Cloning and characterisation of *S*adenosyl-L-homocysteine hydrolase

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester.

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September, 1996

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Acknowledgements

My thanks go to both of my supervisors, Prof. John Draper (University of Leicester) and Dr. Andy Greenland (ZENECA) for their help and suggestions during the course of this study, and for proofreading this thesis. I am grateful to Dr. Simon Warner for his helpful teaching and assistance at the bench, and also for his corrections of this thesis. Thanks to everyone else in the Department of Botany, past and present for their help during my time at Leicester. Thanks must also be extended to Dr. Robert Edwards at the University of Durham for letting me work in his laboratory and providing me with SHH enzyme analysis methodology. Finally, a special mention to Louise for her support at all times.

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Mark Skipsey: Cloning and characterisation of S-adenosyl-L-homocysteine hydrolase

This thesis concerns S-adenosyl-L-homocysteine hydrolase (SHH), the enzyme responsible for the reversible hydrolysis of S-adenosyl-L-homocysteine (SAH) to adenosine and homocysteine. SAH is formed as a direct product of transmethylation reactions involving S-adenosyl-L-methionine (SAM) and is known to be a potent inhibitor of most SAM mediated methyltransfer reactions. An Asparagus officinalis cDNA showing strong homology to previously cloned SHH cDNAs was identified by the random sequencing of cDNAs from a library enriched for wound induced clones. PCR primers were designed which allowed the amplification of a region of the SHH gene from any plant species tested to date. The alignment of sequences from both cloned PCR products, A. officinalis cDNAs, and previously isolated SHH clones, highlights the high level of sequence homology retained between divergent species as well as the presence of an extra polypeptide motif in the amino acid sequence of SHH genes from photosynthetic species. Northern analysis using RNA isolated from asparagus suggested SHH is transcriptionally upregulated from its constitutive low level by a wound stimulus. An Arabidopsis thaliana genomic SHH clone was isolated and used in promoter/reporter gene fusion studies to facilitate a detailed study of the temporal and spatial expression pattern of the SHH gene. Data obtained from this study corroborated evidence obtained from northern, western and enzyme activity analysis, suggesting SHH was expressed in all plant organs. Perturbation of SHH gene expression was also attempted within transgenic plants to try and further elucidate biochemical pathways requiring SHH enzyme activity.

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Abbreviations

- 1-aminocyclopropane-1-carboxylic acid
- Adenosine Triphosphate
- 5-bromo-4-chloro-3-indoyl phosphate
- base pair
- Bovine Serum Albumin
- Degrees Centigrade
- complementary DNA
- Calf Intestinal Alkaline Phosphatase
- centimetre
- counts per minute
- hexadecyltrimethylammonium bromide
- deoxyadenosine triphosphate
- deoxycytosine triphosphate
- sterilised double distilled water
- deoxyguanosine triphosphate
- deoxythymidine triphosphate
- deoxynucleoside triphosphate
- dideoxyadenosine triphosphate
- dideoxycytosine triphosphate
- dideoxyguanosine triphosphate
- dideoxythymidine triphosphate
- dideoxynucleoside triphosphate
- dimethylformamide
- deoxyribonucleic acid

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DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
EtBr	- Ethidium Bromide
g	- gram
8	- gravity
gus	- ß-glucuronidase
HEPES	- (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
IMS	- industrial methylated spirits
IPTG	- isopropyl-ß-D-thiogalactopyranoside
kb	- kilobase pair
kD	- kilodaltons
KMB	- 2-keto-4-methylthiobutyric acid
kV	- kilovolts
1	- litre
М	- Molar
m A	- milliamperes
MES	- 2-(N-morpholine)ethanesulphonic acid
MTA	- methylthioadenosine
MTR	- methylthioribose
MTR-1-P	- methylthioribose-1-phosphate
μF	- microfarads
MOPS	- 3-(N-morpholino)-propane sulphonic acid
mRNA	- messenger RNA
4-MU	- 4-methylumbelliferone
MUG	- 4-methylumbelliferyl glucuronide
NA	- Nutrient Agar
NB	- Nutrient Broth
NBT	- nitroblue tetrazolium
OD	- Optical Density
PCR	- Polymerase Chain Reaction
PEG	- polyethylene glycol
p.f.u.	- Plaque Forming Units
PIPES	- Piperazine-N, N'-bis[2-ethanesulphonic acid]

