequent experiments on the structure of the clone "a" insert. A feature of hybridisation pattern of the  $\alpha$ -cDNA:HindIII probe to clone "a" DNA was the presence of several smaller hybridising fragments (see fig. 3.1) and it is possible therefore that the digestion of the clone "a" DNA was partial and this may explain why the hybridising HindIII/EcoRI fragment was approximately 2.0Kbp and not 900bp. Partial digestion of clone "a" DNA does not however provide a possible explanation of why the  $\alpha$ -cDNA:HindIII probe hybridised to a phage clone "a" HindIII fragment of approximately 2.2Kbp while the gln- $\alpha$  probe hybridised to a phage clone "a" HindIII fragment of approximately 10.0Kbp. Further experimental investigation is therefore required.

Experimental data obtained during the characterisation of the gln- $\alpha$  gene suggests that the 5' 28bp of the  $\alpha$ -cDNA are a cloning artefact. This was however only one of several possible reasons to explain why the gln- $\alpha$  and  $\alpha$ -cDNA sequences diverged 28bp from the 5' end of the  $\alpha$ -cDNA. Initial studies using an RNase protection assay (see section 3.7) suggested that the 5' 28bp were not represented in the  $\alpha$ -RNA from the P. vulgaris cultivar Tendergreen. The genomic DNA used in the construction of the genomic library as isolated from the roots of cultivar Tendergreen plants. The  $\alpha$ -cDNA used to isolate the gln- $\alpha$  clone and to which its sequence was compared, was isolated from the plants of cultivar Bush Blue Lake (Gebhardt, 1986) and it was therefore possible that the 5' 28bp are represented in the Bush

Blue Lake  $\alpha$ -mRNA but are not represented in the Tendergreen  $\alpha$ -mRNA. Subsequent studies however mapped the gln- $\alpha$  transcriptional start site to the point of divergence of the gln- $\alpha$  and  $\alpha$ -cDNA sequences. Possible inaccuracies were present in both the techniques used to map the transcriptional start site. Multiple protected fragments were observed for the RNase protection assay (see fig. 3.8) and it was assumed that the upper, most intense band corresponded to the limit of the hybridisation of the probe to the  $\alpha$ -mRNA. The reverse transcriptase enzyme during primer extension may not have transcribed to the end of the  $\alpha$ -mRNA or may have continued beyond the end of the mRNA molecule and may have possibly formed hair pin loops. Under either circumstance, the extension products would have inaccurately mapped the transcriptional start site. Indeed, a discrepancy of 1bp was observed between total leaf RNA and leaf poly A<sup>+</sup> RNA (see section 3.8.a). Overall though, the data indicates that the transcriptional start site aligns closely to the sequence divergence point and that the 5' 28bp of the  $\alpha$ -cDNA are a cloning artefact. This indication had further ramifications. Firstly, the irregular occurrence of a second start codon in the  $\alpha$ -cDNA was dismissed as it was located within those 5' 28bp. This meant that the  $\alpha$ -RNA molecule complied with the studies of Kosak (1986) and Joshi (1987) who found that in over 90% of genes they studied, the first AUG of the RNA molecule acted as the translational initiation site. Secondly, the occurrence of A at the gln- $\alpha$  transcriptional start site was significant as Joshi



(1987) found that A was at the transcriptional start site in 85% of the plant genes he studied.

The primer extension technique was also limited by the relatively low levels of  $\alpha$ -RNA in 5 $\mu$ g of total RNA extracted from the leaves of P. vulgaris seeds germinated for 24hrs and 5 $\mu$ g of poly A<sup>+</sup> RNA extracted from P. vulgaris leaves. Although the extension products were clearly visible, they were also very weak

Transcriptional regulation of gene expression is mediated by the action of sequence-specific transcriptional factors on regulatory sequences contained within gene promoters. One group of transcriptional factor is defined by basis/leucine zipper (bZIP) motif. The bZIP factor TGA1a from pea and tobacco interacts with the 21bp activation sequence (as)-1 (-83 to -63) of the CaMV 35S enhancer (Lam et al., 1989). This factor also binds to several other DNA sequence elements including the nopaline and octapine synthase genes (the nos and ocs elements, respectively), and the wheat histone 3 promoter (the hex element) (Bouchez et al., 1989; Katagari et al., 1989). The as-1 element has been shown to be responsible for the root-specific expression of the 35S promoter (Lam et al., 1989) and both the as-1 element and the ocs element have been shown to direct uidA reporter gene expression in the root-tips of transgenic plants (Benfey et al., 1989; Fromm et al., 1989). The tandemly repeated TGACG motifs of the as-1 element have been shown to be critical in the binding of TGA1a to the element. The TGAC motifs of the gln-g promoter (position -61 and -173; see fig. 3.5) and the GACG motif (position

-52) of the gln- $\alpha$  promoter although not complete, may be involved in the binding of TGA1a or a related factor and may partly or wholly be responsible for the observed expression of the gln- $\alpha$  promoter in root-tips (see sections 4.3 and 4.4).

Recently, a 22bp element of a pea lectin promoter conferring seed-specific expression contains essential TGAC-like motifs (Pater et al., 1993). Studies suggested a TGA1a-like bZIP protein seed specific factor with a binding specificity similar to that of TGA1a may be involved in the seed-specific expression of the lectin promoter. Furthermore, seed-specific expression has been observed with a reporter gene construct containing a G-box related motif derived from abscisic acid-inducible rice promoters (Salinas et al., 1992). The CACGT motif of the gln- $\alpha$  promoter (position -102 (see fig. 3.5)) is the core sequence of the G-box element (CCACGTGG) and overall therefore, the seed-specific expression of the gln- $\alpha$  promoter (see sections 4.7 and 4.8 and Swarup et al., 1990) may be mediated either individually or co-operatively by the CACGT and TGAC motifs of the gln- $\alpha$  promoter.

It should be noted that the G-box element (CCACGTGG) encompasses the TGACGT sequence found in the as-1, ocs, nos and hex elements and therefore the ACGT core sequence present within the gln- $\alpha$  promoter G-box-like motif may also be involved in the binding of TGA1a or a related bZIP protein. Therefore, the interaction of bZIP proteins to the gln- $\alpha$  promoter may not be clear-cut as outlined above.

Overall therefore, the sequence motifs of the gln- $\alpha$  promoter discussed above may be responsible for certain aspects of the spatial expression pattern of the gln- $\alpha$  promoter observed in P. vulgaris plants and in transgenic plants.

### 3.11 conclusion:

902bp of upstream region of the gln- $\alpha$  gene was cloned from a P. vulgaris genomic library and sequenced. The gln- $\alpha$  clone and  $\alpha$ -cDNA sequences diverged 28bp from the 5' end of the cDNA. An RNase protection assay indicated that these 28 cDNA bp were not represented in the cultivar Tendergreen  $\alpha$ -mRNA. The transcriptional start was mapped using both primer extension and RNase protection to the point of sequence divergence of the cDNA clone and the genomic clone. Together with data relating to gene consensus sequences, the data overall tended to suggest that the 5' cDNA 28bp were in fact a cloning artefact. The gln- $\alpha$  sequence possesses a putative TATA box consensus sequence 28bp upstream of the transcriptional start site. Sequence data is summarised on fig. 3.5.

Chapter 4: Results - Activity of the gln- $\alpha$  promoter in transgenic  
Nicotiana tabacum (tobacco).

#### 4.1 Introduction;

The development of the Agrobacterium tumefaciens Ti plasmid as a vector for the transformation of plants has provided a method for the analysis of the expression of both homologous and heterologous genes in plant cells. The system has been used extensively to analyse the functional sequences of plant gene promoters involved in tissue-specificity of expression with respect to developmental and environmental cues (for example; Miao et al., 1991). Usually, the promoter is fused to a reporter gene in-vitro which allows the accurate measurement of promoter activity in-vivo. Current transgenic plant studies almost exclusively use the E. coli  $\beta$ -glucuronidase reporter gene system.  $\beta$ -glucuronidase is encoded by the uidA locus and as many plants assayed to date lack detectable  $\beta$ -glucuronidase activity, the sensitivity of the system is very much more than that of other reporter-gene systems (Jefferson et al., 1986). Expression of uidA can be measured accurately using fluorimetric assays of very small quantities of transgenic plant tissue and histochemical analysis can be used to study the localisation of uidA gene activity in transformed cells and tissues (Jefferson et al., 1987).

Other transgenic plant studies have focussed on the elucidation of those portions of transit peptides responsible for the proper compartmentalisation of proteins within the plant cell (for example; Schreier et al., 1985). Others have focussed on the elucidation of those regions of the promoter responsible

for the light-regulation of certain genes (for example; Simpson et al., 1985). Finally, other studies have centered around crop improvements, namely the transformation of crop plants with genes that render that plant resistant to specific herbicides (for example; Comai et al., 1985) or viruses (for example Abel et al., 1986).

Because of uncontrollable properties of the A. tumefaciens transformation system, the data from this type of study requires careful interpretation. The site of integration of the chimeric gene has been shown to be random (Chyi et al., 1986) and, combined possibly with other factors, leads to a high degree of quantitative variability between different transgenic plant lines. This "between line variability" has been reported for the majority of transgenic plant studies (for example, Jones et al., 1985).

The gln- $\alpha$  polypeptide is the major GS enzyme component in plumules (Cock et al., 1991), radicles (Ortega et al., 1986) and cotyledons (Swarup et al., 1989) of P. vulgaris dry seeds. Upon seed germination, expression levels decrease and several days after germination, expression in all three plant organs is undetectable. The gln- $\alpha$  gene is also expressed in response to the mechanical wounding of P. vulgaris hypocotyls (Daniell, 1992) and the bacterial infection of P. vulgaris leaves (Daniell, 1992). The purpose of this study was therefore to study gln- $\alpha$  promoter activity in transgenic tobacco plants in response to the developmental and environmental cues discussed above.

#### 4.2 tobacco transformation;

The gln- $\alpha$  translational start site and surrounding sequence (5' CCA TGT 3') was mutated using PCR to an NcoI restriction enzyme recognition site (5' CCA TGG 3'). The primers used were the mismatched " $\alpha$ -4" 23mer anti-sense strand primer and the "SP6" 19mer sense-strand primer (see section 2.12). The PCR product was agarose gel isolated, restricted with EcoRI and NcoI and re-isolated from an agarose gel. The restriction fragment was then subcloned into a plasmid vector containing the uidA gene and the ocs terminator (pSLJ4D4) (see section 2.12). The fusion site between the gln- $\alpha$  and uidA genes was checked to be in-frame by sequencing across the fusion site. The sequence is shown in fig. 4.1 and shows that the fusion was in-frame and that the  $\beta$ -glucuronidase enzyme had a small N-terminal extension of 5 amino acids. Studies have shown that  $\beta$ -glucuronidase can tolerate large N-terminal extensions without any loss in enzymatic activity (Jefferson et al., 1986) and this small extension was not envisaged to be a problem. The gln- $\alpha$ ::uidA fusion was then subcloned into the multiple cloning site of the plant transformation vector Bin19. The Bin19 vector contains two T-DNA border repeats (left and right) which flank both the multiple cloning site and a chimeric nopaline synthase::neomycin phosphotransferase (nos::nptII) gene which confers plant cell resistance to the toxic antibiotic kanamycin and was used as a dominant selectable marker. The resulting vector and those containing two control constructs, 35S::uidA (pBI121.1) and

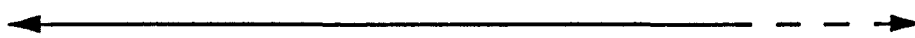
fig. 4.1. DNA sequence of the fusion site between the gln- $\alpha$  gene promoter and the coding sequence of the E. coli uidA gene (ATG = gln- $\alpha$  translational start, ATG = uidA translational start).



*NcoI*

ATC ACC ATG GTT CAG TCC CTT ATG TTA CGT

met gly gln ser leu met leu pro



*uidA*

promoterless uidA (pBI101.1) (Jefferson et al., 1987), were introduced independently into tobacco plants via Agrobacterium tumefaciens-mediated transformation. Plants were transformed using the leaf disc method of Horsch et al. (1984). Shoots were generated from wounded leaf disc tissue plated on shoot induction media containing kanamycin. Shoots were excised from the discs and placed on root induction media containing kanamycin. Shoots were designated both a number (corresponding to the leaf disc from which they were excised) and letter (for when more than one shoot was excised from a single disc, for example, shoots 6A and 6B were both excised from leaf disc number 6). Twenty individually transformed lines were obtained for the gln- $\alpha$ ::uidA construct, twenty four lines for the 35S::uidA construct and eleven lines for the promoterless uidA construct. Plants expressing the uidA gene were morphologically normal, healthy and fertile. Transformed plants were propagated vegetatively in magenta pots on MS media containing kanamycin (referred to as "in-vitro" maintained plants) and also grown in greenhouses in compost and watered with tap water.

#### 4.3 measurement of the level of extractable GUS activity of root-tips of transgenic plants;

GS has been localised in the root tips of P. vulgaris plants using immunocytochemistry (Datta et al., 1991) and a possible explanation of the results of Ortega et al. (1986) is that gln- $\alpha$  gene expression is confined mainly to the root-tip. This root-tip expression was confirmed by preliminary histochemical studies

on the roots of gln- $\alpha$ ::uidA transgenic plants.

Three 0.5cm root tips were cut from each in-vitro maintained transgenic plant line, combined and assayed for extractable GUS activity and soluble protein content. Despite arguments against using soluble protein as the activity denominator when comparing gusA activities of different tissues because of the differences in protein content of different cell-types (Jefferson et al., 1987), the alternative of using DNA content as the denominator is equally unsatisfactory owing to differences in chromosome ploidy that are a common feature of differentiated plant cells (Bryant, 1976). As the same tissues were being analysed and compared, protein content was considered to be the most satisfactory activity denominator.

The 20 gln- $\alpha$ ::uidA lines showed an average GUS activity of 5800 pmol/min/mg soluble protein (see fig. 4.2) and the 24 35S::uidA lines showed an average GUS activity of 6500 . pmol/min/mg soluble protein (see fig. 4.3). The 11 transgenic promoterless uidA plant lines showed approximately 36-fold and 41-fold lower levels of extractable GUS activity as compared to gln- $\alpha$ ::uidA root tips and 35S::uidA root tips respectively (see fig. 4.4) (average activity: 165 pmol/min/mg soluble protein). A striking feature of figs. 4.2 and 4.3 is the high degree of variability in the level of GUS activity between the individual transformants of the transgenic gln- $\alpha$ ::uidA and 35S::uidA lines (up to 100 fold).

#### 4.4 histochemical analysis of roots of transgenic plants:

fig. 4.2. Extractable GUS activity from the root-tips of 20 individual transgenic gln- $\alpha$ ::uidA tobacco plant lines.

GUS activity (pmol/min/mg soluble protein)

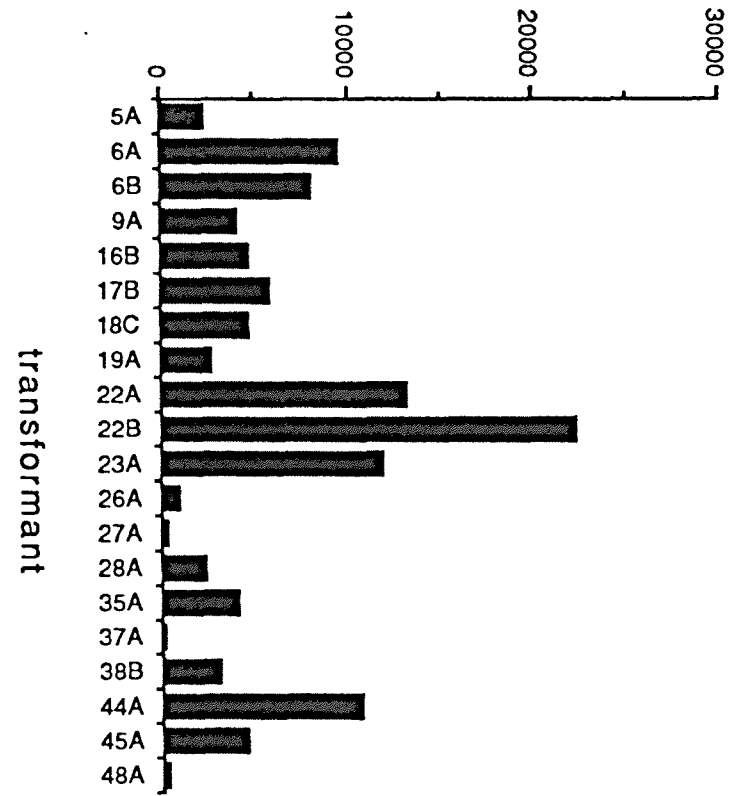


fig. 4.3. Extractable GUS activity from the root-tips of 25 individual transgenic 35S::uidA tobacco plant lines.

GUS activity (pmol/min/mg soluble protein)

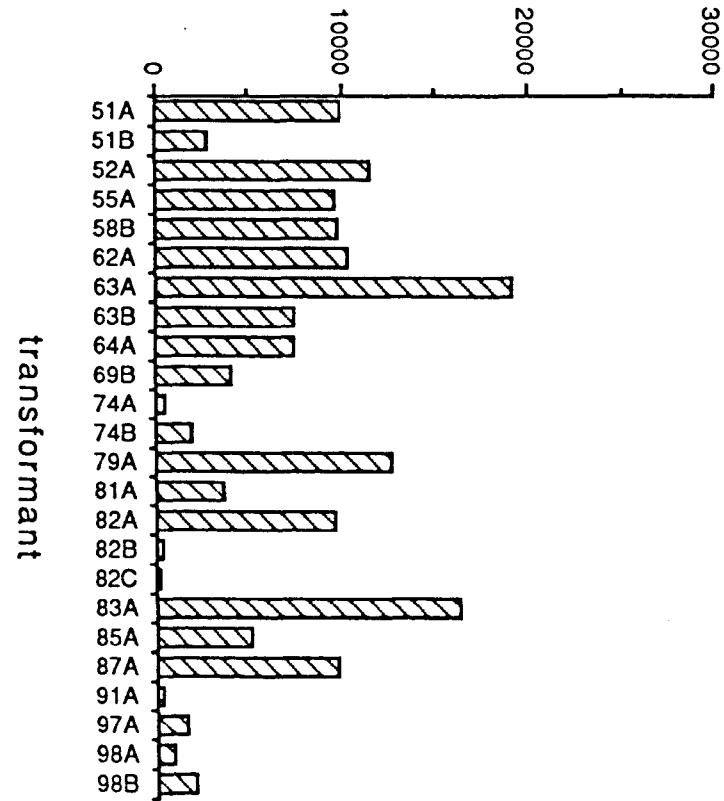
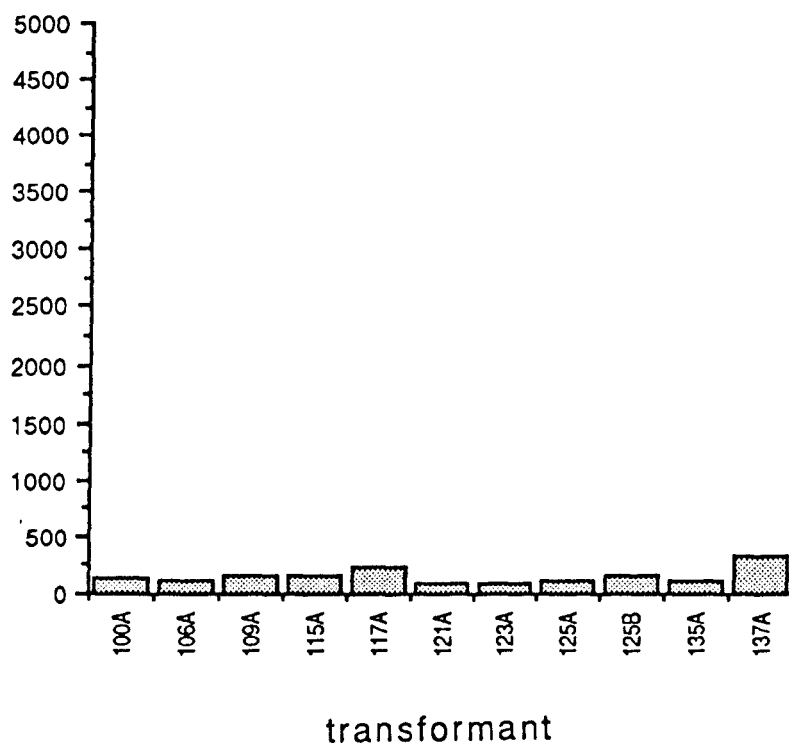


fig. 4.4. Extractable GUS activity from the root-tips of 12 individual transgenic promoterless::uidA tobacco plant lines.



GUS activity (pmol/min/mg soluble protein)



Root samples from each in-vitro maintained transgenic plant line were incubated, without prior fixing, with the histochemical substrate X-gluc O/N at 37°C (see section 2.15). The sites of GUS activity were revealed by the formation of an insoluble indigo-blue product.

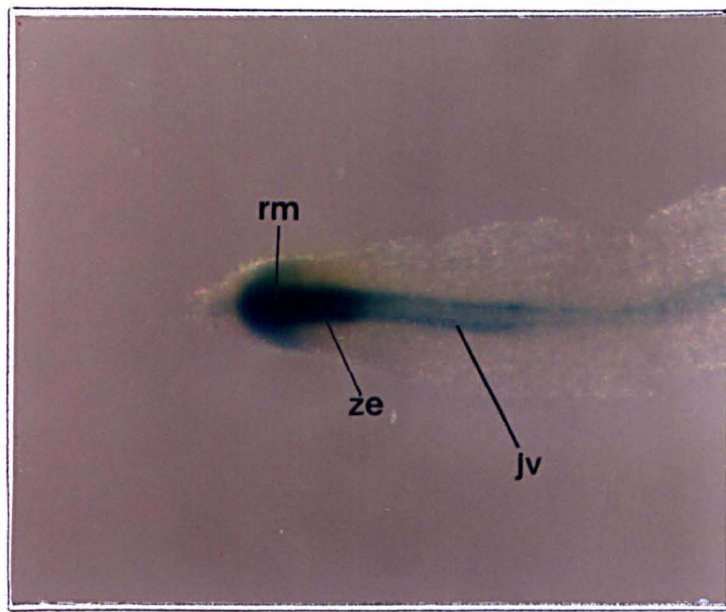
The cell-specific distribution of GUS activity in the root-tips of the 20 transformed gln- $\alpha$ ::uidA plant lines were grouped into three patterns; "central" (lines; 5A, 16B, 28A and 45A), "tip" (lines; 17B, 22A and 22B) and "epidermal" (lines; 6A, 6B, 9A, 18C, 19A, 23A, 35A, 37A, 38B and 44A). In the root-tips showing "central" staining, root-tip meristematic tissues and the zone of cell-elongation stained blue (for example, see fig. 4.5.a). In the root tips showing "tip" staining, cells of the root cap stained blue (for example, see fig 4.5.b) and those root-tips showing "epidermal" staining, root-tip epidermal cells stained blue (for example, see fig 4.5.c). Three transformed lines (26A, 27A and 48A) showed no cell-localised chimeric gene activity and upon reference to fig. 4.2 it was seen that these lines showed low extractable GUS activity.

In order to limit the differences in chimeric gene expression, attempts were made to analyse the roots at a defined developmental stage. All plants were subcultured at the same time and the roots were sampled only when they neared the sides of the magenta pot.

It was noted that the diversity in expression pattern between lines was not due to transient fluctuations in

fig. 4.5. Histochemical localisation of GUS activity in the root tip tissues of transgenic gln- $\alpha$ ::uidA tobacco plants ((a) = "central" (line 16B), (b) = "tip" (line 22B), (c) = "epidermal" (line 23A), rc = root cap, rm = root meristem, ze = zone of cell elongation, jv = juvenile vascular tissue) X40 magnification.

(a)



(b)



(c)

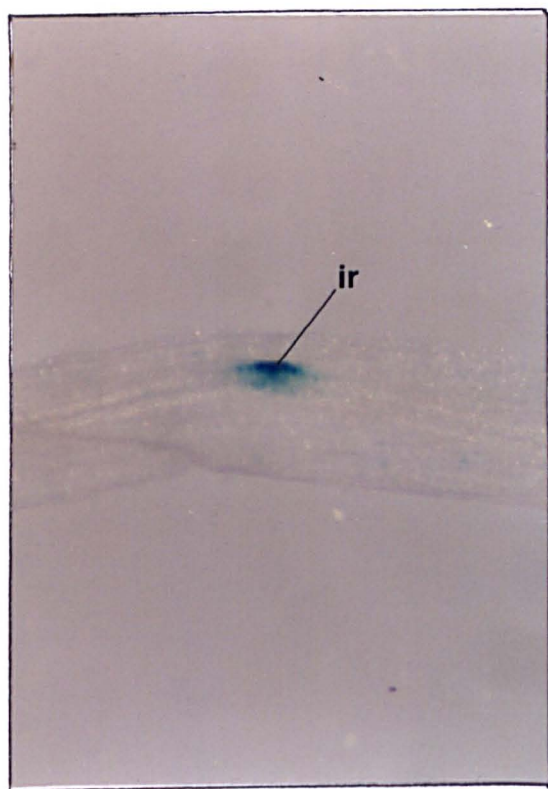


physiological conditions as tissue re-sampled for histochemical staining and sectioning showed patterns of dye deposition identical to those observed previously. Furthermore, all roots sampled from each transgenic plant displayed identical spatial patterns of uidA expression. If physiological conditions were a cause for the observed variation in the spatial pattern of expression between different plant lines, then it would have been expected that different roots sampled from the same plant would vary in the pattern of root-tip chimeric gene expression. Different roots sampled from the same plant did vary considerably in the intensity of stain but the actual pattern of chimeric gene expression was the same. Copy number studies showed lines 22A and 22B to be clonal and both lines showed identical patterns of chimeric gene activity.

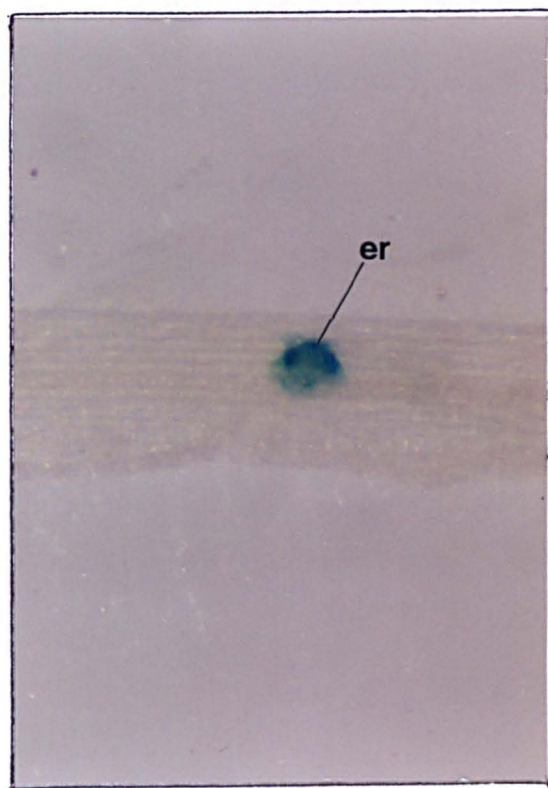
The majority of roots of gln- $\alpha$ ::uidA transformed plants (exceptions; 48A and 27A) showed gln- $\alpha$  promoter activity associated with the sites of lateral root initiation and in emerging lateral roots (see fig. 4.6). Preliminary experiments indicated that gln- $\alpha$  promoter activity is confined to the emerging lateral root outer cortex (data not shown). A proportion of those transformed roots showed uidA expression in those epidermal cells damaged by the emergence of the lateral root (lines; 6A, 6B, 17B, 19A, 22A, 35A and 38B) (see fig. 4.7). Promoter activity was also associated with the vascular tissue of several gln- $\alpha$ ::uidA plant lines (lines; 16B, 17B, 18C, 19A, 22A, 22B, 35A, 44A, 45A) (see fig. 4.5) and root hair GUS activity was

fig. 4.6. Histochemical localisation of GUS activity in lateral root initiation sites and emerging lateral roots of transgenic gln- $\alpha$ ::uidA tobacco plants ((a) to (d) = increasing development stage of lateral root growth, ir = incipient root, er = emerging root) X40 magnification.

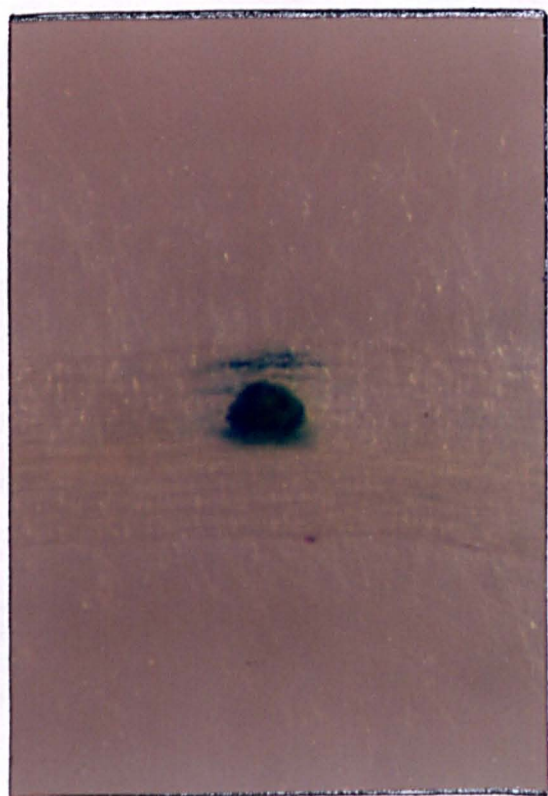
(a)



(b)



(c)



(d)

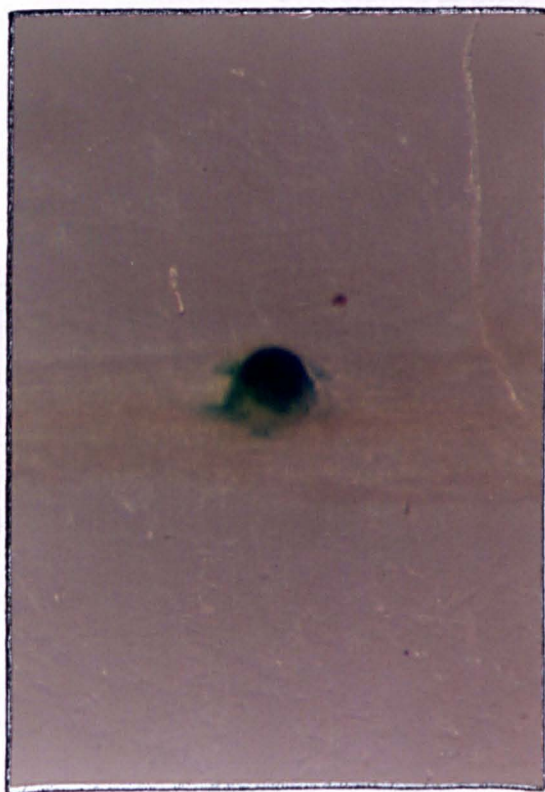
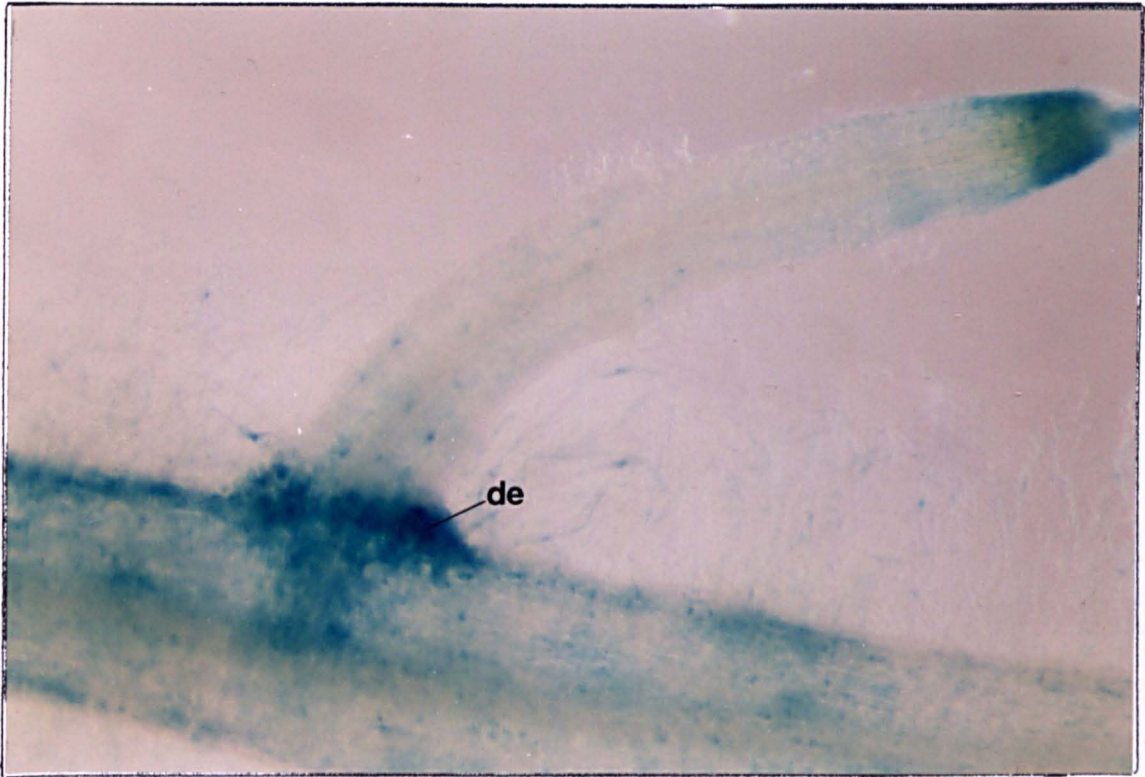


fig. 4.7. Histochemical localisation of GUS activity in those root epidermal cells damaged during lateral root emergence in a transgenic gln- $\alpha$ ::uidA tobacco plant (line 35A) (de = damaged epidermal cells) X40 magnification.





observed on the roots of several transgenic gln- $\alpha$ ::uidA plant lines (6A, 6B, 16B, 17B, 18C, 22A, 22B, 35A and 44A) (see fig. 4.8)

The 35S::uidA transgenic plants displayed a strictly non-uniform distribution of root-tip chimeric gene activity (see fig. 4.9). Intense staining was seen within the vascular tissue and within areas of the root tip. It has been proposed that differences in staining intensity may be due to differences in metabolic activity and size of different types of cell (Jefferson et al., 1987). No staining was seen within the root hairs, lateral root initiation sites nor in the emerging lateral roots. These differences in the spatial-pattern of gln- $\alpha$  and 35S promoter activity indicated that the uidA expression in the roots of gln- $\alpha$ ::uidA plants was due to the regulatory properties of the gln- $\alpha$  promoter. Differences in staining intensity in gln- $\alpha$ ::uidA roots was considered therefore not to be due to differences in cell size and metabolic activity or due to differences in the degree of penetration of the histochemical substrate.

Finally, as expected from the fluorimetric analysis of extractable GUS expression, none of the roots of promoterless ::uidA plant lines exhibited histochemical staining to any degree.

#### 4.5 measurement of the level of extractable GUS activity of leaves of transgenic plants:

Cock et al. (1991) studied extensively the differential expression of the GS genes of P.vulgaris during leaf development.

fig. 4.8. Histochemical localisation of GUS activity in the root hairs of a transgenic gln- $\alpha$ ::uidA tobacco plant (line 35A) (rh = root hairs) X40 magnification.

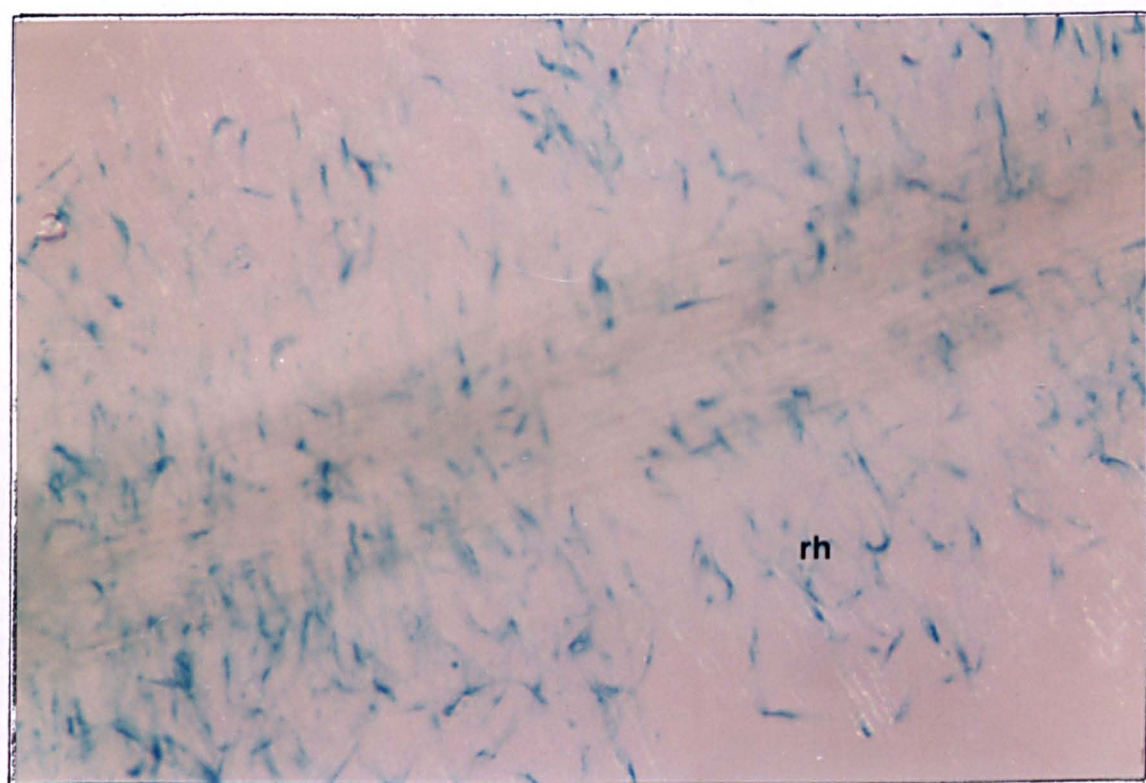


fig. 4.9. Histochemical localisation of GUS activity in the roots of a transgenic 35S::uidA tobacco plant (line 82A) (rt = root tip, vt = vascular tissue) X10 magnification.



They determined that in the dry seed plumule, the  $\alpha$ -mRNA was the only GS mRNA present and after seed germination, the abundance of the  $\alpha$ -mRNA increased to a maximum at day 2, decreased, and was undetectable after day 4 (Cock et al., 1991).

In an attempt to minimise the variability of extractable GUS activity between transgenic lines, the leaves sampled were of approximately the same size and developmental stage. The leaves sampled were all approximately between 4 to 5 cm in length and were the youngest and therefore the most apical leaves that were between those two approximate limits in length. Only one leaf was sampled from each plant and the entire leaf was ground in a mortar and pestle to homogeny and a sample of this used to assay gusA activity and soluble protein content.

The 20 gln- $\alpha$ ::uidA transgenic plant lines had an average leaf GUS activity of 540 pmole/min/mg of soluble protein (see fig. 4.10) but it was apparent that gln- $\alpha$ ::uidA line 9A had an abnormally high leaf GUS activity (7745 pmol/min/mg soluble protein). The remaining 19 gln- $\alpha$ ::uidA lines have an average leaf GUS activity of only 160 pmol/min/mg soluble protein. The 24 35S::uidA transgenic lines had an average GUS leaf activity of 10,030 pmol/min/mg (see fig. 4.11) and the promoterless uidA transgenic lines had an average of 18 pmole/min/mg (see fig. 4.12). The leaf activities of the gln- $\alpha$ ::uidA and 35S::uidA transgenic lines both showed the high degree of variability as experienced with the root-tip GUS activity (see figs. 4.10 and 4.11 respectively).

fig. 4.10. Extractable GUS activity from the leaves of 20 individual transgenic gln- $\alpha$ ::uidA tobacco plant lines.



GUS activity (pmol/min/mg soluble protein)

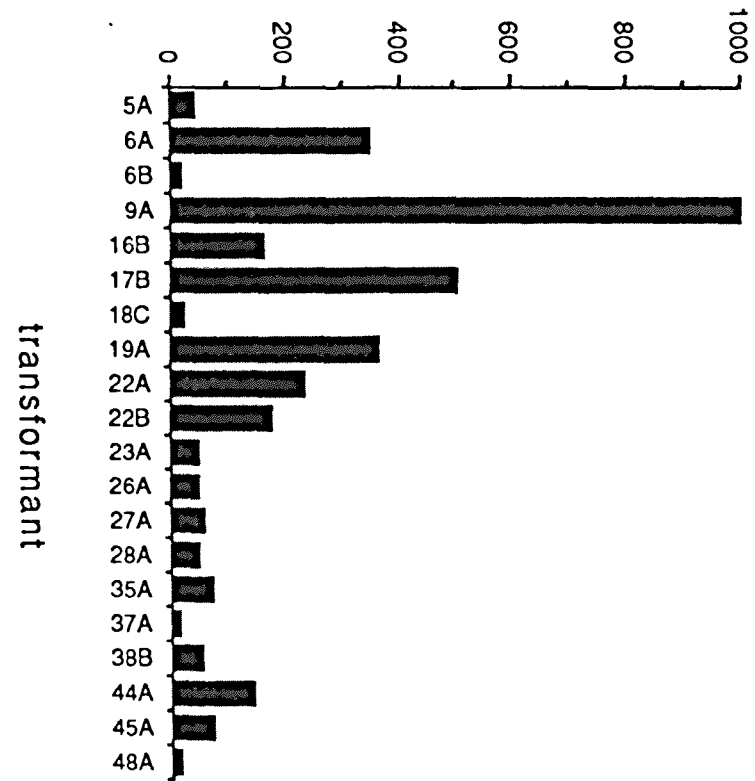


fig. 4.11. Extractable GUS activity from the leaves of 25 individual transgenic 35S::uidA tobacco plant lines.

GUS activity (pmol/min/mg soluble protein)

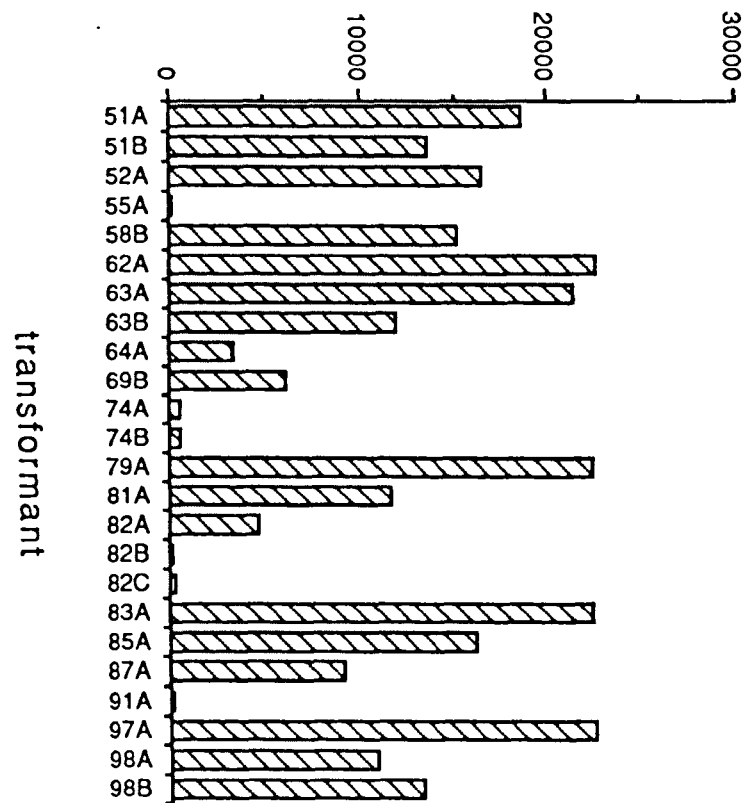
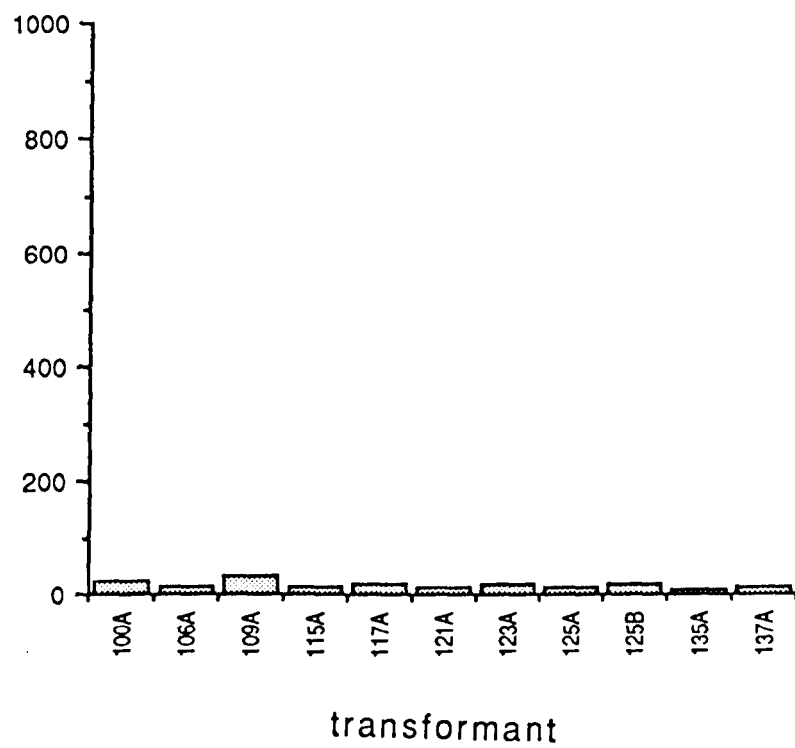


fig. 4.12. Extractable GUS activity from the leaves of 12 individual transgenic promoterless::uidA tobacco plant lines.

GUS activity (pmol/min/mg soluble protein)



Leaves from transgenic plants showing near average extractable GUS activities (lines; 6A, 16B, 19A, 22A, 22B 44A, 51A, 63A, 81A, 87A, 98A and 98B) were re-sampled and the extractable GUS activity measured. The results (see table 2) showed that the activities varied considerably between repeated measurements. The leaves assayed were of similar size and developmental stage to those sampled previously.