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PMSF	- phenylmethylsulfonyl fluoride
PVPP	- polyvinylpolypyrrolidone
RNA	- Ribonucleic Acid
rpm	- revolutions per minute
SAH	- S-adenosyl-L-homocysteine
SAM	- S-adenosyl-L-methionine
SDS	- sodium dodecyl sulphate
SHH	- S-adenosyl-L-homocysteine hydrolase
TCA	- trichloroacetic acid
TEMED	- N,N,N',N'-Tetramethylethylene-diamine
TLC	- thin layered chromatography
UV	- Ultra Violet
V	- volts
X-gal	- 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside
X-gluc	- 5-bromo-4-chloro-3-indoyl-ß-D-glucuronide

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Other abbreviations are explained in the text.

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Chapter 1

General introduction to S-adenosyl-L-homocysteine hydrolase

1.1 General introduction

As plants grow and mature, several developmental processes occur as well as the plant experiencing different environmental stimuli. In order to respond to these stimuli or engage specific developmental pathways, the plant requires mechanisms to alter and control its metabolism. One common chemical modification observed in diverse biochemical pathways is methylation. Methylation is simply the addition of a methyl group [CH₃] to an existing substrate. This process can serve several functions: the addition of a methyl group can be utilised to increase the length of the substrate's carbon backbone; the methyl group may replace a more reactive side group and therefore produce a less reactive product; the reversible addition of a methyl group may inhibit binding of other substrates and lead to a temporary loss of substrate function.

Methylation has been observed to occur in many biochemical pathways as well as being utilised to modify mature products of several pathways. The majority of methylation reactions use *S*-adenosyl-L-methionine [SAM] as the methyl group donor and due to the diverse utilisation of the process of methylation, the enzymes involved in this process are considered to be Figure 1.1 Reaction equation for S-adenosyl-L-homocysteine hydrolase

S-adenosyl-L-homocysteine hydrolase (SHH) is the enzyme responsible for the reversible, thermodynamically unfavourable, hydrolysis of S-adenosyl-L-homocysteine to adenosine and Lhomocysteine.



encoded by housekeeping genes. One of these enzymes is *S*-adenosyl-L-homocysteine hydrolase [SHH].

1.1.1 Introduction to S-adenosyl-L-homocysteine hydrolase

S-adenosyl-L-homocysteine hydrolase [EC 3.3.1.1, SHH] was first detected in rat liver extracts as the activity responsible for the reversible, thermodynamically unfavourable, hydrolysis of S-adenosyl-L-homocysteine [SAH] to adenosine and homocysteine (de la Haba and Cantoni, 1959). This reaction is illustrated in figure 1.1. SAH is formed as a direct product of Sadenosyl-L-methionine [SAM] dependent transmethylation reactions catalysed by a large variety of methyltransferase enzymes (Cantoni and Scarano, 1954). The affinity of many methyltransferase enzymes, is much greater towards SAH than SAM, with SAH exhibiting a potent product inhibition (Deguchi and Barchas, 1971). To alleviate this product inhibition, SAH produced as a consequence of transmethylation is metabolised to homocysteine and adenosine by the action of SHH.

1.2 The methyl cycle and methionine salvage pathway

The hydrolysis of SAH to homocysteine and adenosine by SHH enzyme activity (as represented in figure 1.1) is only one reaction within a cyclic process known as the methyl cycle. A related cyclic process also exists to recycle methionine from a separate biosynthetic by-product, and this is known as the methionine salvage pathway. These two cycles are shown within figure 1.2 and are described in detail in sections 1.2.1 and 1.2.2 with reference to this figure. Two salient features of the scheme shown in figure 1.2 should be noted.

Firstly, of the pathways illustrated, incorporation of methionine into protein (reaction 6) during protein synthesis, is the only pathway consuming the entire methionine molecule; the methyl, sulphur and 4-carbon moieties (reviewed by Giovanelli, 1987).

Secondly, as the methyl and methionine salvage pathways simply recycle methionine, no net increase in methionine quantities can occur using these pathways. The only pathway to produce extra methionine is *de novo* synthesis of homocysteine (reaction 1), the direct precursor of methionine.

Figure 1.2 The methyl cycle and methionine salvage pathway

This figure is adapted from previous figures by Miyazaki and Yang (1987), Theologis (1992), and Giovanelli *et al.* (1985).