#### 4.6 histochemical analysis of leaves of transgenic plants;

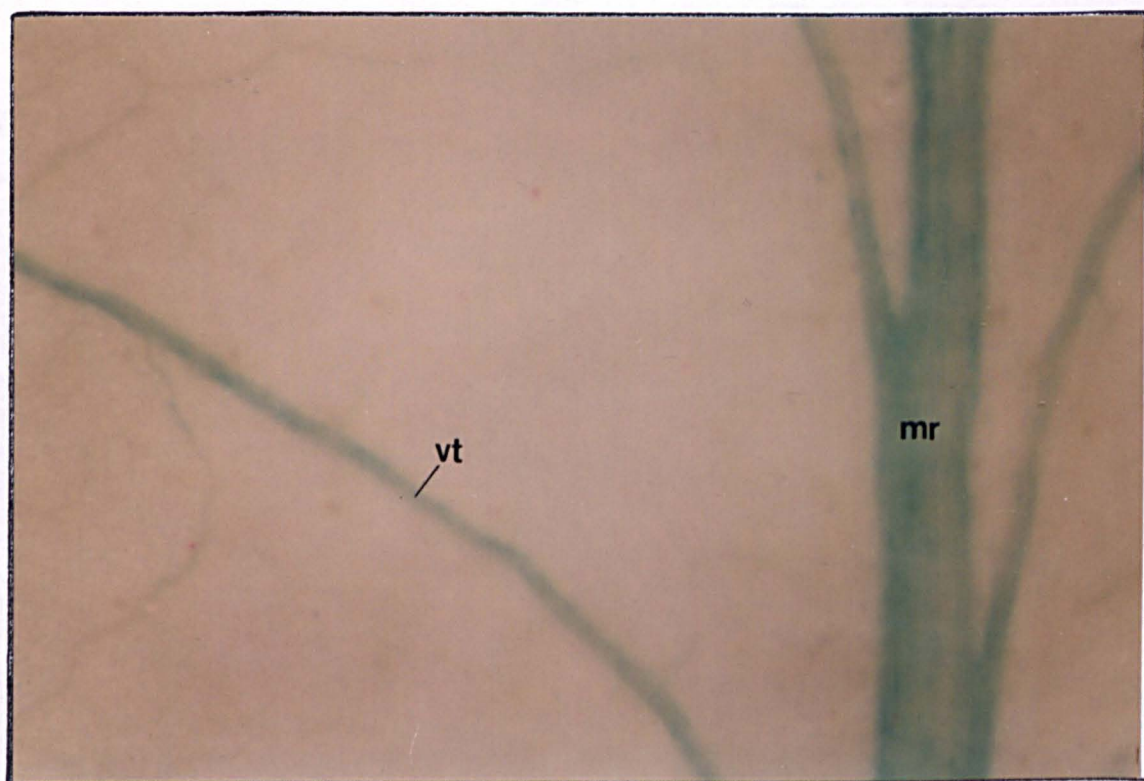
Those gln- $\alpha$ ::uidA lines exhibiting a near average leaf GUS activity (lines; 6A, 16B, 19A, 22A, 22B and 44A) were analysed histochemically to study the localisation of chimeric gene activity in the cells and tissues of the transgenic leaf. A proportion of the leaves analysed displayed staining within the leaf vascular tissue (see fig. 4.13). It was observed that both the transgenic lines showing stain and the resulting stain intensity differed between repeated histochemical analyses, although the actual spatial-pattern of staining remained the same. This may be due to either differences in degrees of infiltration of histochemical substrate or in the variability in gusA activity. Although the leaves sampled were not mature, they were several days old and from the work of Cock et al. (1991), it was possible that gln- $\alpha$  promoter activity in leaves of this developmental stage would have ceased. The resulting localisation of GUS activity and the measurement of significant levels of extractable GUS activity (average GUS leaf activity (excluding line 9A) = 160 pmol/min/mg soluble protein

table 2. variability in transgenic gln- $\alpha$ ::uidA tobacco plant leaf extractable GUS activity within the same transgenic plant line with repeated sampling;

line	GUS activity (pmol/min/mg soluble protein) with repeated sampling			
6A	108.0	9.0	2.5	60.0
16B	109.0	154.0	-	161.5
22A	320.5	283.0	138.0	234.0
44A	89.5	307.0	8.0	143.0



fig. 4.13. Histochemical localisation of GUS activity in a leaf of a transgenic gln- $\alpha$ ::uidA tobacco plant (vt = vascular tissue, mr = mid-rib) X25 magnification.



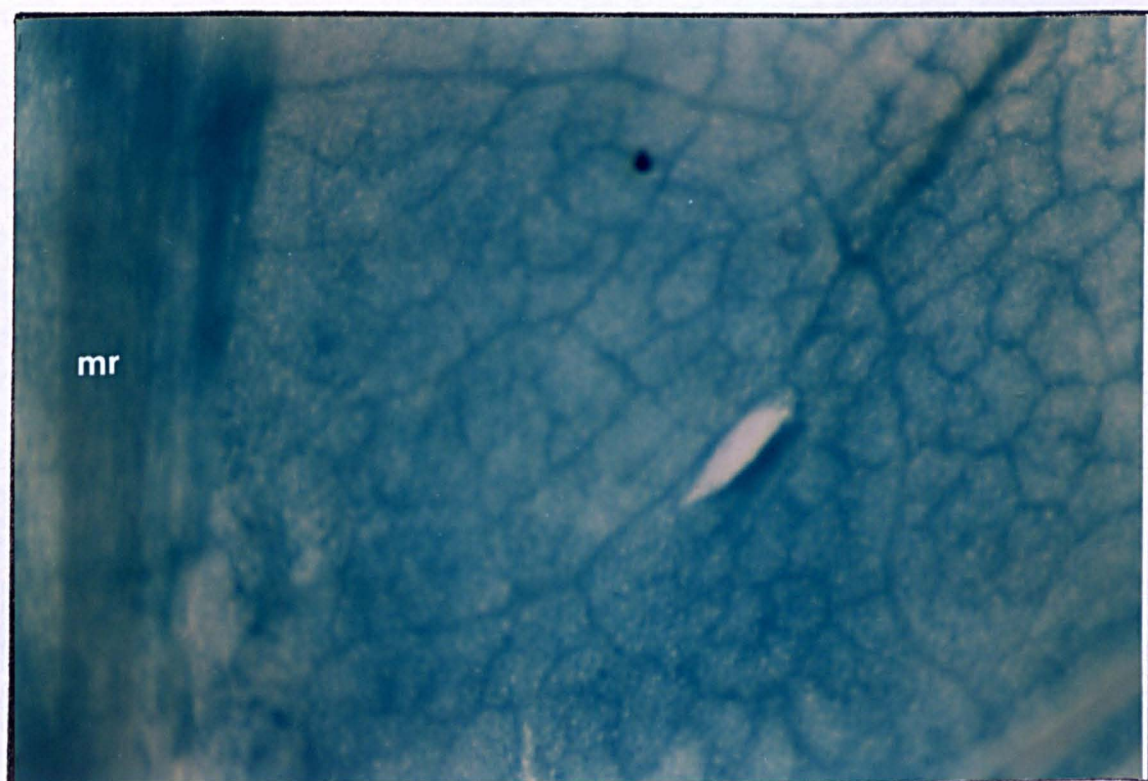
(promoterless::uidA average leaf GUS activity = 18 pmol/min/mg soluble protein)) in leaves of this developmental stage may reflect the prolonged translation of the uidA mRNA synthesised during earlier stages of leaf development and/or the high stability of the GUS protein (Jefferson et al., 1987).

Histochemical analysis of the spatial-pattern of  $\beta$ -glucuronidase activity in 35S::uidA plants showed a uniform distribution across all cell types (see fig. 4.14) as predicted from the studies of Jefferson et al. (1987). As expected from the measurement of extractable GUS activity, none of the promoterless uidA plant leaves displayed any staining to any degree. We can therefore conclude that the staining pattern seen for the leaves of gln- $\alpha$ ::uidA and 35S::uidA plants was due to promoter activity and was not due to a staining artefact.

#### 4.7 histochemical analysis of flowers of transgenic plants:

Flowers of gln- $\alpha$ ::uidA (lines; 6A, 17B, 22A, 44A) and 35S::uidA (lines; 51A, 63B and 87A) greenhouse-grown plants were sampled at three stages of development. Koltunow et al. (1990) divided tobacco flower development into 19 stages (-7 to +12) and the three stages at which we sampled flowers were (6 or 7), (9 or 10) and (12), as according to the system devised by Koltunow et al. (1990). These correspond to; (6 or 7) - corolla tube pale green, petal tips closed, anthocyanin beginning to accumulate in petal tips, (9 or 10) - corolla tube beginning to open, petal tips pink and expanding, tube becoming white, and (12) - flower open, corolla limb fully expanded and deep pink, tube white in

fig. 4.14. Histochemical localisation of GUS activity in a leaf of a transgenic 35S::uidA tobacco plant (mr = mid-rib) X25 magnification.



appearance. For convenience, the three developmental stages were referred to as "early", "middle" and "late" respectively. The flowers were longitudinally sectioned by hand before immersion in histochemical buffer. Prior to O/N incubation at 37°C, the samples were vacuum infiltrated to aid the penetration of the histochemical substrate. Because of recent reports of high levels of endogenous GUS activity in various anther structures and pollen of non-transgenic tobacco plants (Plegt and Bino, 1989), methanol was added to the histochemical buffer to a final concentration of 20% (v/v) to suppress endogenous GUS activity as recommended by Kosugi et al., (1990).

Anthers of early and middle stage flowers of gln- $\alpha$ ::uidA plants were seen to stain blue (see figs. 4.15 and 4.18). The intensity of stain over the anther surface was non-uniform with the intensity of the stain increasing over the anther wall as it approached the stomium, which stained the most intensely blue (see figs. 4.15 and 4.18). The stomium is derived from the anther connective tissue and is not part of the anther wall. Only a proportion of the anthers sampled displayed stain and both the stain intensity and the extent of the stain coverage varied considerably. This may reflect either differences in the degree of infiltration of the histochemical substrate or differences in gusA activities inherent within each anther. Anthers from early and middle stage flowers of 35S::uidA plants were also seen to stain blue and in a manner similar to that seen for gln- $\alpha$ ::uidA anthers (data not shown).

fig. 4.15. Histochemical localisation of GUS activity in the anthers of a transgenic gln- $\alpha$ ::uidA tobacco plant (line 16A, "middle" stage) (s = stomium, w = wall) X10 magnification.







From longitudinal sections of early, middle and late stage flowers taken from gln- $\alpha$ ::uidA plants, the majority of tissues of the pistil were seen to stain intensely blue. These included the placenta, the ovary vascular bundle and wall, the ova and the tissues of the receptacle adjacent to the ovary (see fig. 4.16), the tissues of the style adjacent to the stigma and the stigma itself (see fig. 4.17). The pistils of early, middle and late flowers of 35S::uidA plants were seen to stain in a similar manner with the peduncle vascular tissue, all tissues of the receptacle and those tissues of the sepal adjacent to the receptacle also staining blue (data not shown). The differences in spatial-pattern of stain were observed for the flowers of both transgenic plant types sampled for each developmental stage sampled.

No staining was observed in the pigmented regions of the corolla of neither the middle nor the late stage gln- $\alpha$ ::uidA plant flowers stained to any degree (see fig. 4.18).

Pollen from both transgenic gln- $\alpha$ ::uidA and 35S::uidA plants (lines; 6A, 17B, 22A, 44A, 51A, 63B, 87A) were histochemically analysed to localise GUS activity. Pollen was taken from both immature and mature anthers. A very high proportion of the pollen from all four gln- $\alpha$ ::uidA plants studied stained blue but the stain intensity varied considerably between different pollen grains (see fig. 4.19). Conversely, none of the pollen from 35S::uidA plants showed any staining (data not shown) and clearly demonstrates differences between the regulatory properties of the

fig. 4.16. Histochemical localisation of GUS activity in the ovary of a transgenic gln- $\alpha$ ::uidA tobacco plant flower (line 6A, "late" stage) (sectioned longitudinally prior to incubation with the histochemical substrate, p = placenta, vb = vascular bundle, w = wall, o = ovule, ft = flower tube, s = sepal, r = receptacle) X6.5 magnification.

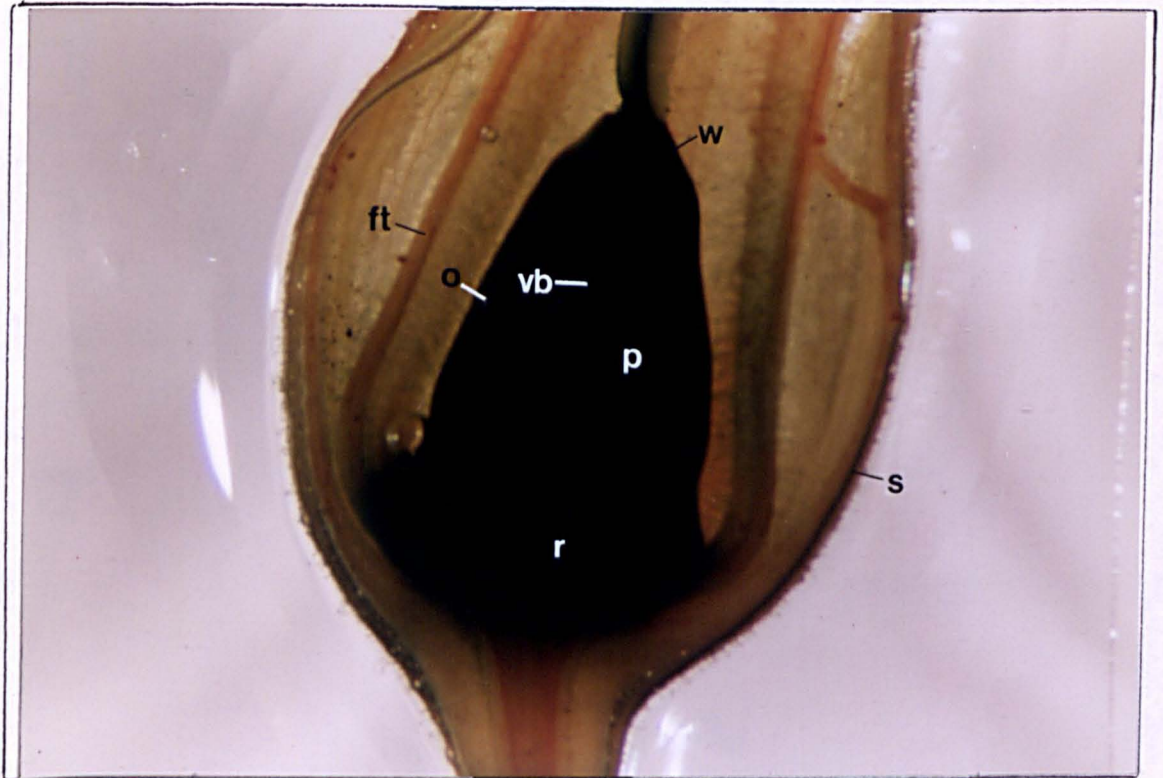


fig. 4.17. Histochemical localisation of GUS activity in the stigma and style of a transgenic gln- $\alpha$ ::uidA tobacco plant flower (sti = stigma, sty = style) X10 magnification.

Figure 4.17 shows the histochemical localisation of GUS activity in the stigma and style of a transgenic gln- $\alpha$ ::uidA tobacco plant flower. The figure is divided into two panels, (a) and (b). Panel (a) shows the stigma (sti) and panel (b) shows the style (sty). Both panels show a dark blue/purple color, indicating GUS activity. The magnification is X10.

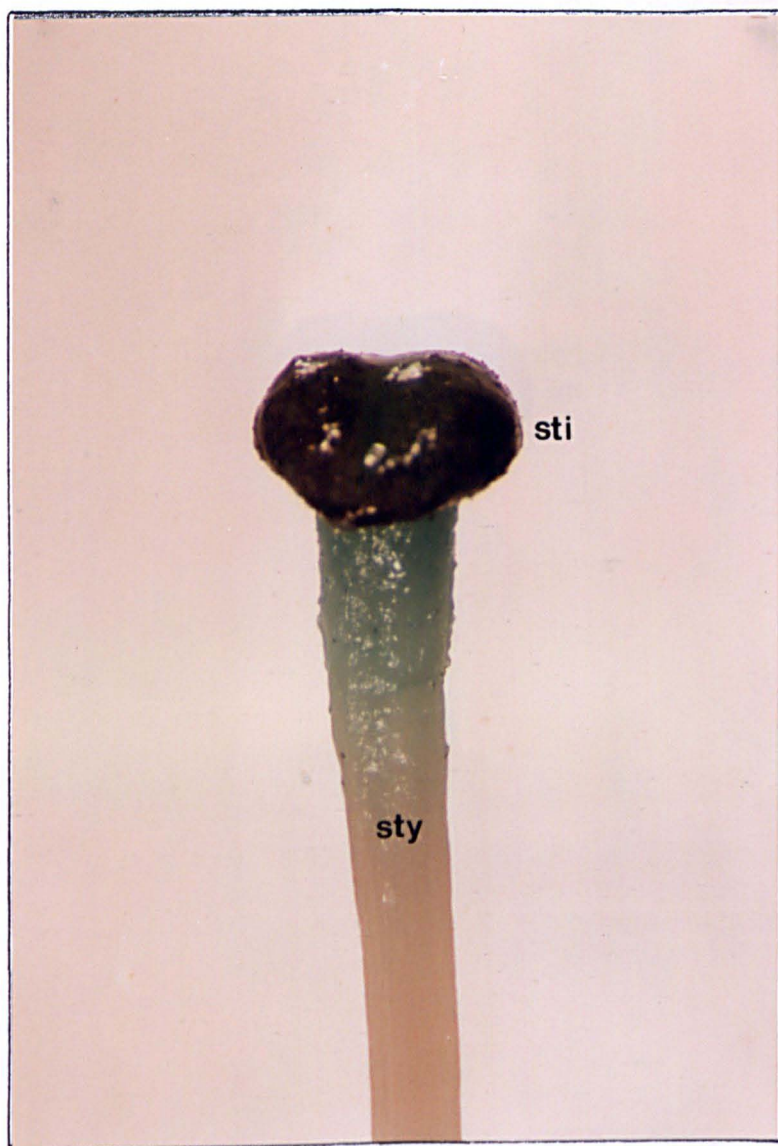


fig. 4.18. Histochemical localisation of GUS activity in the anthers and stigma and style of a transgenic gln- $\alpha$ ::uidA tobacco plant flower (a = anther, sti = stigma, sty = style, c = corolla) X6.5 magnification.



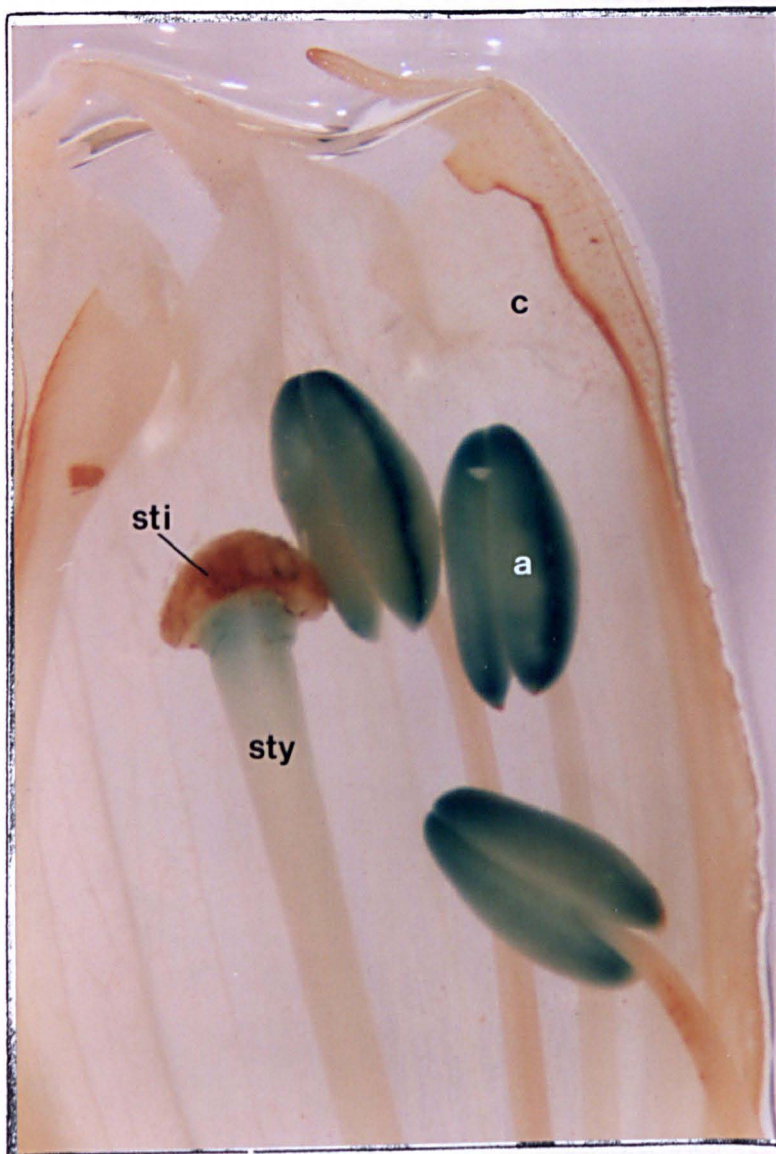
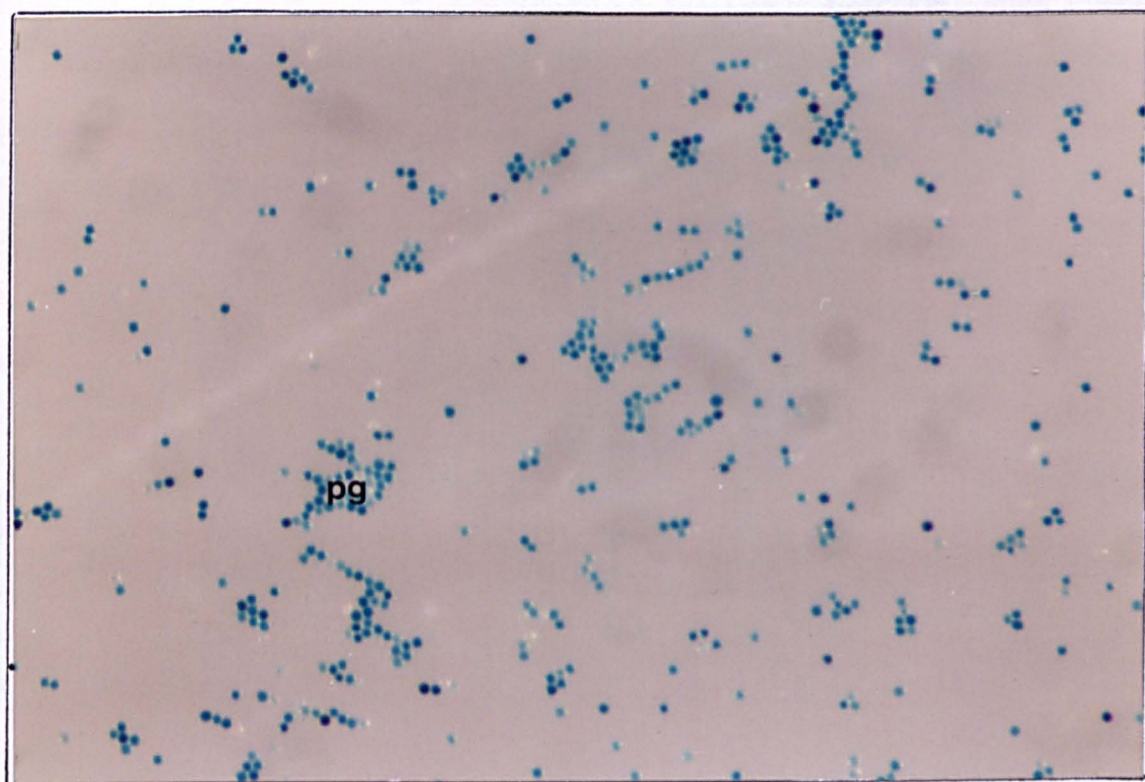


fig. 4.19. Histochemical localisation of GUS activity in transgenic gln- $\alpha$ ::uidA tobacco plant pollen (line 44A) (pg = pollen grains) X40 magnification.





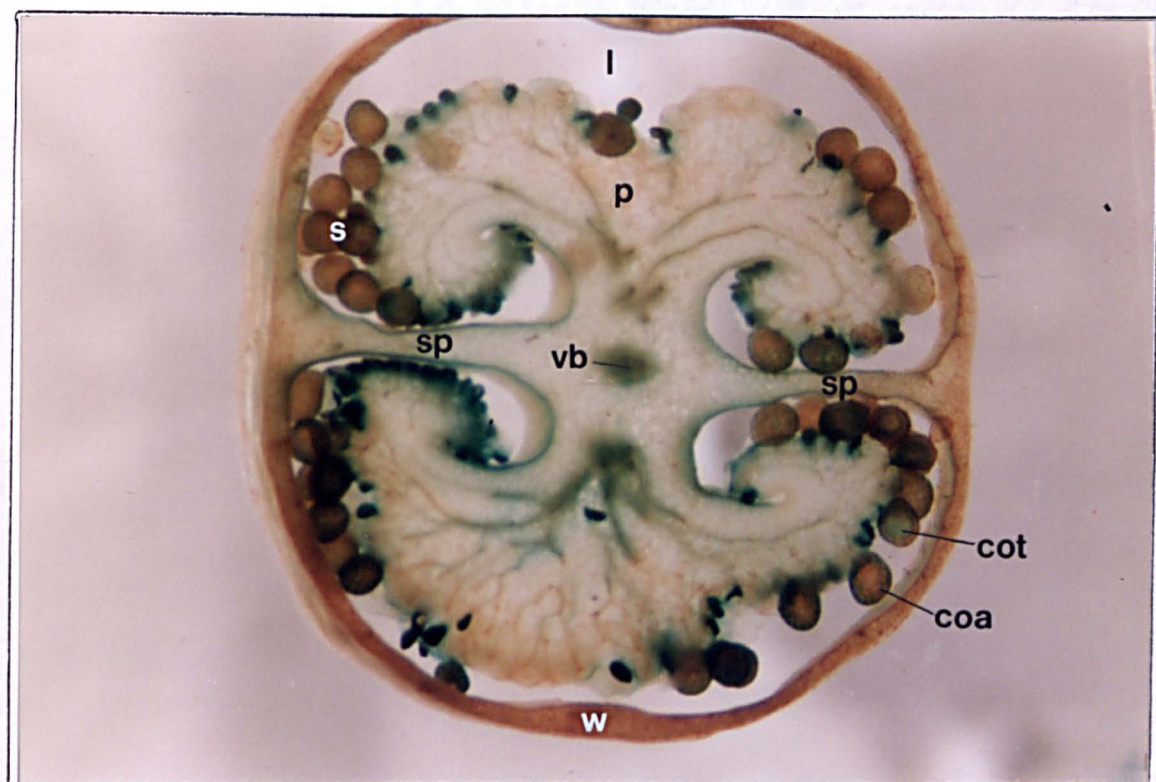
gln- $\alpha$  and 35S promoters.

The fruit of several transgenic plants (lines; 6A, 17B, 22A, 44A, 51A, 63B, 87A) were sampled at two stages of development; 11 days and 21 days after anthesis. The fruits were longitudinally sectioned by hand at several points along the length of the fruit and the sections histochemically analysed, with pre-fixing, to localise GUS activity. Figs. 4.20 and 4.21 show respectively the resulting spatial-pattern of GUS activity in 11 day old and 22 day old fruits of gln- $\alpha$ ::uidA and 35S::uidA plants.

Although staining was observed in the developing seed coat and seed cotyledon of both 11 day old gln- $\alpha$ ::uidA and 35S::uidA fruits, it was apparent that there were several differences in the spatial-pattern of GUS activity between the two transgenic plant types 11 days after anthesis (see fig. 4.20). Fruits of 35S::uidA plants showed staining within the placenta, the placental vascular tissue, the septa and within the fruit wall (see fig. 4.20 (b)) whereas placental and fruit wall staining were absent from gln- $\alpha$ ::uidA fruits and septa staining was limited to those tissues adjacent to the locule (see fig 4.20 (a)). Staining of those septal tissues adjacent to the locule may have been the result of the uptake of stain from the histochemical buffer. Sectioning of the fruits would have released GUS protein from severed cells expressing the uidA gene and it was noted that after incubation, the histochemical buffers had turned varying intensities of blue. The same process may also account for the limited vascular tissue staining observed in the

fig. 4.20. Histochemical localisation of GUS activity in the 11 day-old fruits of transgenic (a) gln- $\alpha$ ::uidA (line 22A) and (b) 35S::uidA (line 63A) tobacco plants (sectioned transversely prior to incubation with the histochemical substrate, s = seed, vt = vascular tissue, vb = vascular bundle, p = placenta, w = wall, cot = developing seed cotyledon, coa = developing seed coat, l = locule, sp = septum) X6.5 magnification.

(a)



(b)

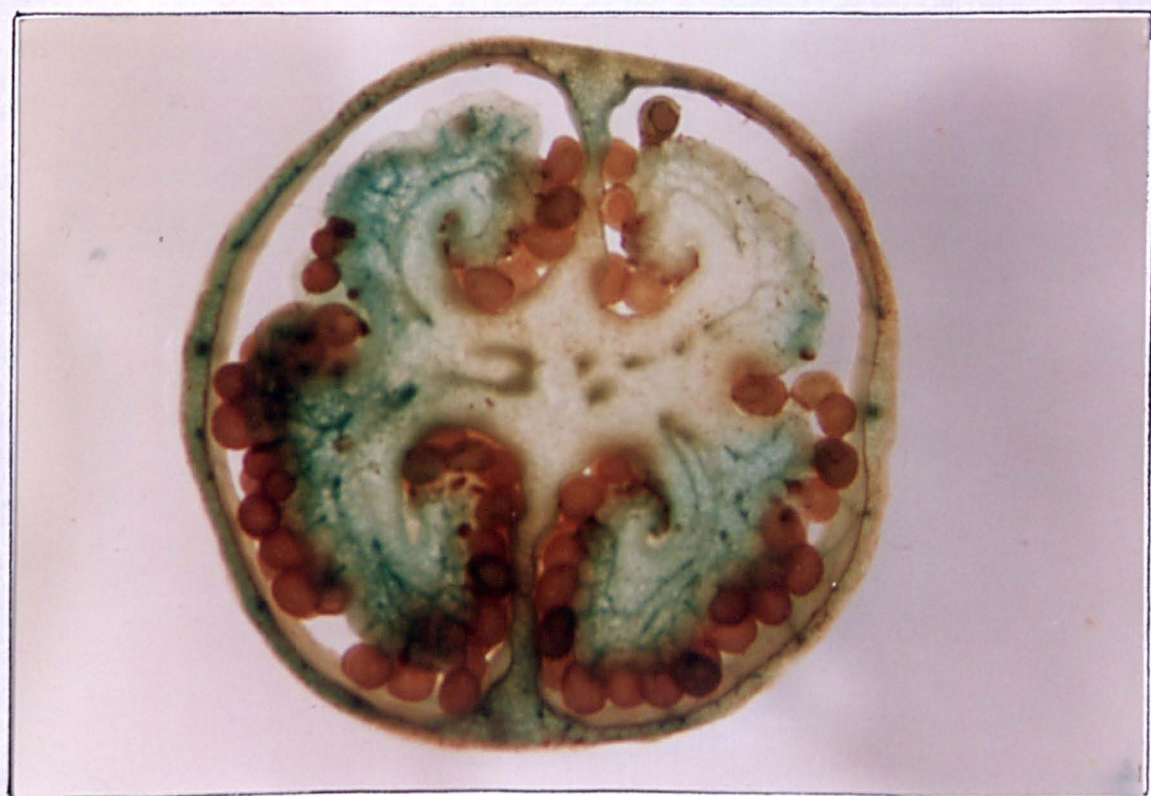
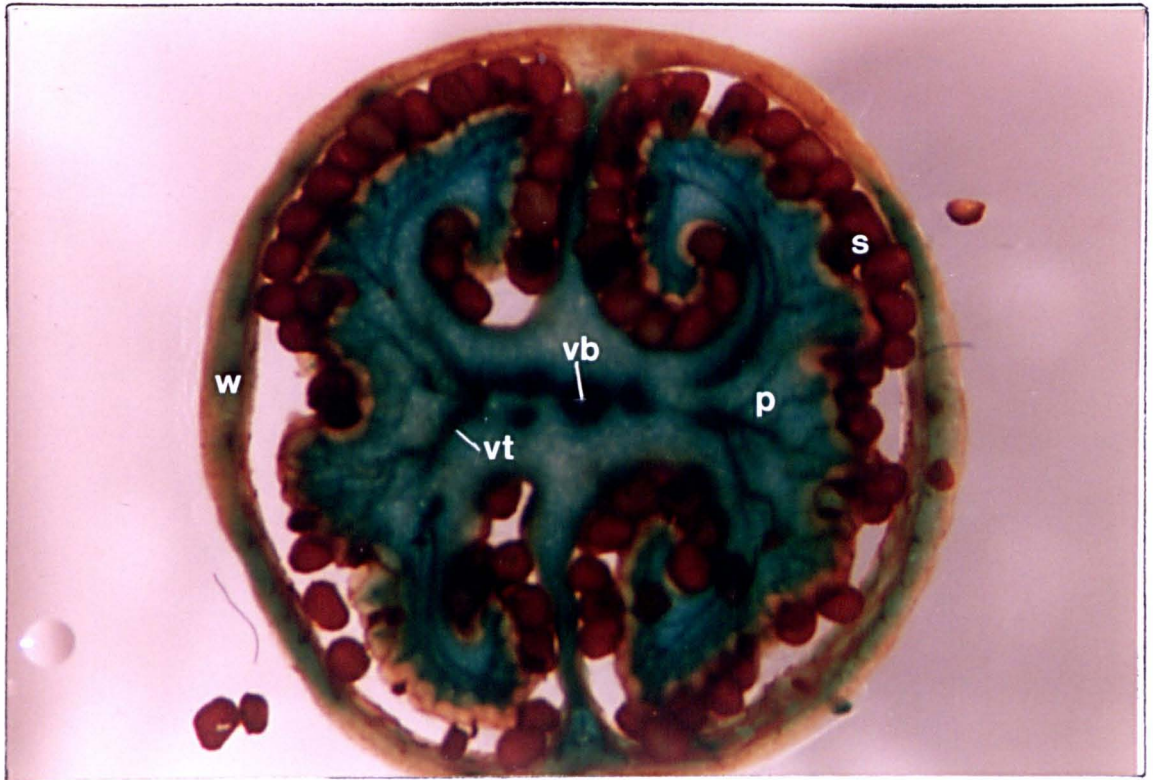


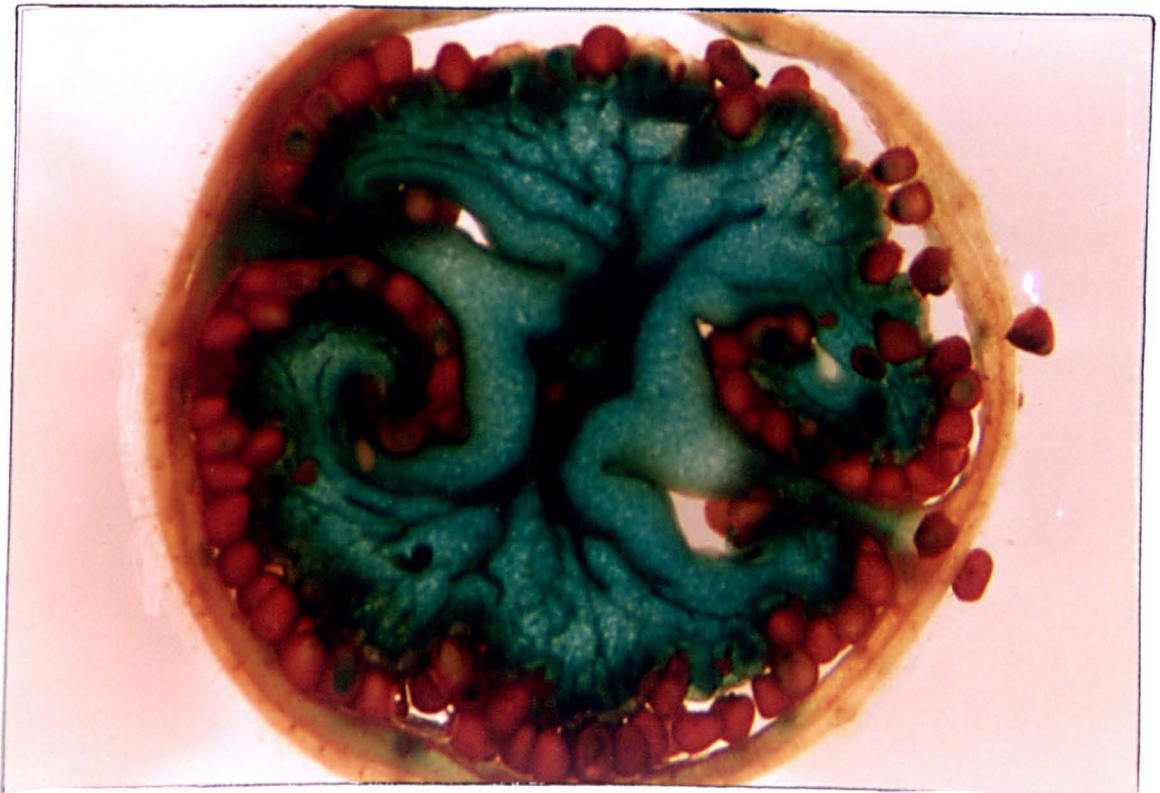
fig. 4.21. Histochemical localisation of GUS activity in the 21 day-old fruits of transgenic (a) gln- $\alpha$ ::uidA (line 17B) and (b) 35S::uidA (line 51A) tobacco plants (sectioned transversely prior to incubation with the histochemical substrate, s = seed, vt = vascular tissue, vb = vascular bundle, p = placenta, w = wall) X6.5 magnification.



(a)



(b)



placenta of 11 day old gln- $\alpha$ ::uidA fruits.

The staining pattern of 11 day old fruits of gln- $\alpha$ ::uidA and 35S::uidA plants differed in another respect. It was observed for gln- $\alpha$ ::uidA fruits that small bodies associated with the periphery of the placenta stained intensely blue (see fig. 4.20 (a)). Such bodies were absent from 35S::uidA fruits (see fig. 4.20 (b)). These bodies may possibly be unfertilised ova or be seeds that have not developed beyond a relatively early stage.

21 days after anthesis, the spatial-pattern of stain of fruits of both transgenic plant types were apparently identical. Intense staining was observed within the placenta, wall, septa, vascular bundle and placental vascular tissue, and within the developing seed coat and seed cotyledon (see fig. 4.21).

It must be noted that the present transgenic fruit and flower studies were only preliminary and are subject to more detailed experimentation.

#### 4.8 histochemical analysis of transgenic F1 seedlings;

Transgenic plant seeds (lines; 5A, 9A, 16B, 17B, 18C, 22A, 28A, 35A, 38B and 51A) were germinated on MS media containing 100 mg/ml kanamycin. After 2 weeks growth, seedlings were analysed histochemically.

The root spatial-pattern of GUS activity for both gln- $\alpha$ ::uidA and 35S::uidA seedlings were as observed previously in section 4.4. Promoter activity was associated with the root-tip (see fig. 4.22) (lines; 5A, 9A, 17B, 19A, and 22A), juvenile root vascular tissue (see fig. 4.22) (lines 17B and 22A) and with emerging lateral roots (see fig. 4.23) (lines: 5A, 9A, 17B, 19A and 35A) of roots of gln- $\alpha$ ::uidA seedlings. It can be seen from fig. 4.22 that the spatial-pattern of root-tip GUS activity of the transgenic seedlings analysed were seen to vary considerably between different seedling lines as was observed with the root-tips of transgenic F0 tobacco plants.

The chimeric gln- $\alpha$ ::uidA gene was also weakly expressed within the cotyledon vascular tissue of the gln- $\alpha$ ::uidA seedling line 5A (data not shown) and highly expressed in the cotyledon vascular tissue of the gln- $\alpha$ ::uidA seedling 9A (see fig. 4.24). This confirmed preliminary histochemical analysis of gln- $\alpha$ ::uidA seedlings indicating uidA expression in cotyledon vascular tissue.

High levels of GUS activity was observed in the root vascular tissue and in all cotyledon and primary leaf tissues of 35S::uidA seedlings (see fig. 4.25).



fig. 4.22. Histochemical localisation of GUS activity in the root-tip tissues of 2 week-old transgenic gln- $\alpha$ ::uidA seedlings ((a) = line 5A, (b) = line 9A, (c) = line 17B, (d) = line 19A, (e) = line 22A) X40 magnification.

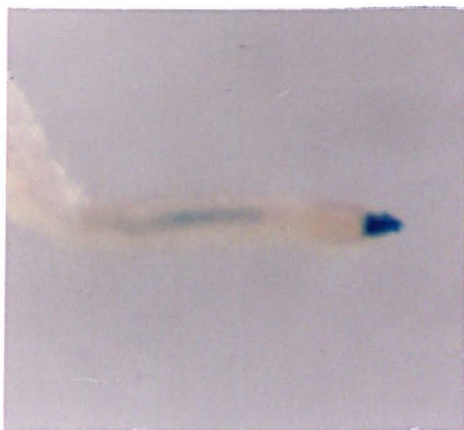
(a)



(b)



(c)



(d)

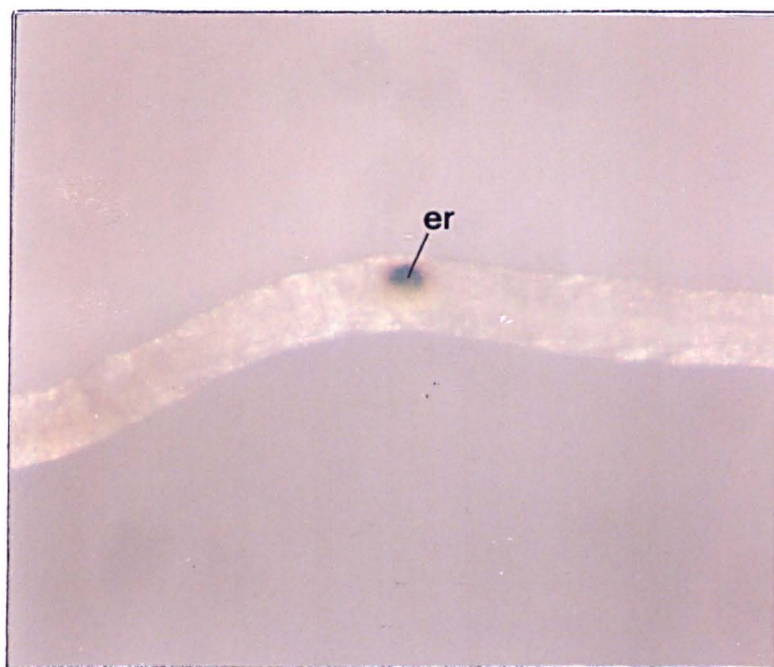


(e)



fig. 4.23. Histochemical localisation of GUS activity in emerging lateral root tissues of 2 week-old transgenic gln- $\alpha$ ::uidA seedlings ((a) = line 5A, (b) = line 35A, er = emerging root) X40 magnification.

(a)



(b)

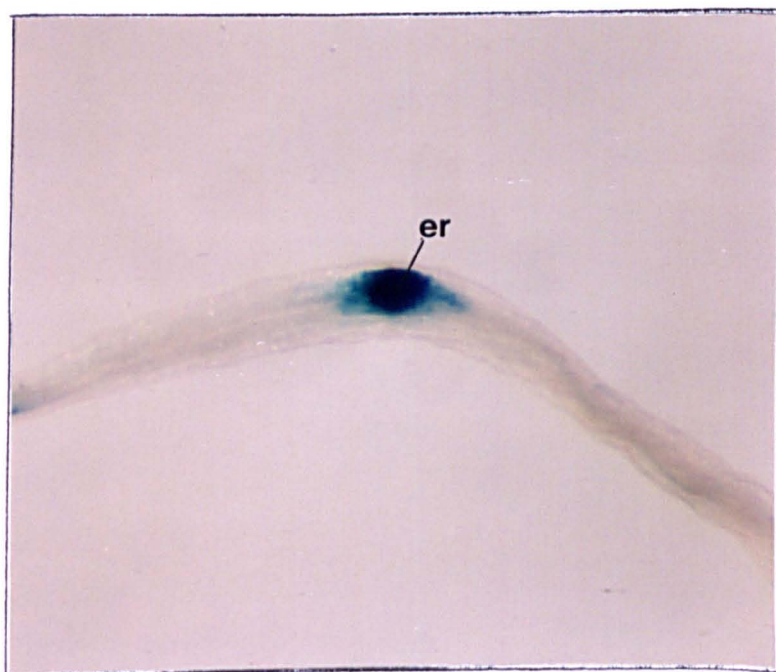


fig. 4.24. Histochemical localisation of GUS activity in the vascular tissue of a cotyledon of a 2 week-old transgenic gln- $\alpha$ ::uidA seedling (line 9A) (vt = vascular tissue) X40 magnification.