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; KMB, 2-keto-4-methylthiobutyric acid; MTA, methylthioadenosine; MTR, methylthioribose; MTR-1-P, methylthioribose-1-phosphate; N⁵-Methyl-H 4Pte Glu3, tetrahydropteroyltriglutamate; SAH, S-adenosyl-Lhomocysteine; SAM, S-adenosyl-L-methionine.

Enzymes are as follows: 1= cysteine synthase, cystathionine γ synthase and cystathionine β -lyase; 2= tetrahydropteroyltriglutamate methyltransferase; 3= SAM synthetase; 4= any SAM-dependent transmethylase enzyme; 5= S-adenosyl-L-homocysteine hydrolase; 6= methionine incorporation into proteins via protein synthesis; 7= ACC synthase; 8= ACC oxidase; 9= SAM decarboxylase; 10= spermidine synthase; 11= spermine synthase; 12= MTA nucleosidase; 13= MTR kinase; 14= Conversion of MTR-1-P to KMB by a proposed three step process utilising unidentified enzymes, the first of these steps is thought to be an isomerase to convert MTR-1-P into methylthioribulose-1-phosphate; 15= Conversion of KMB to methionine by a transaminase.



Biosynthesis of homocysteine occurs by transsulphuration from cysteine by the enzymes cystathionine γ -synthase and cystathionine β -lyase [EC 4.4.1.8] (reviewed by Giovanelli, 1987). The resultant homocysteine is methylated to form methionine by tetrahydropteroyltriglutamate methyltransferase [EC 2.1.1.14] (reaction 2). This is an example of a methyltransferase enzyme which does not require SAM for activity, as it uses the triglutamyl derivative of N⁵-methyltetrahydrofolic acid. *In vivo* studies have shown methionine feedback to regulate its own *de novo* synthesis (Giovanelli *et al.*, 1985).

1.2.1 The methyl cycle

Within figure 1.2 the methyl cycle is represented by reactions 2-5. When the methyl group of methionine is utilised in a SAM-dependent transmethylase reaction, the homocysteinyl moiety is recycled to reform methionine. Reaction 2 within this cycle is also common to the de novo synthesis pathway for methionine, which is due to the recycling process using homocysteine as an intermediate. Therefore all homocysteinyl moieties recycled, simply add to the homocysteine available for methylation to methionine. Reaction 3 is common to the methionine salvage pathway, as it essentially activates methionine to allow its utilisation in several reactions due to its conversion to S-adenosyl-L-methionine [SAM]. The biosynthesis of SAM from methionine is catalysed by S-adenosyl-L-methionine synthetase [EC 2.5.1.6] using ATP as an energy source (reviewed by Tabor and Tabor, 1984; see section 1.3). Reaction 4 represents the numerous SAM-dependent methyltransferase reactions (see section 1.4.3) in which a transmethylated product is produced with the concomitant production of SAH as a by-product. As previously described, SAH is hydrolysed into homocysteine and adenosine by the action of S-adenosyl-L-homocysteine hydrolase [SHH] (reaction 5). The homocysteine produced can be utilised in the same reaction observed in denovo synthesis of methionine (reaction 2), to produce methionine and complete the cycle. This cycle therefore results in no net gain or loss of methionine, requires ATP as an energy source and produces both adenosine and inorganic phosphate groups as by-products (reviewed by Giovanelli, 1987). It has been shown that the adenosine by-product is rapidly metabolised to adenine nucleotides (Giovanelli *et al.*, 1985). This removal of adenosine is mechanistically important as both homocysteine or adenosine accumulation will inhibit SAH hydrolysis by SHH enzyme activity due to the unfavourable equilibrium reaction (de la Haba and Cantoni, 1959).

1.2.2 The methionine salvage pathway

Within figure 1.2 the methionine salvage pathway is represented by reactions 3 and 12-15. When the 4-carbon moiety of methionine is utilised for ethylene or polyamine synthesis, the methylthio moiety is recycled to reform methionine. The initial substrate of this pathway is methylthioadenosine [MTA] which is produced as a by-product following the initial reaction of ethylene synthesis (Yang and Hoffman, 1984; reviewed by Theologis, 1992) or following spermidine or spermine synthesis from decarboxylated SAM (Miyazaki and Yang, 1987). These reactions and biosynthetic pathways are discussed in section 1.4. Therefore two diverse biosynthetic plant pathways utilise an identical substrate, SAM, and produce an identical by-product MTA, hence allowing a single pathway to salvage methionine from both biosynthetic reactions.