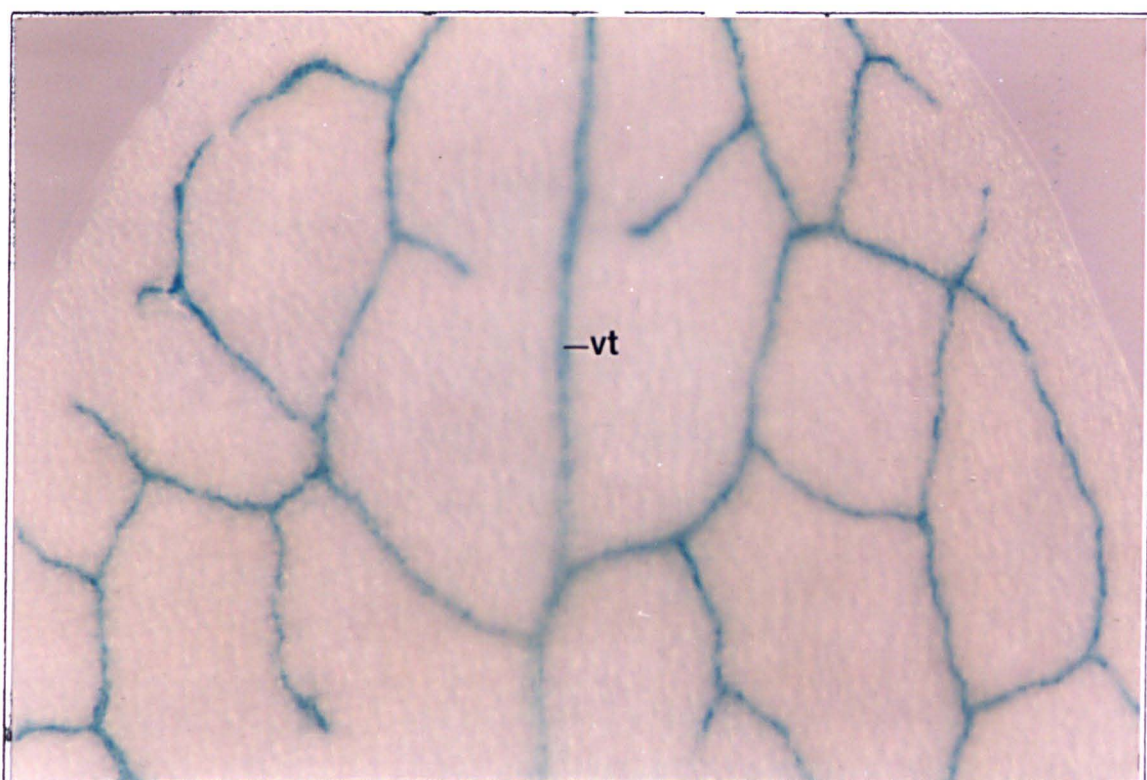
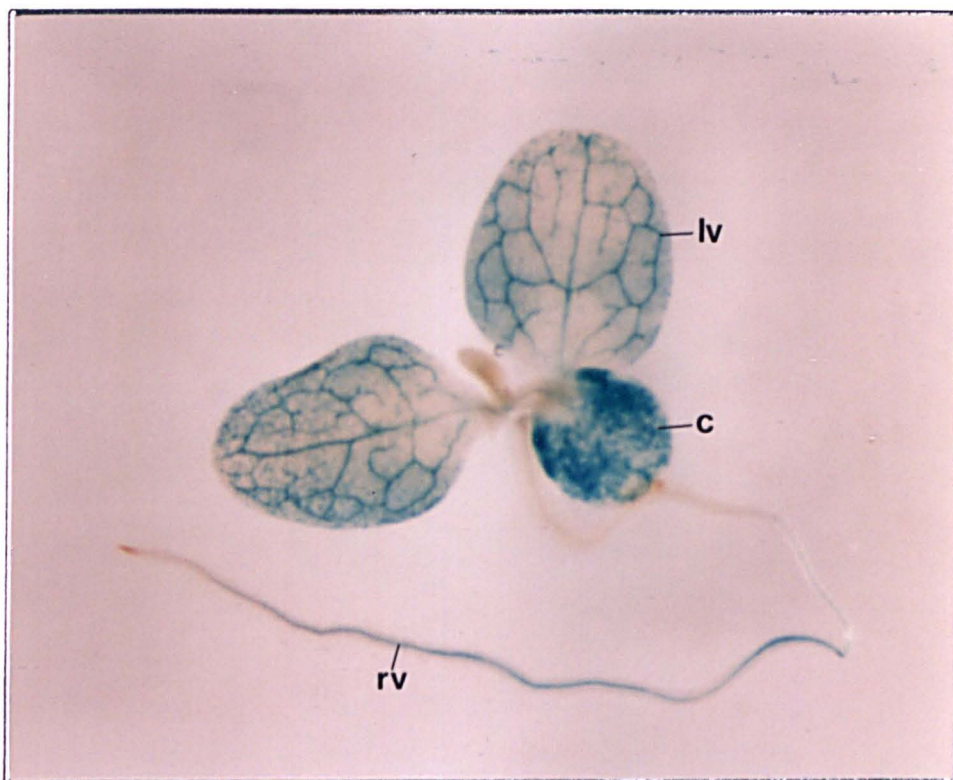


fig. 4.25. Histochemical localisation of GUS activity in the 2 week-old transgenic 35S::uidA seedling (line 51A) (rv = root vascular tissue, lv = primary leaf vascular tissue, c = cotyledon) X6.5 magnification.





#### 4.9 the effect of mechanical wounding on the level of extractable GUS activity of the leaves of transgenic plants;

Mechanical wounding of P. vulgaris hypocotyls has previously been shown to elicit an induction in PAL1 gene expression (Lawton and Lamb, 1987; Daniell, 1992) and has been correlated with an induction in gln- $\alpha$  expression (Daniell, 1992). A coordinate expression of the PAL and gln- $\alpha$  genes was proposed with gln- $\alpha$  expression functioning in the reassimilation the ammonium produced by the catalytic action of PAL (Daniell, 1992).

Leaves were cut from in-vitro maintained gln- $\alpha$ ::uidA and 35S::gusA transgenic tobacco plants (lines; 6A, 16B, 19A, 22A, 22B, 44A, 51A, 63B, 81A, 87A, 98A and 98B). Plant lines were chosen on their near average extractable leaf GUS activities. The leaves were wounded under sterile conditions as described in section 2.17 and then floated on sterile MS media containing 1mM reduced glutathione. Studies suggest that glutathione may function in mediating the response of plant cells to biological and physical stress and has been shown to cause a massive and selective induction of the plant defence genes PAL and CHS (Wingate et al., 1988). Daniell (1992) demonstrated that in the absence of 1mM reduced glutathione, the induction of gln- $\alpha$  transcription resulting from the mechanical wounding of hypocotyls is significantly less than in the presence of 1mM glutathione. Wounded leaf tissue were taken at 6 hrs and 24 hrs after wounding, frozen in liquid nitrogen and stored at -80°C. Because of the high stability of the GUS enzyme (a half life in

living mesophyll cells of approximately 50 hrs Jefferson et al., 1987), it was assumed that the 24 hrs time point would reflect the total GUS activity produced in response to the wounding up to that point at which the sample was taken.

Extractable GUS activities and soluble protein content of the wounded leaves were measured. It was observed that after 6 hrs of wounding, the GUS activity of wounded gln- $\alpha$ ::uidA leaves had not changed significantly from their time 0 activity except for line 22B that had increased approximately 2-fold (data not shown). After 24 hrs of wounding however, the GUS activity for each wounded gln- $\alpha$ ::uidA plant leaf had increased between 2- and 5-fold (see fig. 4.26.a) as compared to the time 0 activity. The GUS activity of wounded 35S::uidA plant leaves remained either approximately the same or fell by up to approximately half their time 0 leaf activity (see fig. 4.26.b). The results of this experiment were in agreement with the results of preliminary wounding experiments and overall suggested that gln- $\alpha$  promoter activity is specifically induced by wounding.

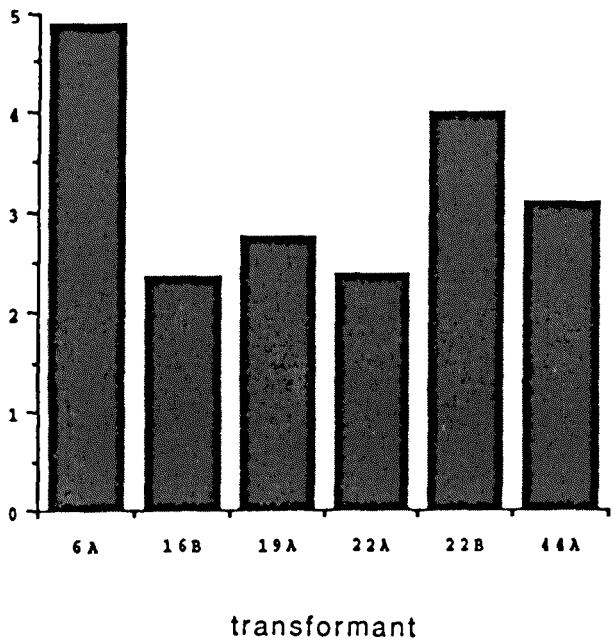
#### 4.10 histochemical analysis of wounded leaves of transgenic plants:

Leaves of gln- $\alpha$ ::uidA plants were wounded for 24 hrs as described above and incubated with histochemical substrate O/N at 37°C with pre-fixing. Vacuum infiltration was used to aid the penetration of the histochemical substrate. After incubation, the wounded leaves showed no stain in those tissues adjacent to the wound site (see fig. 4.27). The only difference seen was that

fig. 4.26. Fold-induction in extractable GUS activity 24 hours after wounding of (a) gln- $\alpha$ ::uidA leaves and (b) 35S::uidA leaves.

(a)

fold induction in GUS activity 24hrs after wounding



(b)

fold induction in GUS activity 24hrs after wounding

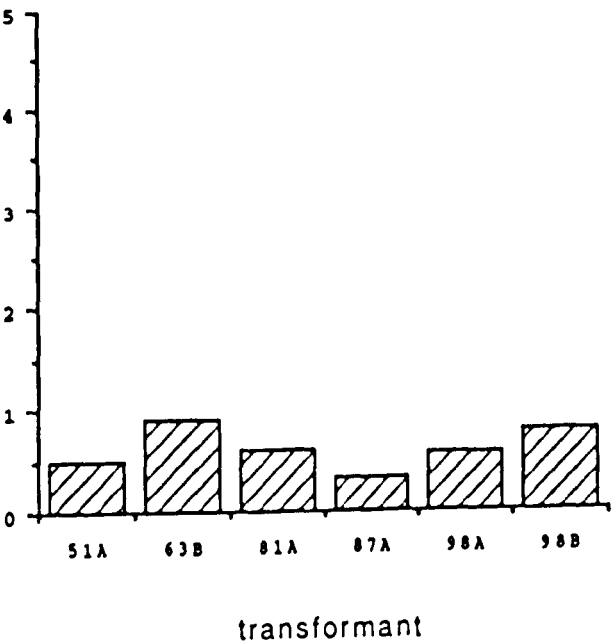
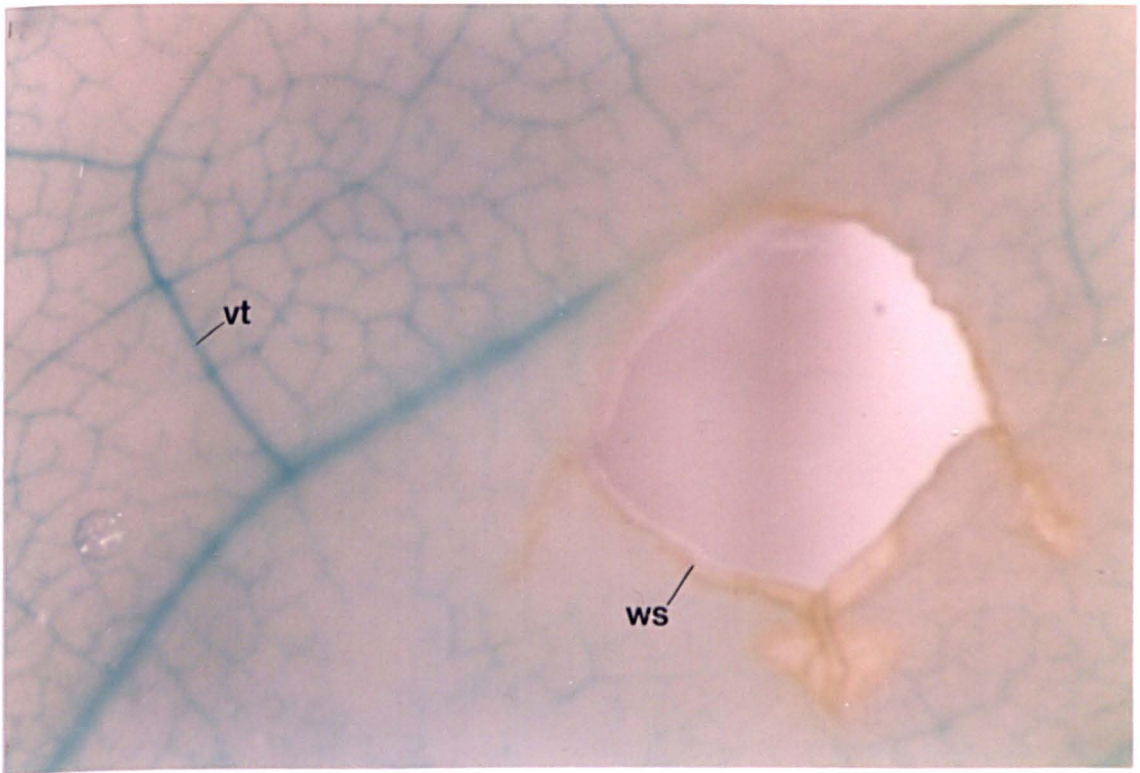


fig. 4.27. Histochemical localisation of GUS activity in the vascular tissue of a transgenic gln-α::uidA tobacco plant leaf 24 hours after mechanical wounding (ws = wound surface, vt = vascular tissue) X10 magnification.



the number of leaves showing vascular tissue staining were significantly more than the number of non-wounded control leaves. Out of the twelve leaves sampled (2 leaves from each of 6 individual transgenic plant lines giving a total of 6 wounded and 6 non-wounded leaves), 4 out of 6 wounded leaves displayed vascular tissue staining whereas only 2 out of 6 non-wounded leaves displayed vascular staining.

#### 4.11 discussion:

Overall, the regulatory properties of the gln- $\alpha$  promoter in transgenic plants were similar to those previously observed in P. vulgaris plants and implies that the expression of the gln- $\alpha$  gene is caused by sequences within 819bp of upstream region relative to the transcriptional start site.

##### 4.11.a between-line variation in GUS activity:

A major feature of this study has been the variability in the level of uidA expression between the same tissues of different transgenic plant lines (for example, see fig. 4.2). Such variability has been reported for the majority of introduced genes in transgenic plants (for example, Jones et al., 1985). Several studies have shown no correlation between this variability and the copy number of the introduced DNA at a given locus (Jones et al., 1985; Odell et al., 1987). It has been proposed that the variability in expression may in part be a consequence of "position effects" caused by influences of adjacent plant genomic DNA on the expression of the integrated gene fusion. Other possible contributory factors varying the level of gene fusion expression include the physiological state of the plant material, the number of independent T-DNA loci in different transformants, and the inhibitory effects of certain T-DNA structures on gene expression (Jones et al., 1985). The variability in level of GUS activity was also observed for the same transgenic tissues sampled from the same transgenic plant line but at different times (see table 2). This variability was



observed despite attempts to standardise the developmental stage at which tissue were sampled and the variation in activity may reflect the sensitivity of the gln- $\alpha$  promoter to physiological conditions.

As well as the observed variability in GUS activity levels between the same tissues of different transgenic plant lines and between the same samples of the same plant line, the spatial-pattern of GUS activity within certain plant organs was also seen to vary considerably between different lines. This was especially true for root-tip uidA expression (see fig. 4.5). The spatial-pattern of expression did not however alter between different roots of the same plant nor between re-sampled roots. These differences may be due to differences in the site of insertion within the tobacco genome of the chimeric gene. It has been discussed earlier in section 2.12 that the influence of the surrounding DNA on the transcriptional activity of an introduced chimeric gene acts over several kilobases (Odell et al., 1987) and it is therefore possible that the chimeric gene expression can be influenced by a nearby enhancer element of a gene encoding for, for example, a root-cap specific protein or a root-tip epidermal protein. This may explain the occurrence of lines that differ in their spatial-pattern of promoter activity.

#### 4.11.b possible physiological functions relating to gln- $\alpha$ expression:

Specific physiological functions regarding gln- $\alpha$  gene expression would better be elucidated by the study of, for

example, plants in which gln- $\alpha$  expression was absent. Such studies may involve mutant P. vulgaris plants lacking the gln- $\alpha$  gene or, the transformation of plants with an  $\alpha$ -cDNA antisense construct. Possible functions regarding gln- $\alpha$  expression were however suggested by these transgenic plant studies and involved the comparison of the sites of gln- $\alpha$  promoter activity with data relating to the sites of PAL and CHS expression, nitrogen cycling and primary nitrogen assimilation in plants.

#### 4.11.b.1 possible physiological functions for gln- $\alpha$ expression in roots;

The gln- $\alpha$  promoter was active within the juvenile vascular tissue of roots of transgenic gln- $\alpha$ ::uidA plants (see figs. 4.5.a, b and c) and within the root-tip zone of cell-elongation (see fig. 4.5.a). The promoter was also active within the vascular tissue of older sections of transgenic root (data not shown). Expression of the gln- $\alpha$  gene within the root vascular tissue may function in the reassimilation of ammonium produced by the catalytic action of PAL. PAL activity has been associated with xylem development as phenylpropanoids derived from cinnamic acid are polymerised in the cell walls of xylem vessels to form lignin (Rubery and Northcote, 1968). Furthermore, the P. vulgaris PAL2 gene promoter has been shown to direct high levels of uidA reporter gene activity in the developing xylem vessels of transgenic tobacco plants (Bevan et al., 1989; Liang et al., 1989b).

Alternatively, vascular tissue gln- $\alpha$  expression may function

in the generation of glutamine for intercellular transport, a role proposed for the cytosolic GS of pea (Edwards et al., 1990). The pea cytosolic GS gene promoter directed uidA reporter gene expression within the phloem elements of all organs of the mature transgenic tobacco plant studied (Edwards et al., 1990). Forde et al. (1989) demonstrated that the P. vulgaris gln- $\beta$  gene promoter directed uidA reporter gene expression in the vasculature of roots of transgenic Lotus corniculatus plants. However, the study was aimed at the spatial and developmental regulation of the gln- $\beta$  and gln- $\gamma$  genes during nodule development and only a small section of root either side of the nodule was taken for the histochemical localisation of GUS activity. Therefore, the data on gln- $\beta$  expression in transgenic roots was limited to those sections bordering the nodule.

Activity of the gln- $\alpha$  promoter was associated with the root-tips of gln- $\alpha$ ::uidA plants (see fig. 4.5) and gln- $\alpha$  gene expression in these tissues may function in the assimilation of ammonium from a variety of different sources. The promoter region of a root cytosolic GS gene from soybean has also been shown to direct uidA reporter gene expression in the root tips of transgenic tobacco and L. corniculatus plants (Miao et al., 1991). As ammonium is available from the reduction of soil nitrate by nitrate reductase, which has been localised in the root-tip and as it has recently been shown that GS and nitrate reductase are coordinately regulated in mustard seedling root tips in the presence of nitrate and light, it was suggested that the

expression of the cytosolic GS gene in the soybean root tip may function in the assimilation of the ammonium from nitrate reduction (Miao et al., 1991). Therefore root-tip gln- $\alpha$  expression may similarly function in root-tips in converting ammonium from nitrate reduction into glutamine.

However, as the promoter fragment of the P. vulgaris PAL2 gene has been shown to direct uidA reporter gene activity in the zone of cell proliferation adjacent to the root apical meristem of transgenic tobacco plants (Liang et al., 1989b), root-tip meristematic tissue gln- $\alpha$  expression may alternatively function in the reassimilation of ammonium from PAL activity. The root-tip PAL2 expression was suggested to function in the synthesis of flavonoids that are regulators of polar auxin transport and/or the synthesis of certain phenylpropanoid derivatives that have cytokinetic properties and/or the synthesis of lignin within the juvenile tissues of the xylem (Liang et al., 1989b).

It has been proposed that the root-tip may be considered as an active nitrogen sink as well as an active nitrogen source (Miao et al., 1991). The presense of GS in root-tips suggested that ammonium may be transported to these cells from other parts of the plant and converted into glutamine (Miao et al., 1991). Therefore, gln- $\alpha$  root-tip expression may alternatively function in the reassimilation of ammonium transported to the root-tip from other parts of the plant.

As the function of root hairs is to increase root surface area for the efficient uptake of solutes from the environment,

root hair gln- $\alpha$  expression (see fig. 4.8) may possibly function in primary nitrogen assimilation. However, as activity of the P. vulgaris PAL2 promoter has been associated with the root hairs and the root-tip of transgenic tobacco plants, root-hair gln- $\alpha$  expression may function in the reassimilation of ammonium from PAL. PAL2 gene expression in root hairs was suggested to be involved in the production of low molecular weight phenolics that have recently been shown to be transcriptional regulators of Rhizobium and Agrobacterium genes (Bevan et al., 1989).

Activity of the gln- $\alpha$  promoter activity was also associated with lateral root initiation sites on the roots of transgenic gln- $\alpha$ ::uidA plants (see fig. 4.6). Expression of the gln- $\alpha$  gene at these sites may function in the synthesis of nitrogenous compounds for root growth. Lateral roots are initiated by cell-division in the pericycle of the parent root. Alternatively, activity of a P. vulgaris chalcone synthase (CHS) gene promoter has been associated with the site of lateral root initiation of transgenic tobacco plant roots and it was suggested that CHS gene activity may be involved in the production of phenylpropanoid metabolites involved in polar auxin transport and the subsequent modulation of root morphogenesis (Schmid et al., 1990). CHS is, like PAL, a key regulatory enzyme of phenylpropanoid biosynthesis and catalyses the first reaction in the branch pathway specific to flavonoid synthesis. Flavanoids have a range of biological functions including the pigmentation of flowers and fruits (anthocyanins and flavonols; Brouillard, 1988), the defence

against microbial attack (isoflavonoids; Lamb et al., 1989), defence against UV irradiation (flavonols; Schmetzer et al., 1989), the induction of nodulation (flavones; Long, 1989) and have been implicated as modulators of polar auxin transport (flavonols; Jacobs and Rubery, 1988). Recent studies have shown that transcriptional activation of the phenylpropanoid genes is a major factor in regulating enzyme level and hence product accumulation (Chappell and Hahlbrock, 1984; Lawton and Lamb, 1987) and suggests therefore an alternative function for gln- $\alpha$  expression during lateral root initiation, that is, the reassimilation of the ammonium produced by PAL during the synthesis of morphogenic signals.

A proportion of transgenic gln- $\alpha$ ::uidA plant lines showed promoter activity associated with those epidermal cells damaged by the emergence of the lateral root (see fig. 4.7). A P. vulgaris CHS gene promoter has been shown to direct uidA reporter gene expression in those cells damaged by lateral root emergence in transgenic tobacco plants and it was suggested that the cell damage may initiate a defence response against microbial infection at the site of lateral root emergence (Schmid et al., 1990). This suggests that the function of gln- $\alpha$  expression in these cells may be to reassimilate the ammonium produced by PAL during the activation of a defence mechanism(s).

4.11.b.2 possible physiological functions for gln- $\alpha$  expression in flowers and fruits;

Flavonoids have been associated with certain pollen

structures and their synthesis has been attributed to the anther tapetum. Both PAL and CHS have been localised predominantly in the tapetum cells using immunochemical techniques (Kelvrel and Wierman, 1985). Moreover, the P. vulgaris PAL2 promoter has been shown to direct high levels of uidA reporter gene expression in transgenic tobacco plant anthers (Liang et al., 1989b) and a petunia CHS gene promoter has been shown direct high levels of reporter gene expression in the anthers and ovaries of transgenic petunia plants (Koes et al., 1990). Expression of the petunia CHS gene was linked to the synthesis of the colourless flavonoid, flavanol within the ovary and the synthesis of the flavonoid pigment, anthocyanin in the anthers of the petunia plant (Koes et al., 1990). Flavonoid synthesis has also been shown to be important for the pigmentation of the seed coat (Brouillard, 1988) and CHS gene activity has been associated with the developing seed coat (Koes et al., 1990). Therefore, gln- $\alpha$  expression in these flower and fruit structures may function in the reassimilation of the ammonium produced by PAL during flavonoid synthesis (see figs. 4.15, 4.16, 4.18, 4.20 and 4.21).

Alternatively, as with the root-tip, parts of the developing flower and fruit may be considered to be an active nitrogen sink. Ammonium may be released from other parts of the plant and reassimilated by GS and used in the synthesis of various flower and fruit cell components.

Swarup et al. (1990) demonstrated that the  $\alpha$ -mRNA and polypeptide were the only GS mRNA and polypeptide present in the

cotyledons of dry P. vulgaris seeds and the major GS mRNA and polypeptide in the cotyledons of P. vulgaris seeds up to 2 days after germination. They proposed that both the  $\alpha$ -mRNA and polypeptide are expressed during cotyledon development and persist during desiccation and into the mature seed (Swarup et al., 1990). Our studies have shown that the gln- $\alpha$  promoter was active in the cotyledons of developing transgenic gln- $\alpha$ ::uidA seeds (see figs. 4.20 and 4.21) and also within the vasculature of cotyledons of 2-week old transgenic gln- $\alpha$ ::uidA seedlings (see fig. 4.24). Swarup et al. (1990) proposed that expression of the gln- $\alpha$  gene in cotyledons of germination seeds functioned in the reassimilation of the ammonium produced during the proteolysis of seed storage proteins. Indeed, Edwards et al. (1990) have shown that the promoter of a cytosolic GS gene from pea was highly expressed in the cotyledon vascular tissue of 7-day old transgenic tobacco seedlings and proposed the same function for the expression of the GS gene in these tissues.

The P. vulgaris PAL1 gene has shown to be only very weakly expressed in the corolla P. vulgaris plant flowers (Liang et al., 1989a) and the absence of gln- $\alpha$  promoter activity within these tissues (see fig. 4.17) helps confirm the possible co-regulation of these genes. It is worth noting that the P. vulgaris PAL2 has been shown to be very highly expressed in the corolla of P. vulgaris plant flowers and transgenic plant studies have shown the P. vulgaris PAL2 gene promoter to direct intense staining within the pigmented regions of transgenic plant flower with



promoter activity suggested to be linked to flavonoid pigment synthesis (Bevan et al., 1989; Liang et al., 1989b).

Overall therefore, gln- $\alpha$  expression may function in the reassimilation of the ammonium produced through PAL activity and/or the metabolism of nitrogen transport compounds in all tissues displaying gln- $\alpha$  promoter activity and/or in the assimilation of ammonium produced through primary nitrogen assimilation in root-tips and root hairs. As previously mentioned, it is difficult to derive function just from promoter activity and further experimentation would be required. Moreover, the scope of this discussion is limited in that it is not known which cells are producing ammonium from which reactions.

#### 4.11.c the effect of mechanical wounding on gln- $\alpha$ promoter activity.

Mechanical wounding was shown to induce gln- $\alpha$  promoter activity between 2- to 5-fold over 24 hrs and decrease control (35S) promoter activity by approximately half over the same time period (see fig. 4.26). This observation was in agreement with earlier wounding studies which demonstrated an induction of gln- $\alpha$  gene expression by wounding in P. vulgaris plants (Daniell, 1992). P. vulgaris PAL1 gene expression was also induced by wounding and such observations were the basis for the proposed co-regulation of the gln- $\alpha$  and PAL1 genes (Daniell, 1992). However, as P. vulgaris PAL1 promoter sequences have not yet been cloned, no data is available on the cell-specific expression of the PAL1 gene with respect to wounding. Comparisons of the cell-

specific expression patterns of the PAL1 and gln- $\alpha$  genes with respect to wounding is not possible. The P. vulgaris PAL2 promoter sequences have however been cloned and have been shown to direct reporter gene expression within a well defined set of tissues adjacent to the wound site in transgenic plants (Bevan et al., 1989; Liang et al., 1989b). The present study revealed gln- $\alpha$  promoter activity in wounded tissue not to be associated with these tissues (see fig. 4.27) and instead wounding induced leaf vascular tissue activity. It has been shown that wounding induces the expression of all three P. vulgaris genes (Liang et al., 1989a) and it is possible that the PAL1 gene follows a cell-specific pattern of expression in response to wounding in a manner similar to that shown for the gln- $\alpha$  gene. As gln- $\alpha$  and PAL2 gene expression patterns differ with respect to wounding, reassimilation of the ammonium produced as a result PAL2 gene expression may possibly be through the expression of other GS gene(s).

The co-regulation of the PAL1 and gln- $\alpha$  genes may be investigated further by the inhibition of PAL enzyme activity by the exogenous application of the enzyme's product, cinnamic acid. Studies have shown both the activity of the PAL enzyme and the rate of synthesis of PAL subunits are highly susceptible to the level of cinnamic acid (Bolwell et al., 1986). The effect of cinnamic acid on GUS activity in transgenic gln- $\alpha$ ::uidA plant tissues may have therefore helped confirm the proposed co-regulation.

#### 4.12 conclusion:

Approximately 900bp of upstream region of the P. vulgaris GS gln- $\alpha$  gene was able to direct tissue-specific uidA reporter gene expression in transgenic tobacco plants. Promoter activity was associated with root tips, root hairs, lateral root initiation sites, emerging lateral roots and root vascular tissue. Low levels of promoter activity was also associated with immature leaves, possibly within the vascular tissue. Further studies demonstrated that promoter activity was associated with the anthers, pollen, corolla tube and ovaries of transgenic tobacco flowers studied at early, middle and late stages of flower development. Mechanical wounding was shown to induce gln- $\alpha$  promoter activity between 2- and 5-fold and decrease control (35S) promoter activity. Activity of the gln- $\alpha$  promoter was not associated with those tissues adjacent to the leaf wound site and instead, wounding appeared to induce the expression pattern observed in non-wounded leaves, that is, within the leaf vascular tissue.

Chapter 5: Results - Activity of the gln- $\alpha$  promoter in transgenic  
Lotus corniculatus.

### 5.1 Introduction:

Bennett et al. (1989) studied extensively the differential expression of P. vulgaris GS genes during nodule development in P. vulgaris plants. The gln- $\alpha$  mRNA was confirmed to be present in isolated nodules but that its abundance in young nodules was higher than in older nodules (Bennett et al., 1989). In mature nodules, the gln- $\gamma$  and gln- $\beta$  mRNAs and polypeptides are approximately of equal abundance and are approximately 3- and 12-fold more abundant than the gln- $\delta$  and gln- $\alpha$  mRNAs respectively (Bennett et al., 1989). Cock et al., (1990) demonstrated that during nodule senescence, the abundances of both gln- $\alpha$  and PAL1 gene mRNA increased co-ordinately with a concomitant decrease in gln- $\beta$ , gln- $\gamma$ , and gln- $\delta$  mRNA abundance.

The cell-specific expression of a number of cytosolic GS genes have been studied during nodule development using transgenic plant systems. Forde et al. (1989) studied the spatial and developmental patterns of P. vulgaris gln- $\beta$  and gln- $\gamma$  gene expression during nodule development on transgenic Lotus corniculatus roots. Using histochemical localisation of reporter gene activity they determined that activity of the gln- $\gamma$  promoter was associated specifically within the rhizobially infected cells of the nodule and that the activity of the gln- $\beta$  promoter was associated with both the cortical and infected regions of the young nodule but became restricted to the vascular tissue as the nodule matured (Forde et al., 1989). Brears et al. (1991) studied the cell-specific expression of a cytosolic pea GS gene

in transgenic alfalfa plants. The promoter conferred reporter gene expression within the nodule primordia as well as meristem, symbiotic zone and vascular tissue of the mature nodule (Brears et al., 1991). Finally, Miao et al. (1991) studied the cell-specific expression of a cytosolic soybean GS gene in the root nodules of transgenic Lotus corniculatus plants. The gene is expressed in the roots and root nodules of soybean and gene expression is inducible by the availability of ammonium (Miao et al., 1991). Subcellular location of reporter gene expression revealed gene promoter activity to be associated with emerging nodules and in incipient nodules before any morphological demarcation on the root surface. In mature nodules, promoter activity was associated with the infected zone containing both infected and uninfected cells and the cells of the inner cortex. No promoter activity was associated with the nodule outer cortex but treatment of the nodule with ammonia was seen to induce promoter activity in the outer cortex (Miao et al., 1991).

The present study was aimed at localising gln- $\alpha$  promoter activity in transgenic hairy roots of L. corniculatus plants with respect to nodule development and senescence.

## 5.2 plant transformation:

The constructs gln- $\alpha$ ::uidA, 35S::uidA and promoterless uidA were individually introduced into competent A. rhizogenes. The resulting cultures were used to stab inoculate hypocotyls of 10 day old dark-grown L. corniculatus seedlings (Hansen et al., 1989). Unfortunately, the A. rhizogenes strains containing the two control constructs failed to induce hairy-root growth and were therefore absent from the study. The absence of control lines was to a degree counteracted by the availability of extensive data regarding the expression pattern of the 35S promoter in transgenic plant systems. After 2 to 3 weeks of hairy-root growth, the plants (consisting of a single shoot and multiple individually transformed hairy-roots) were transferred to growth pouches and nodulated with Rhizobium loti. It was noted that the hairy-roots produced were morphologically abnormal with respect to L. corniculatus roots due possibly to the presence of Agrobacterial genes. The hairy-roots were thinner and more "twisted". A total of 40 individual nodulated plants were obtained for the gln- $\alpha$ ::uidA construct.

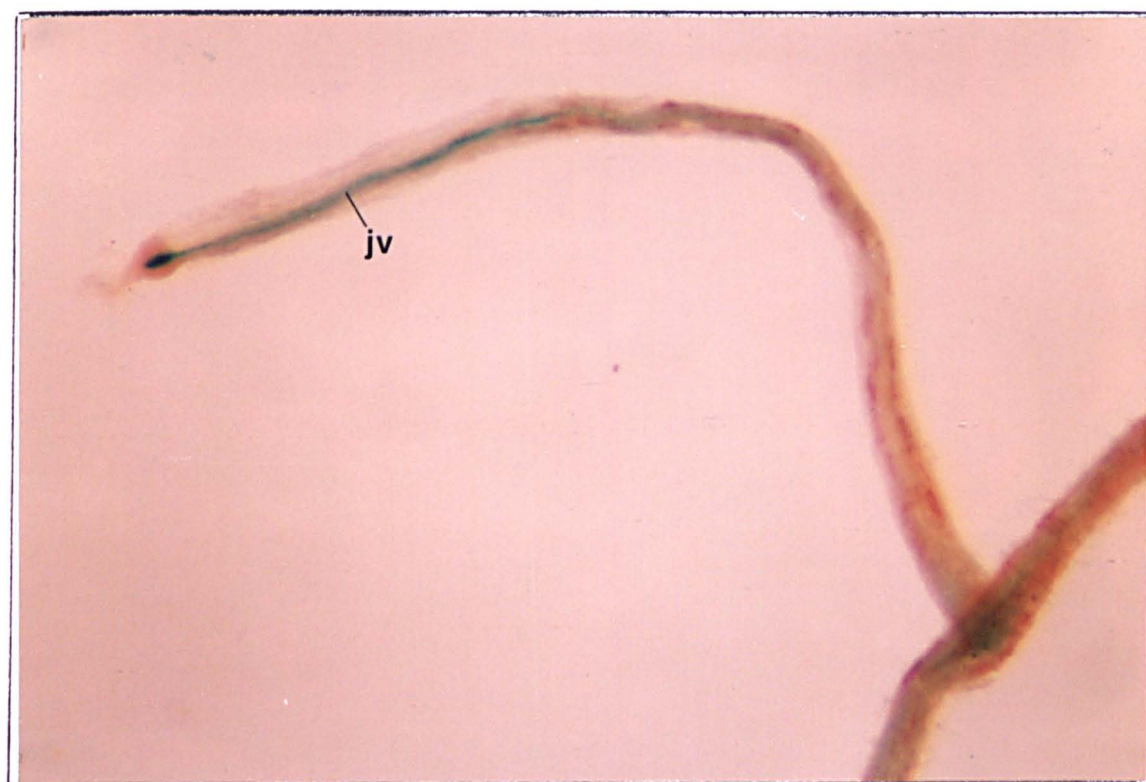
## 5.3 histochemical analysis of nodulated transgenic roots:

The entire nodulated transgenic root system of several L. corniculatus plants were incubated with the histochemical substrate O/N at 37°C (see section 2.15).

The meristematic and cell-elongation root-tip tissues of gln- $\alpha$ ::uidA L. corniculatus transgenic roots stained blue (see fig. 5.1). In contrast to the transgenic tobacco plant root-

fig. 5.1. Histochemical localisation of GUS activity in a transgenic gln-α::uidA Lotus corniculatus root (jv = juvenile vascular tissue) X40 magnification.



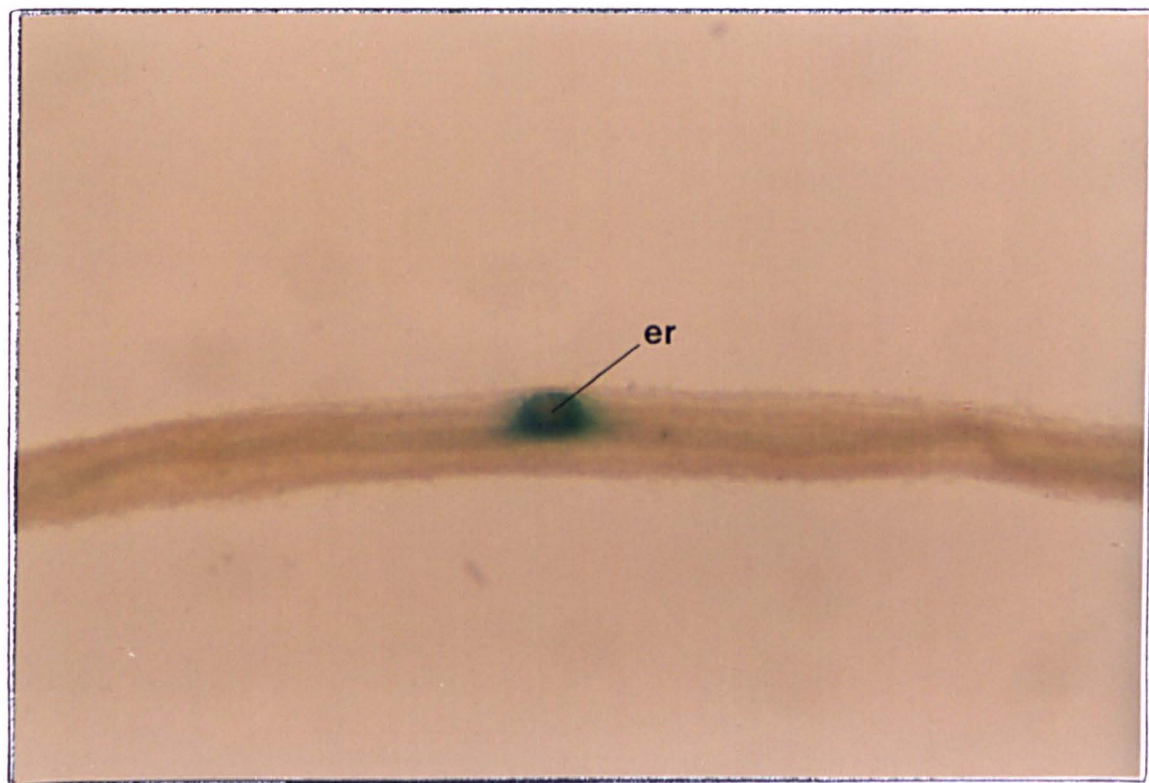


tips, the L. corniculatus root-tips showed only one spatial-pattern of stain which closely resembled that of the "central" root-tip pattern of promoter activity for tobacco plants (see fig. 4.5.a). Juvenile vascular tissue adjacent to the zone of cell-elongation was seen to stain blue (see fig. 5.1) as did sections of vascular tissue of older sections of the transgenic root (data not shown). Finally, emerging lateral roots were also seen to stain blue on transgenic gln- $\alpha$ ::uidA L. corniculatus roots (see fig. 5.2).

Only a very small number of root structures of the types described above were seen to stain (approximately 5 root structures of each type per hairy root system). Indeed, it was noted that the majority of root structures of the types described above known to be transgenic (because one or a few of the root structures on that individual hairy-root stained blue), failed to stain. As a proportion of the transgenic root structures of each type stained, histochemical substrate infiltration was not considered to be the problem. Furthermore, a very high proportion of the individual hairy-roots produced were apparently non-transgenic on the criterion that none of the above root structures were seen to stain. Previous studies which employed a method of transformation that was essentially identical to that of the method used in the present study recorded up to 80% of individual hairy-roots produced to be transgenic (Shen et al., 1992).

#### 5.4 chimeric gene activity during nodule development and

fig. 5.2. Histochemical localisation of GUS activity in emerging lateral root tissues of a transgenic gln- $\alpha$ ::uidA Lotus corniculatus root (er = emerging root) X40 magnification.



### senescence;

Although nodule primordia are structurally distinct from lateral root primordia in that they involve a broad region of undifferentiated tissue as opposed to the narrower, more conically shaped undifferentiated tissue of a lateral root primordia, it was difficult to discern between what may have been nodule primordia and what may have been sites of lateral root initiation. The problem was further compounded by the very low numbers (approximately 5 in total) of root structures of this type staining, an example of which is shown in fig. 5.3. Activity of the gln- $\alpha$  gene promoter was not apparently associated with young or mature nodules (data not shown).

Twelve nodulated plants were treated with high levels of  $\text{KNO}_3$  in order to induce nodule senescence and 3 days after treatment, the entire root systems were incubated with histochemical substrate O/N at 37°C. Transgenic senescing nodules were seen to display staining restricted to the inner cortex and the infected region of the nodule (see figs. 5.4.a and 5.4.b). No stain was seen within the nodule outer cortex.

As described in section 5.3, the numbers of senescing nodules showing stain were small (approximately 5 in total).

fig. 5.3. Histochemical localisation of GUS activity in nodule primordia/lateral root initiation site tissues of transgenic gln- $\alpha$ ::uidA Lotus corniculatus root (in/ir = incipient nodule/lateral root initiation site) X40 magnification.

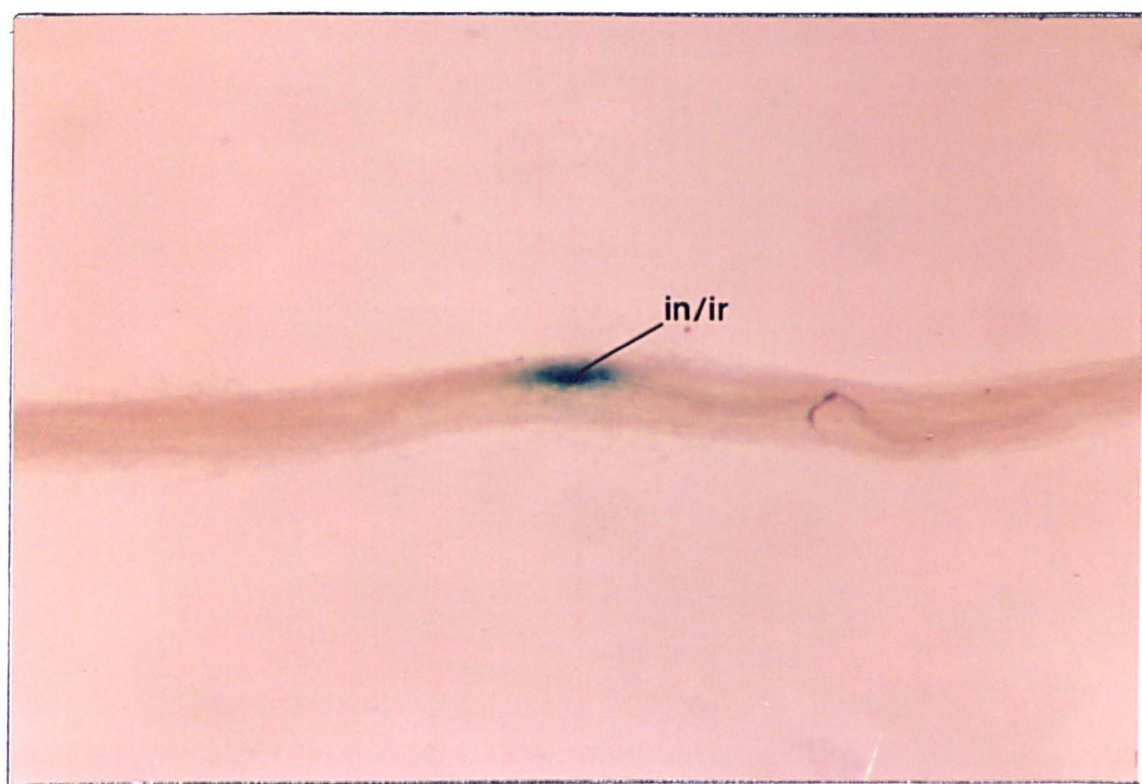
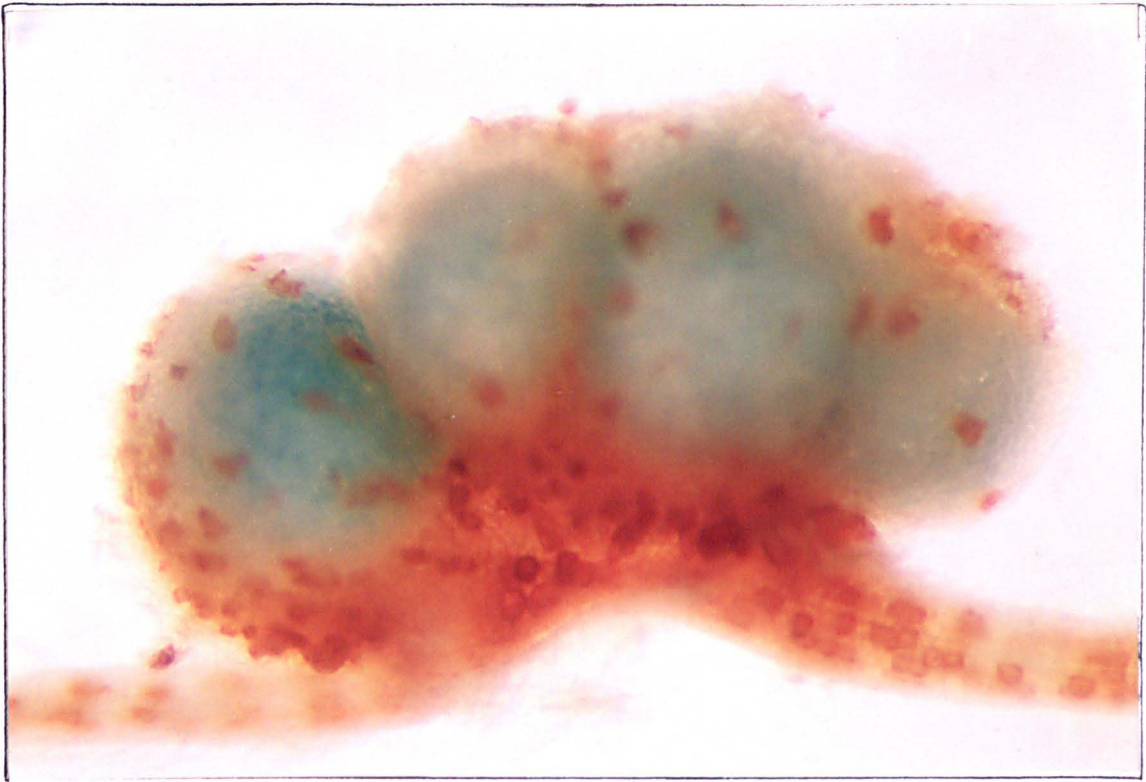


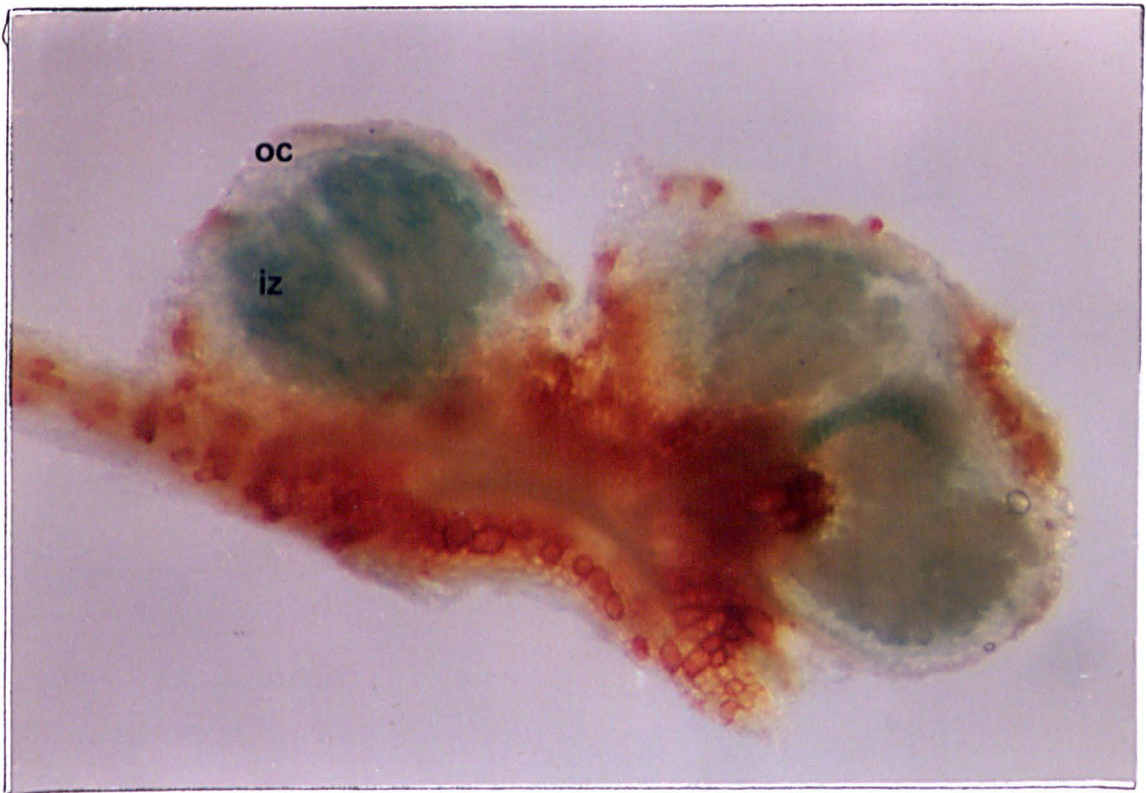
fig. 5.4. Histochemical localisation of GUS activity in senescing transgenic gln- $\alpha$ ::uidA Lotus corniculatus nodules ((a) non-sectioned, (b) longitudinally sectioned after incubation with the histochemical substrate, iz = infected zone, oc = outer cortex) X40 magnification.



(a)



(b)



### 5.5 discussion:

The limiting aspects of the L. corniculatus system were firstly, the numbers of transgenic hairy roots which exhibited staining upon incubation with the histochemical substrate were very small. Similar numbers were seen for those senescing nodules displaying infected zone staining. Although it was expected that only a proportion of the hairy roots would prove to be transgenic and hence stain, the actual numbers were much lower than those recorded previously (Shen et al., 1992). This was further compounded by the fact that the majority of root-tips, emerging lateral roots and root vasculature known to be transgenic (because one or several of these root structures on that individual hairy-root stained) failed to stain. As a proportion of root structures of each type stained, infiltration of the histochemical substrate was not considered to the reason for this. The low numbers encountered in the present study limited the applicability of the results obtained and therefore the study may only be considered as preliminary.

Secondly, the two control constructs used in the tobacco study were absent from the L. corniculatus study. This was due to the failure of those strains of A. rhizogenes containing the control constructs to produce hairy-roots upon stab inoculation of L. corniculatus hypocotyls. This may possibly be due to the loss of the helper Ti plasmid from the Agrobacterium. The helper plasmid has been shown to be susceptible to the temperature at which the Agrobacterium culture is grown and may be rapidly lost

from the Agrobacterium. However, the plasmid only becomes unstable at temperatures above 30°C and the cultures were grown at 28°C.

Despite the limiting numbers of root structures exhibiting staining, gln-α promoter activity was associated with root-tips (see fig. 5.1), root vasculature (see fig. 5.1), emerging lateral roots (see fig. 5.2) and possibly lateral root initiation sites (see fig. 5.3) of transgenic L. corniculatus roots and overall the expression pattern observed was similar to that observed for the roots of transgenic tobacco plants. This similarity in expression pattern counteracted to some degree the limitations of the present L. corniculatus study and implied that the gln-α expression pattern observed were due to gln-α promoter sequences.

The duality in root-specific expression patterns between the two transgenic plant systems did not extend to root hairs; the L. corniculatus system failed to show any root-hair chimeric gene expression. The L. corniculatus plants in the present study were grown in the presence of nitrate and despite the plants being nodulated, they were expected to reduce nitrate. It was suggested in section 4.10 that root-hair gln-α expression may function in primary nitrogen assimilation and it was therefore unexpected that gln-α promoter activity was not associated with transgenic L. corniculatus root-hairs. As it is not known whether the gln-α gene is expressed in the root hairs of P. vulgaris plants, it is possible that either L. corniculatus plants lack those factors responsible for root-hair gln-α expression or that tobacco plants

contain factors responsible for the abnormal expression of the gln- $\alpha$  gene in root hairs.

Functions for gln- $\alpha$  expression in those root tissues common to both transgenic systems have been detailed within section 4.10. In each case it was impossible to discern the exact source of ammonium when considering data from previous studies. For each tissue displaying gln- $\alpha$  promoter activity, PAL activity had also been associated. In other tissues, nitrate reductase had been previously localised or the tissues had simply been suggested to be an active nitrogen sink and gln- $\alpha$  expression could therefore be required for the reassimilation of the ammonium transported to these tissues from other parts of the plant. Nevertheless, it must not be overlooked that gln- $\alpha$  expression may function in the assimilation of ammonium derived from a combination of two or three of the above within a specific tissue.

Activity of the gln- $\alpha$  gene promoter was possibly associated with transgenic nodule primordia (see fig. 5.3). Studies have shown that subsets of both the PAL and CHS gene families are specifically induced during the early stages of soybean-Bradyrhizobium japonicum symbiosis (Estabrook and Sengupta-Golopan, 1991). Furthermore, the subsets of PAL and CHS genes induced early in symbiosis are different from gene members induced as a result of a host defence or stress response during the later stages of ineffective symbiosis using a Nod<sup>+</sup> Fix<sup>-</sup> strain of B. japonicum. Estabrook and Sengupta-Golopan suggested that the results agreed with the model of Van Brussel et al.,

(1990) who proposed that after induction of the Rhizobial nod genes, the bacteria produce a factor that signals the plant to produce more flavonoids and maintain nod gene expression during infection. However, they suggested that the induction of PAL and CHS during nodulation may alternatively function in the production of those flavonoids involved in cortical cell proliferation (Estabrook and Sengupta-Golopan, 1991). Overall therefore, the induction of gln- $\alpha$  expression during the early stages of nodule formation may function in the reassimilation of the ammonium produced by PAL.

Miao et al. (1991) demonstrated that a cytosolic soybean GS gene promoter directed reporter-gene expression in the nodule primordia of transgenic L. corniculatus plants. They proposed that the expression of this GS gene was involved in the reassimilation of the ammonium transported to the nodule primordia from other parts of the plant, converted into glutamine and used in nodule development. Therefore the developing nodule was considered to be an active nitrogen sink (Miao et al., 1991). Expression of the gln- $\alpha$  gene may therefore alternatively be involved in the reassimilation of the ammonium transported to the nodule primordia from other parts of the plant.

Histochemical analysis of senescing transgenic nodules revealed that the gln- $\alpha$  gene expression was induced in response to senescence and that the expression was restricted to the inner cortex and infected zones of the nodule (see fig. 5.4). Nodule senescence has previously been shown to induce both PAL and gln- $\alpha$

gene expression (Cock et al., 1990). PAL gene expression may be a host-defence response as senescence leads to the breakdown of the peribacteroid membrane (a plant derived membrane which encloses the bacterium) and the release of bacteria into the plant cell cytosol. Expression of the gln- $\alpha$  gene in the infected zone of the senescing nodule may therefore be reassimilatory.

However, senescing tissues become a source of nitrogen for the plant (Thomas, 1978) and the tissues' constituent protein is hydrolysed and the protein moiety is redistributed throughout the plant. The gln- $\alpha$  gene expression in senescing nodules may therefore function in the reassimilation of the ammonium produced during protein hydrolysis and the metabolism of the resulting amino acids. However, as protein hydrolysis would be expected to occur throughout the entire senescing nodule and as the present study restricted gln- $\alpha$  expression to the inner cortex and the infected zone of the senescing nodule (see fig. 5.4.b), nodule gln- $\alpha$  expression is more probably induced through a defence response than in response to protein hydrolysis.

With respect to root-specific chimeric gene expression, the L. corniculatus system may be considered as more applicable. The transgenic tobacco roots studied were taken from "in-vitro" maintained plants grown on a media containing sucrose and ammonium nitrate. Transgenic plants grown under such conditions have been shown to affect greatly the expression of the introduced chimeric gene (Hemon et al., 1990). The L. corniculatus plants were on the other hand grown on a liquid

media absent of sucrose and the only supplemented nitrogen source was  $\text{KNO}_3$ . Therefore the L. corniculatus system reflected more those physiological conditions experienced by a field grown plant although it must not be overlooked that the roots were clearly morphologically abnormal due to the introduction of the A. rhizogenes genes. It was noted that the root-tip spatial-pattern of chimeric gene activity was of only one type as opposed to the several observed for the tobacco system. This may be a reflection of the conditions under which the plants of the two different systems were grown.

## 5.6 conclusions:

Approximately 900bp of the P. vulgaris GS gln- $\alpha$  gene promoter was able to direct tissue-specific uidA reporter gene expression in transgenic L. corniculatus hairy roots. Promoter activity was associated with root-tips, root vascular tissue, emerging lateral roots and possibly with lateral root initiation sites. Furthermore, activity of the promoter was possibly associated with the early stages of nodule development but was apparently absent from young and fully mature nodules. Nodule senescence induced promoter activity in the inner, infected zone of the nodule.



Chapter 6: Concluding discussion.

Although previous attempts to clone gln- $\alpha$  sequences proved to be unsuccessful (Cullimore, unpublished results; Daniell, 1992), in the present study approximately 900bp of the upstream region of the P. vulgaris GS gln- $\alpha$  gene was isolated from a P. vulgaris genomic library. The seemingly critical alteration to the cloning protocol was in the use of a different and more appropriate E. coli host strain and packaging mix. The present study made the use of an mcr- host strain and mcr- packaging mix. The nucleases encoded by the E. coli mcr genes recognise and restrict at sites of eukaryotic DNA methylation, 5-methylcytosine in certain sequence contexts to be exact (Raleigh and Wilson, 1986). As some plant DNAs contain high levels of 5-methylcytosine, the effects of mcr activity are especially relevant to cloning plant genomic DNAs. Indeed, several reports have shown that the use of mcr- packaging mixes and host strains had led to the cloning of sequences which had been previously considered as "unclonable" (for example, see Westaway et al., 1987).

Sequencing of the gln- $\alpha$  upstream region revealed that the gln- $\alpha$  sequence and the  $\alpha$ -cDNA sequence diverged. The divergence point was 28 bp from the 5' end of the  $\alpha$ -cDNA clone and those 5'  $\alpha$ -cDNA 28 bp were not represented in the gln- $\alpha$  sequence. The results of further investigations into this sequence divergence suggested that the 5' 28 bp of the  $\alpha$ -cDNA were a cloning artefact. These investigations included mapping of the gln- $\alpha$

transcriptional start site to the sequence divergence point which in turn revealed the existence of a TATA box consensus sequence - 28bp relative to the transcriptional start site (see fig. 3.5).

Following characterisation of the gln- $\alpha$  clone, the cell- and tissue-specific activity of the gln- $\alpha$  gene promoter was studied in transgenic plants. The gln- $\alpha$  translational start site was mutated to an NcoI restriction enzyme site using PCR and a mismatched primer. This NcoI site was then used to fuse the gln- $\alpha$  promoter to the coding region of the E. coli  $\beta$ -glucuronidase uidA reporter gene (see fig. 2.3). The construct was then subcloned into the plant transformation vector Bin19 and the resulting T-DNA was introduced into both the non-legume tobacco and the legume L. corniculatus using Agrobacterium-mediated DNA transfer. GUS activity was measured accurately in cell free extracts using a fluorimetric assay of various transgenic plant tissues and histochemical analysis was used to study the localisation of GUS activity in the transgenic cells and tissues.

The measurement and localisation of GUS activity within the transgenic plant helped confirm and further elaborate on the expression pattern of the gln- $\alpha$  gene as determined for P. vulgaris. Ortega et al. (1986) showed that for P. vulgaris the  $\beta$ -polypeptide was the main component of the root GS in the embryo and in to 5 day old roots but from day 5, the  $\alpha$ -polypeptide becomes the predominant root GS subunit. A possible explanation of these results is that gln- $\alpha$  expression is confined to the root-tip, and indeed more recently, immunocytochemical techniques

have located GS in the root-tips of P. vulgaris plants (Datta et al., 1991). In the present study, measurement of GUS activity in the root-tips of transgenic gln- $\alpha$ ::uidA tobacco plants showed that the gln- $\alpha$  promoter and upstream regions directed root-tip expression (see fig. 4.2).

From the studies of Cock et al. (1991), it was shown that the  $\alpha$ -mRNA was abundant in both the plumules of P. vulgaris dry seeds and in the young leaf up to 3 days after germination. Beyond this time point however,  $\alpha$ -mRNA became undetectable and was absent from mature P. vulgaris leaves. Relatively immature transgenic gln- $\alpha$ ::uidA tobacco leaves were shown to contain low but significant levels of GUS activity as compared to transgenic promoterless::uidA leaves (see fig. 4.10). This may have reflected the prolonged translation of the uidA mRNA synthesised during earlier stages of leaf development and/or the high stability of the GUS protein. Subsequent histochemical analysis of immature transgenic gln- $\alpha$ ::uidA leaves revealed that expression of the gln- $\alpha$  gene was confined to the leaf's vascular tissue (see fig. 4.13).

Extensive studies regarding the differential expression of P. vulgaris GS genes during nodule development showed that gln- $\alpha$  mRNA was present in isolated nodules but that its abundance was greater in younger nodules than in older nodules (Bennett et al., 1989). Histochemical analysis of nodulated transgenic roots of L. corniculatus plants demonstrated that gln- $\alpha$  expression was not however associated with either young or mature nodules but

was possibly associated with the nodule primordia (see fig. 5.3).

The work of Daniell (1992) and Cock et al. (1990) demonstrated that gln- $\alpha$  gene expression was induced by mechanical wounding and during nodule senescence respectively. Measurement of GUS levels in wounded transgenic gln- $\alpha$ ::uidA plant leaves showed a 2- to 5-fold induction after 24 hours as compared to non-wounded transgenic leaf tissue (see fig. 4.26.a). Conversely, histochemical analysis of mature senescing transgenic gln- $\alpha$ ::uidA L. corniculatus nodules revealed gln- $\alpha$  promoter activity was associated with the inner cortex and rhizobially infected zone of the nodule (see fig. 5.4.a). Activity of the gln- $\alpha$  promoter was not associated with any tissue of non-senescing mature gln- $\alpha$ ::uidA nodules.

Finally, Swarup et al. (1990) demonstrated that  $\alpha$ -mRNA and polypeptide were the only GS mRNA and polypeptide present in the cotyledons of P. vulgaris dry seeds and the major GS mRNA and polypeptide in the cotyledons of P. vulgaris seeds up to 2 days after germination. They proposed that both the  $\alpha$ -mRNA and polypeptide are expressed during cotyledon development and persist into the mature seed (Swarup et al., 1990). Histochemical analysis revealed that the gln- $\alpha$  promoter was active within the cotyledons of developing transgenic gln- $\alpha$ ::uidA seeds (see fig. 4.20) and within the vasculature of cotyledons of 2-week-old transgenic gln- $\alpha$ ::uidA seedlings (see fig. 4.24).

The transgenic plant studies revealed sites of gln- $\alpha$  expression previously undetected. Promoter activity was

associated within root hairs, lateral root initiation sites, emerging lateral roots and within the root vascular tissue (see figs 4.8, 4.6, and 4.5, respectively). With the exception of root hairs, the pattern of root-specific expression was similar for both plant systems. Analysis of the transgenic tobacco plant system further revealed the gln- $\alpha$  promoter was active within various floral and fruit structures including the developing flower stamen and pistil and the developing fruit placenta, vascular tissue, and wall (see figs. 4.15, 4.16 and 4.20 respectively).

The present study did however have its limitations. Firstly, only approximately 900bp of promoter sequence was cloned. Although a cytosolic GS promoter from pea has been shown to be active even when deleted to -132bp relative to the start of transcription (Brears et al., 1991), it may be possible that certain upstream regulatory sequences were not included in the promoter fragment cloned.

Secondly, the promoter fragment was isolated from the original bacteriophage clone "a" using the PCR technique. The technique uses the thermostable Tag DNA polymerase which cannot proofread as it lacks a 3' to 5' exonuclease activity. Tag DNA polymerase is therefore an error prone DNA polymerase with a measured rate of  $10^{-5}$  to  $10^{-4}$  error per nucleotide synthesised (Saiki et al., 1988). This will of course be compounded further by the re-use of the PCR technique in the mutation of the promoter fragment during the construction of the gln- $\alpha$ ::gusA

fusion. If critical regulatory sequences had been altered during PCR amplification, it would have been expected that the expression pattern of the gln- $\alpha$  promoter clone in transgenic plants would have been greatly different to that previously determined for the gln- $\alpha$  gene within its original plant, that is, P. vulgaris. As the pattern of expression was similar for both, it appears that the PCR amplification had not substantially altered the promoter fragment's regulatory properties, if at all.

Thirdly, a major feature of the tobacco plant transformation work was the "between-line" variability in GUS activity. As this "between-line" variability has been recorded for the majority of transgenic plant studies (for example, Jones et al., 1985), it was expected to occur. However, the variability in quantitative measurements of gln- $\alpha$ ::uidA activity extended to the same tissues of the same transgenic plant sampled at different times (see table 2). This may reflect the sensitivity of the gln- $\alpha$  promoter to physiological conditions. What was of greater concern however, was the differences in the spatial-pattern of GUS activity in the root-tips of different transgenic gln- $\alpha$ ::uidA tobacco lines. The spatial-patterns of expression were identical between different root-tips of the same plant line and between repeated sampling of different plants of the same transgenic line. One possible explanation for this may be the influence of tobacco genomic sequences flanking the introduced chimeric gene. Studies have shown that sequences several kilobases from the introduced gene can alter its transcriptional activity (Odell et al., 1987).

Alternatively, the gln- $\alpha$  upstream region cloned may only contain part of a regulatory element rendering the gln- $\alpha$ ::uidA construct particularly sensitive to its genomic environment. Indeed, in other studies, qualitative differences between transgenic lines was not generally seen.

Fourthly, and finally, the number L. corniculatus plant root structures displaying histochemical staining were very small, from approximately 5 per hairy root system for root-tip, emerging lateral root and root vascular tissue staining to approximately 5 in total for nodule primordia/lateral root initiation site and senescing nodule staining. Even root structures known to be transgenic and known to be sites of gln- $\alpha$  promoter activity failed to stain upon incubation with the histochemical substrate. The lack of high numbers limited the applicability of the results obtained from the transgenic L. corniculatus system. However, it is noteworthy that the spatial-pattern of gln- $\alpha$  gene expression in the transgenic L. corniculatus and tobacco systems showed some similarities.

Future studies regarding gln- $\alpha$  gene expression may involve the cloning and use in transgenic plant studies of a larger gln- $\alpha$  promoter fragment. Future studies may also involve the elucidation of those regions of the promoter involved in tissue-specificity. Such studies for example may involve a series of 5'-end deletions of the promoter and the fusion of these deletions to a reporter-gene such as uidA. The different constructs would then be analysed in transgenic plants. Sequencing of the gln- $\alpha$



promoter revealed no suitable restriction enzyme sites for the deletion of sections of the promoter and deletions may therefore involve the incubation of the promoter fragment with an exonuclease enzyme for differing periods of time. In addition, protein-DNA interaction studies may elucidate those promoter sequences involved in the binding of transcriptional activators. These sequences may then be deleted or mutated and the effect of this monitored in transgenic plants.

Future studies may also be aimed at determining specific functions of the gln- $\alpha$  gene although the present transgenic plant studies did reveal several possible functions. As discussed in section 4.10.b, the elucidation of the specific function(s) for gln- $\alpha$  expression within certain tissues and cell-types may involve the study of plants lacking gln- $\alpha$  expression either through plant mutation or through  $\alpha$ -cDNA antisense transgenic plant techniques. Furthermore, the proposed co-regulation of the gln- $\alpha$  and PAL genes may be further investigated using studies involving the inhibition of PAL gene expression and/or the inhibition of PAL enzyme activity in P. vulgaris. As mentioned in section 4.10.c, PAL activity and the rate of synthesis of PAL subunits are inhibited by the exogenous application of the end-product of the PAL reaction, cinnamic acid (CA) to elicitor treated P. vulgaris suspension cultures (Bolwell, et al., 1986). Studies suggest that the removal of PAL activity by CA may involve the inactivation of the enzyme through modification of the enzymes active site and this inactivation is accompanied by

an inhibition of the rate of synthesis of PAL subunits (Bolwell, et al., 1986). The effect of CA on gln- $\alpha$  gene transcription (and PAL1 gene transcription) in for example, wounded P. vulgaris hypocotyls may therefore indicate at which level the possible co-induction of the PAL1 and gln- $\alpha$  genes operates. Studies have shown that GS expression in P. vulgaris is not induced either indirectly or directly by the addition of ammonium, the enzymes substrate. Therefore, the production of ammonium through PAL activity is unlikely to be the regulatory factor in the co-induction of the PAL and gln- $\alpha$  genes in response to various environmental stimuli.

## Chapter 7: References.

- Abel, P.P., Nelson, R.S., Hoffmann, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232, 738-743.
- An, G., Ebert, P.R., Mitra, A., and Ha, S.B. (1988). Binary vectors. In "Plant Molecular Biology Manual" A3, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds. (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1-19.
- Benfey, N.B., Ren, L., and Chua, N-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO J.* 8, 2195-2202.
- Bennett, M.J. and Cullimore, J.V. (1989). Glutamine synthetase isoenzymes of Phaseolus vulgaris L.: subunit composition in developing root nodules and plumules. *Planta* 179, 433-440.
- Bennett, M.J., Lightfoot, D.A. and Cullimore, J.V. (1989). cDNA sequence and differential expression of the gene encoding the glutamine synthetase polypeptide of Phaseolus vulgaris L. *Plant Mol. Biol.* 12, 553-565.
- Bevan, M. (1984). Binary Agrobacterium vectors for plant transformation. *Nucl. Acids Res.* 12, 8711-8721.
- Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R., and Schuch, W. (1989). Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. *EMBO J.* 8, 1899-1906.

- Bickle, T. (1982). The ATP-dependent restriction nucleases. In: Nucleases, S.M. Lynn, and R.J. Roberts eds., Cold Spring Harbour Lab. Cold Spring Harbor, NY, pp. 85-108.
- Birnboim, B.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7, 1513-1523.
- Blackwell, R.D., Murray, A.J.S., and Lea, P.J. (1987). Inhibition of photosynthesis in barley with decreased levels of chloroplastic glutamine synthetase activity. J. Expt. Biol. 38, 1799-1809.
- Blotting and hybridisation protocols for Hybond membranes. Amersham International plc.
- Blumenthal, R.M. (1989). Cloning and restriction of methylated DNA in Escherichia coli. Focus 11, 41-46.
- Bolton, G.W., Nester, E.W., and Gordon, M.P. (1986). Plant phenolic compounds induce expression of the Agrobacterium tumefaciens loci needed for virulence. Science 232, 983-985.
- Bolwell, G.P., Cramer, C.L., Lamb, C.J., Schuch, W., and Dixon, R.A. (1986). L-Phenylalanine ammonia-lyase from Phaseolus vulgaris: Modulation of the levels of active enzyme by trans-cinnamic acid. Planta 169, 97-107.
- Bouchez, D., Tokkuhisa, J.G., Llewellyn, D.J., Dennis, E.S., and Ellis, J.G. (1989). The ocs-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J. 8, 4197-4200.
- Bradford, M.M. (1976). A rapid and sensitive method for the

- quatitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Brears, T., Walker, E.L., and Coruzzi, G.M. (1991). A promoter sequence involved in cell-specific expression of the pea glutamine synthetase GS3A gene in organs of transgenic tobacco and alfalfa. *Plant J.* 1, 235-244.
  - Brouillard, R. (1988). Flavonoids and flower colour. In "The flavonoids, advances in research since 1980". J.B. Harborne, ed. (London: Chapman and Hall), pp. 525-538.
  - Bryant, J.A. (1976). Nuclear DNA. In "Molecular aspects of gene expression in plants". J.A. Bryant, ed. (London: Academic Press), pp. 1-51.
  - Chandler, G., Ladley, P., McNally, S.F., Stewart, G.R., and Sumar, N. (1985). The activity and isoform complement of glutamine synthetase in Panicum species differing in photosynthetic pathway. *J. Plant Physiol.* 121, 13-21.
  - Casanova, J-L., Pannetier, C., Jaulin, C., and Kourilsky, P. (1990). Optimal conditions for directly sequencing double-stranded PCR products with Sequenase. *Nuc. Acids. Res.* 18, 4028. cDNA cloning systems booklet, Amersham International p.l.c.
  - Chappell, J., and Hahlbrock, K. (1984). Transcription of plant defence genes in response to UV light or fungal elicitor. *Nature* 311, 76-78.
  - Chen, F.L., Bennett, M.J., and Cullimore, J.V. (1990). Effect of nitrogen supply on the activities of isoenzymes of NADH-dependent glutamate synthase and glutamine synthetase in root nodules of

- Phaseolus vulgaris L. J. Exp. Bot. 41, 1215-1221.
- Chyi, Y-S., Jorgensen, R.A., Goldstein, D., Tanksley, S.D., and Loaiza-Figueroa, F. (1986). Locations and stability of Agrobacterium-mediated T-DNA insertions in the Lycopersicon genome. Mol. Gen. Genet. 204, 64-69.
  - Cock, J.M., Brock, I.W., Watson, A.T., Swarup, R., Morby, A.P., and Cullimore, J.V. (1991). Regulation of glutamine synthetase in leaves of Phaseolus vulgaris. Plant Mol. Biol. 17, 761-771.
  - Cock, J.M., Mould, R.M., Bennett, M.J., and Cullimore, J.V. (1990). Expression of glutamine synthetase genes in roots and nodules of Phaseolus vulgaris following changes in the ammonium supply and infection with various Rhizobium mutants. Plant Mol. Biol. 14, 549-560.
  - Comai, L., Facciotti, D., Hiatt, W.R., Thompson, G., Rose, R.E., and Stalker, D.M. (1985). Expression in plants of a mutant aroA gene from Salmonella typhimurium confers tolerance to glyphosate. Nature 317, 741-744.
  - Cramer, C.L., Edwards, K., Dron, M., Liang, X., Dildine, S.L., Bolwell, G.P. Dixon, R.A., Lamb, C.L., and Schuch, W. (1989). Phenylalanine ammonia-lyase gene organisation and structure. Plant Mol. Biol. 12, 367-383.
  - Cullimore, J.V., Gebhardt, C., Saarelainen, R., Miflin B.J., Ilder, K.B., and Barker, R.F. (1984). Glutamine synthetase of Phaseolus vulgaris L.: Organ-specific expression of a multigene family. J. Mol. Appl. Genet. 2, 589-599.
  - Cullimore, J.V., and Bennett, M.J. (1988). The molecular biology

- and biochemistry of plant glutamine synthetase from root nodules of Phaseolus vulgaris L. and other legumes. J. Plant Physiol. 132, 387-393.
- Daniell, T. (1992). Phd thesis, University of Warwick, UK.
  - Datta, B.D., Cai, X., Wong, P.P., and Triplett, E.W. (1991). Immunocytochemical localisation of glutamine synthetase in organs of Phaseolus vulgaris L.. Plant Physiol. 96, 507-512.
  - Dellaporta, S., Wood, J., Hicks, J. (1983). A rapid method for DNA extraction from plant tissue. Plant Mol. Biol. Rep. 1, 19-21.
  - Edwards, K., Cramer, C.L., Bolwell, G.P., Dixon, R.A., Schuch, W., and Lamb, C.J. (1985). Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells. Proc. Natl. Acad. Sci. USA 82, 6731-6735.
  - Edwards, J.E., and Coruzzi, G.M. (1989). Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. Plant Cell 1, 241-248.
  - Edwards, J.E., Walker, E.L., and Coruzzi, G.M. (1990). Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc. Natl. Acad. Sci. USA 87, 3459-3463.
  - Estabrook, E.M., and Sengupta-Golopan, C. (1991). Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3, 299-308.
  - Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Genet. Microbiol. 16, 374-381.



- Feinberg and Vogelstein. (1983). A technique of radiolabelling DNA restriction endonuclease fragments to a high specific activity. Anal. Biochem. 132, 6-13.
- Forde, B.G., and Cullimore, J.V. (1989). The molecular biology of glutamine synthetase in higher plants. Oxf. Surv. Plant Mol. Biol. 6, 247-296.
- Forde, B.G., Day, H.M, Turton, J.F., Shen, W-J., Cullimore, J.V., and Oliver, J.E. (1989). Two glutamine synthetase genes from Phaseolus vulgaris L. display contrasting developmental and spatial patterns of expression in transgenic Lotus corniculatus plants. Plant Cell 1, 391-401.
- Forde, B.G., Freeman, J., Oliver, J.E., and Pineda, M. (1990). Nuclear factors interact with conserved A/T-rich elements upstream of a nodule-enhanced glutamine synthetase gene from French bean. Plant Cell 2, 925-939.
- Fromm, H., Katagari, F., Chua, N-H. (1989). An octapine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell 1, 977-984.
- Gebhardt, C., Oliver, J.E., Forde, B.G., Saarelainen, R., and Mifflin, B.J. (1986). Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of Phaseolus vulgaris. EMBO J. 5, 1429-1435.
- Goodall, G.J., and Filipowicz, W. (1989). The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58, 473-483.

- Graham, M.W., Doherty, J.P. and Woodcock, D.M. (1990). Efficient construction of plant genomic libraries requires the use of mcr-host strains and packaging mixes. Plant Mol. Rep. 8, 18-27.
- Groat, R.G., and Vance, C.P. (1981). Root nodule enzymes of ammonia assimilation in alfalfa (Medicago sativa L.). Plant Physiol. 67, 1198-1203.
- Groat, R.G. and Vance, C.P. (1982). Root and nodule enzymes of ammonium assimilation in two plant-conditioned ineffective genotypes of alfalfa (Medicago sativa L.) Plant Physiol. 69, 614-618.
- Handa, S., Huber, D.M., Warren, H.L., and Tsai, C.Y. (1985). Nitrogen nutrition and N-assimilation in maize seedlings. Can. J. Plant Sci. 65, 87-93.
- Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166, 557-580.
- Hansen, J., Jorgensen, J-E., Stougaard, J., Marcker, K.A. (1989). Hairy roots - a short cut to transgenic root nodules. Plant Cell Rep. 8, 12-15.
- Hemon, P., Robbins, M.P., and Cullimore, J.V. (1990). Targeting of glutamine synthetase to the mitochondria of transgenic tobacco. Plant Mol. Biol. 15, 895-904.
- Hirel, B., Bouet, B., King, B., Layzell, D., Jacobs, F., and Verma, D.P.S. (1987). Glutamine synthetase genes are regulated by ammonia provided externally or by symbiotic nitrogen fixation. EMBO J. 6, 1167-1171.
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers,

- S.G., Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science* 227, 1229-1231.
- Jacobs, M., and Rubery, P.H. (1988). Naturally occurring auxin transport regulators. *Science* 241, 346-349.
  - Jacobsen, K., Laursen, N.B., Jensen, E.O., Marckerf, A., Poulsen, C., and Marcker, K.A. (1990). HMG I-like proteins from leaf and nodule nuclei interact with different AT motifs in soybean nodulin promoters. *Plant Cell* 2, 85-94.
  - Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986).  $\beta$ -glucuronidase from Escherichia coli as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* 83, 8447-8451.
  - Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions;  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901-3907.
  - Jones, D.H. (1984). Phenylalanine ammonia lyase: regulation of its induction, and its role in plant development. *Phytochem.* 23, 1349-1359
  - Jones, J.D.G., Dunsmuir, P., and Bedbrook, J. (1985). High level expression of introduced chimaeric genes in regenerated transformed plants. *EMBO J.* 10, 2411-2418.
  - Joshi, C.P. (1987). An inspection of the putative TATA box and translation start in 79 plant genes. *Nucl. Acids Res.* 15, 6643-6653.
  - Joy, K.W. (1988). Ammonia, glutamine, and asparagine: a carbon-nitrogen interface. *Can. J. Bot.* 66, 2103-2109.
  - Karlin-Neumann, G.A., and Tobin, E.M. (1986). Transit peptides of

nuclear-encoded chloroplast proteins share a common amino acid framework. EMBO J. 5, 9-13.

- Katagari, F., Lam, E., and Chua, N-H. (1989). Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature 340, 727-730.
- Kawakami, N., and Watanabe, A. (1988). Senescence-specific increase in cytosolic glutamine synthetase and its mRNA in radish cotyledons. Plant Physiol. 88, 1430-1434.
- Kehrel, B., and Wiermann, R. (1985). Immunochemical localisation of phenylalanine ammonia-lyase and chalcone synthase in anthers. Planta 163, 183-190.
- Kendall, A.C., Wallsgrave, R.M., Hall, N.P., Turner, J.C., and Lea, P.J. (1986). Carbon and nitrogen metabolism in barley (Hordeum vulgare L.) mutants lacking ferredoxin-dependent glutamate synthase. Planta 168, 316-323.
- Keys, A.J., Bird, I.F., Cornelius, M.J., Lea, P.J., Wallsgrave, R.M., and Mifflin, B.J. (1978). Photorespiratory nitrogen cycle. Nature 275, 741-743.
- Kleinhofs, A., and Warner, R.L. (1990). Advances in nitrate assimilation. In "The Biochemistry of Plants" Vol 16, B.J. Mifflin ed., (New York: Academic Press) pp. 89-120.
- Koes, R.E., van Blokland, R., Quattrocchio, F., van Tunen, A.J., and Mol, J.N.M. (1990). Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types. Plant Cell 2, 379-392.
- Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., and

- Goldberg, R.B. (1990). Different temporal and spatial gene expression patterns occur during anther development. *Plant Cell* 2, 1201-1224.
- Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283-292.
  - Kosaki, A., Sakamoto, A., Tanaka, K., and Takeba, G. (1991). The promoter of the gene for glutamine synthetase from rice shows organ-specific and substrate-induced expression in transgenic tobacco plants. *Plant Cell Physiol.* 32, 353-358.
  - Kosugi, S., Ohashi, Y., Nakajima, K., and Arai, Y. (1990). An improved assay for  $\beta$ -glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous  $\beta$ -glucuronidase activity. *Plant Sci.* 70, 133-140.
  - Kreig, P.A. and Melton, D.A. (1987). In-vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* 155, 397-415.
  - Lara, M., Porta, H., Padilla, J., Folch, J., and Sanchez, F. (1984). Heterogeneity of glutamine synthetase polypeptides in Phaseolus vulgaris L. *Plant Physiol.* 76, 1019-1023.
  - Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. (1989). Signals and transduction mechanisms for activity of plant defences against microbial attack. *Cell* 56, 215-224.
  - Lawton, M.A., and Lamb, C.J. (1987). Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. *Mol. Cell. Biol.* 7, 335-341.
  - Lea, P.J., Robinson, S.A., and Stewart, G.R. (1990). The

- enzymology and metabolism of glutamine, glutamate, and asparagine. In "The Biochemistry of Plants" Vol 16, B.J. Mifflin ed., (New York: Academic Press) pp. 121-159.
- Liang, X., Dron, M., Schmid, J., Dixon, R.A., and Lamb, C.J. (1989a). Developmental and enviromental regulation of a phenylalanine ammonia-lyase- $\beta$ -glucuronidase gene fusion in transgenic tobacco plants. Proc. Nalt. Acad. Sci. USA 86, 9284-9288.
  - Liang, X., Dron, M., Cramer, C.L., Dixon, R.A., and Lamb, C.J. (1989b). Differential regulation of phenylalanine ammonia-lyase genes during plant development and by enviromental cues. J. Biol. Chem. 264, 14486-14492.
  - Lightfoot, D.A., Green, N.K., and Cullimore, J.V. (1988). The chloroplast-located glutamine synthetase of Phaseolus vulgaris L.: nucleotide sequence, expression in different organs and uptake into isolated chloroplasts. Plant Mol. Biol. 11, 191-202.
  - Logemann, J., Schell, J., Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Analyt. Biochem. 163, 16-20.
  - Lois, R., Dietrich, A., Hahlbrock, K., and Schulz, W. (1989). A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis- acting elements. EMBO J. 8, 1641-1648.
  - Long, S.R. (1989). Rhizobium-legume nodulation: life together in the underground. Cell 56, 203-214.
  - McNally, S., and Hirel, B. (1983). Glutamine synthetase isoforms

- in higher plants. *Physiol Veg.* 21, 761-774.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, NY: Cold Spring Harbour Laboratory).
  - McNally, S.F., Hirel, B., Gadai, P., Mann, A.F., Stewart, G.R. (1983). Glutamine synthetases of higher plants. *Plant Physiol.* 72, 22-25.
  - Meeks, J.C., Wolk, C.P., Schilling, N., Shaffer, P.W., Avissar, Y., and Chein, W-S. (1978). Initial organic products of fixation of ( $^{15}\text{N}$ ) dinitrogen by root nodules of soybean (*Glycine max*). *Plant Physiol* 61, 980-983.
  - Miao, G-H, Hirel, B., Marsolier, Ridge, R.W., and Verma, D.P.S. (1991). Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. *Plant Cell* 3, 11-22.
  - Mifflin, B.J. and Lea, P.J. (1980). Ammonia Assimilation. In "The Biochemistry of Plants", Vol. 5, B.J. Mifflin, ed., (New York: Academic Press), pp. 169-202.
  - Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiol* 15, 473-497.
  - Odell, J.T., Nagy, F., Chua, N-H. (1987). In "Plant gene systems and their biology", Vol. 62, J. Key and L. McIntosh eds., (A.R. Liss, New York), pp. 321-329.
  - Ohl, S., Hedrick, S.A., Chory, J., and Lamb, C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter

- from *Arabidopsis*. *Plant Cell* 2, 837-848.
- Ortega, J-L., Campos, F., Sanchez, F., and Lara, M. (1986). Expression of two different glutamine synthetase polypeptides during root development in *Phaseolus vulgaris* L.. *Plant Physiol* 80, 1051-1054.
  - Ortega, J-L., Sanchez, F., Soberon, M., and Flores, M.L. (1992). Regulation of nodule glutamine synthetase by CO<sub>2</sub> levels in bean (*Phaseolus vulgaris* L.). *Plant Physiol.* 98, 584-587.
  - Padilla, J.E., Campos, F., Conde, V., Lara, M., and Sanchez, F. (1987). Nodule-specific glutamine synthetase is expressed before the onset of nitrogen fixation in *Phaseolus vulgaris* L. *Plant Mol. Biol.* 9, 65-74.
  - Pat, J.S., and Atkins, C.A. (1983). In "Nitrogen Fixation" (W.J. Broughton, ed.), Vol. 3, pp 245-325. Cambridge Univ. Press, London.
  - Pate, J.S., and Layzell, D.B. (1990). Energetics and biological costs of nitrogen assimilation. In "The Biochemistry of Plants", Vol. 16, B.J. Mifflin ed. (New York: Academic Press), pp. 1-42.
  - Pater, S., Pham, K., Chua, N-H., Memelink, J., and Kijne, J. (1993). A 22-bp fragment of the pea lectin promoter containing essential TGAC-like motifs confers seed-specific gene expression. *Plant Cell* 5, 877-886.
  - Pearse, A.G.E. (1972). In "Histochemistry. Theoretical and Applied", Vol. 2, (Churchill Livingstone: Edinburgh).
  - Peterman, T.K., and Goodman H.M. (1991) The glutamine synthetase gene family of *Arabidopsis thaliana*: light-regulation and



- differential expression in leaves, roots and seeds. Mol. Gen. Genet. 230, 145-154.
- Plegt, L., and Bino, R.J. (1989).  $\beta$ -Glucuronidase activity during development of the male gametophyte from the transgenic and non-transgenic plants. Mol. Gen. Genet. 216, 321-327.
  - Raliegh, E.A., and Wilson, G. (1986). Escherichia coli K-12 restricts DNA containing 5-methylcytosine. Proc. Natl. Acad. Sci. USA 83, 9070-9076.
  - Rawsthorne, S., Minchin, F., Summerfield, R.J., Cookson, C., and Coombs, J. (1980). Carbon and nitrogen metabolism in legume root nodules. Phytochem. 19, 341-355.
  - Robertson, G., and Farnden, K.J.F. (1980). Ultrastructure and metabolism of the developing legume root nodule. In "The Biochemistry of Plants", Vol. 5, B.J. Mifflin, ed., (New York: Academic Press), pp. 65-113.
  - Rubery, P.H., and Northcote D.H. (1968). Sites of phenylalanine ammonia-lyase activity and synthesis of lignin during xylem differentiation. Nature 219, 1230-1234.
  - Ryder, T.B., Hedrick, S.A., Bell, J.N., Liang, X., Clouse, S., and Lamb, C.J. (1987). Organisation and differential activation of a gene family encoding the plant defence enzyme chalcone synthase in Phaseolus vulgaris. Mol Gen. Genet. 210, 219-233.
  - Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 237, 487-491.

- Sanger, F., Milken, S., Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- Salinas, J., Oeda, K., and Chua, N-H. (1992). Two G-box related sequences confer different expression patterns in transgenic tobacco. Plant Cell 4, 1485-1493.
- Sengupta-Golopan, C., and Pitas, J.W. (1986). Expression of nodule-specific glutamine synthetase genes during nodule development in soybeans. Plant Mol. Biol. 7, 189-199.
- Schmeltzer, E., Jahnen, W., and Halbrock, K. (1988). In situ localisation of light-induced chalcone synthase mRNA, chalcon synthase, and flavonoid end products in epidermal cells of parsley leaves. Proc. Natl. Acad. Sci. 85, 2989-2993.
- Schmid, J., Doerner, P.W., Clouse, S.D., Dixon, R.A., and Lamb, C.J. (1990). Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell 2, 619-631.
- Schreier, P., Seftor, E.A., Schell, J., and Bohnert, H.J. (1985). The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. 4, 25-32.
- Schubert, K.R., and Boland, M.J. (1990). The Ureides. In "The Biochemistry of Plants". Vol. 16, B.J. Mifflin ed. (New York: Academic Press), pp. 197-282.
- Sengupta-Gopalan, G., and Pitas, J.W. (1986). Expression of nodule-specific glutamine synthetase genes during nodule

- development in soybeans. *Plant Mol. Biol.* 7, 189-199.
- Shen, W-J., Williamson, M., and Forde, B.G. (1992). Functional analysis of the promoter region of a nodule-enhanced glutamate synthetase gene from Phaseolus vulgaris L.. *Plant Mol. Biol.* 19, 837-846.
  - Sieciechowicz, K.A., Joy, K.W., and Ireland, R.J. (1988). The metabolism of asparagine in plants. *Phytochem.* 27, 663-671.
  - Simpson, J., Timko, M.P., Cashmore, A.R., Schell, J., Montagu, M.V., and Herrera-Estrella, L. (1985). Light-inducible and tissue-specific expression of a chimaeric gene under control of the 5'-flanking sequence of a pea chlorophyll a/b-binding protein gene. *EMBO J.* 4, 2723-2729.
  - Sommerville, C.R., and Ogren, W.L. (1980). Inhibition of photosynthesis in Arabidopsis mutants lacking leaf glutamate synthase activity. *Nature* 286, 257-259.
  - Sorge, J.A. (1988). In "vectors: A survey of molecular cloning vectors and their uses". R.L. Rodriguez and D.T. Denhardt eds. (Butterworth Publishers, Stoneham), pp. 43-59.
  - Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503-517.
  - Stewart, G.R. Mann, A.F. and Fentem, P.A. (1980). Enzymes of glutamate formation: glutamate dehydrogenase, glutamate synthase, and glutamine synthetase. In "The Biochemistry of Plants", Vol. 5, Miflin, B.J. ed. (Academic Press, New York), pp. 271-327.
  - Stratagene Cloning Systems.

- Swarup, R., Bennett, M.J., and Cullimore, J.V. (1990). Expression of glutamine synthetase genes in cotyledons of germinating Phaseolus vulgaris L. *Planta* 183, 51-56.
- Thomas, H. (1978). Enzymes of nitrogen mobilisation in detached leaves of Lolium temulentum during senescence. *Planta* 142, 161-169.
- Tingey, S.V. and Coruzzi, G.M. (1987). Glutamine synthetase of Nicotiana plumbaginifolia. *Plant Physiol.* 84, 366-373.
- Tingey, S.V., Tsai, F-Y., Edwards, J.W., Walker, E.L., and Coruzzi, G.M. (1988). Chloroplastic and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed in-vivo. *J. Biol. Chem.* 263, 9651-9657.
- Tingey, S.V., Walker, E.L., and Coruzzi, G.M. (1987). Glutamine synthetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. *EMBO J.* 6, 1-9.
- Tischer, E., Shiladitya, D., and Goodman, H.M. (1986). Nucleotide sequence of an alfalfa glutamine synthetase gene. *Mol. Gen. Genet.* 203, 221-229.
- Tobin, A.K., Sumar, N., Patel, M., Moore, A.L., and Stewart, G.R. (1988). Development of photorespiration during chloroplast biogenesis in wheat leaves. *J. Expt. Bot.* 204, 833-843.
- Turton, J.F., Hopley, A.P., and Forde, B.G. (1988). 5'-flanking sequence of a glutamine synthetase gene specifying the  $\alpha$ -subunit of the cytosolic enzyme from Phaseolus vulgaris L.. *Nucl. Acids Res.* 16, 11367.

- Van-Brussel, A.A., Recourt, K., Pees, E., Spaink, H.P., Tak, T., Wijffelman, C.A., Wijne, J.N., and Lugtenberg, B.J.J. (1990). A biovar-specific signal of Rhizobium leguminosarum bv. viciae induces increased nodulation gene-inducing activity in root exudate of Vicia sativa subsp. nigra. J. Bacteriol. 172, 5394-5401.
- Wallsgrave, R.M., Turner, J.C., Hall, N.P., Kendall, A.C., and Bright, S.W.J. (1987). Barley mutants lacking chloroplast glutamine synthetase - biological and genetic analysis. Plant Physiol. 83, 155-158.
- Walker, E.L., and Coruzzi, G.M. (1989). Developmentally regulated expression of the gene family for cytosolic glutamine synthetase in Pisum sativum. Plant Physiol. 91, 702-708.
- Westaway, D., Goodman, P.A., Mirenda, C.A., McKindley, M.P., Carlson, G.A., and Pruisiner, S.B. (1987). Distinct prion proteins in short and long scrapie incubation period mice. Cell 51, 651-662.
- Wingate, V.P.M., Lawton, M.A., and Lamb, C.J. (1988). Glutathione causes a massive induction of plant defence genes. Plant Physiol. 87, 206-210.
- Winkler, R.G., Blevins D.G., Polacco, J.C., and Randall, D.D. (1988). Ureide catabolism in nitrogen-fixing legumes. TIBS. 13, 97-100.
- Yu, L.M. Lamb, C.J., and Dixon, R.A. (1993). Purification and biochemical characterisation of proteins which bind to the H-box cis-element implicated in transcriptional activation of plant

defence genes. Plant J. 3, 805-816.