The first reaction in the methionine salvage pathway is the hydrolytic cleavage of MTA by-product by methylthioadenosine nucleosidase [3.2.2.16] (reaction 12) to form methylthioribose [MTR] and adenine. MTR is then phosphorylated in an ATP-dependent manner by methylthioribose kinase [2.7.1.100] (reaction 13) to form methylthioribose-1-phosphate [MTR-1-P]. MTR-1-P undergoes an isomerization into methylthioribulose-1-P due to the action of methylthioribose-1-phosphate isomerase [5.3.1.23] (reaction 14). Following two further unidentified reactions (also represented by reaction 14), 2-keto-4-methylthiobutyrate [KMB] is formed which is finally transaminated into methionine by a transaminase (reaction 15). This cycle therefore results in no net gain or loss of methionine, requires ATP as an energy source and produces adenine as one of the by-products. When the five-carbon ribose moiety of MTR is converted to the four-carbon ketobutyrate chain of KMB, one carbon is lost as formate. In avocado extracts, L-glutamine is the most efficient amino acid in

effecting the conversion of KMB to methionine (see Miyazaki and Yang (1987) for an overview of this scheme). It has been shown that the adenine by-product is rapidly metabolised to adenine nucleotides (Giovanelli *et al.*, 1983) as occurred with the adenosine by-product of the methyl cycle.

The described pathway will allow high rates of ethylene and polyamine production without high intracellular concentrations of methionine, due to its recycling in an ATP-dependent manner.

1.2.3 Fluxes through the methyl and methionine salvage cycles

In a series of papers by Giovanelli's group, individual rates of the metabolism of the sulphur, methyl and 4-carbon moieties of methionine were estimated in Lemna paucicostata and used to quantitate pathways of methionine metabolism. Synthesis of SAM is the major pathway of methionine metabolism with over 4 times as much methionine metabolised by this route as accumulates in protein. The pools of soluble methionine and SAM are rapidly metabolised with turnover times of 3-4 minutes. More than 90% of the SAM is utilised for transmethylation with methyl groups accumulating in choline and its derivatives accounting for the major portion of flux through transmethylation. As phosphatidycholine is the dominant phospholipid in most plants, phosphatidycholine methyl groups have been proposed to be a major product of transmethylation in the plant kingdom in general (Giovanelli et al., 1985; see section 1.4.3.3). Flux through methylthio recycling accounts for one third the amount of methionine accumulating in protein, with spermidine synthesis accounting for at least 60% of the flux through methylthio recycling.

Although this data refers to a single plant species, with significantly little ethylene production, which was grown in specific environmental conditions, the results clearly emphasise the substantial use of methionine within transmethylase reactions in comparison to protein synthesis or polyamine synthesis. This scenario cannot be extrapolated to all individual plant tissues, species or environmental conditions due to the specialised utilisation of SAM within certain situations. In certain plant tissues, such as ripening fruit, ethylene production may rise from less than 0.01 to as high as 10nmol [g fresh weight]⁻¹ h⁻¹. In such tissues, the flux through the methionine salvage pathway would greatly increase relative to the other methionine-metabolising pathways (Miyazaki and Yang,1987).

As both polyamine and ethylene biosynthesis depend on the methionine salvage pathway to maintain a constant supply of methionine, the regulation of this cycle may be a control mechanism for the biosynthesis of these compounds. Studies have shown that MTR kinase specific activity varied over the course of tomato ripening, with an activity rise corresponding to the burst of ethylene production (Kushad *et al.*, 1985).

Since both ethylene and polyamines share SAM as a common precursor, and are therefore alternative products of SAM, it has been hypothesised that they may regulate each other's synthesis, either directly or by metabolic competition for SAM (reviewed by Evans and Malmberg, 1989). Such a hypothesis is supported by studies of inhibition of ACC synthesis from SAM leading to increased incorporation of SAM into spermidine (Even-Chen *et al.*, 1982), however examples in disagreement with this hypothesis also exist. Therefore it appears that in some experimental systems polyamines and ethylene clearly interact through the intermediate SAM, while in others the evidence contradicts this metabolic competition; this may reflect instances where pools of SAM may be limiting or non-limiting.

1.3 S-adenosyl-L-methionine synthetase

As all pathways discussed to date involve the utilisation of SAM, the following section reviews the current data available on SAM synthetase [EC 2.5.1.6], the only enzyme known to synthesise SAM. This enzyme utilises methionine and ATP to produce SAM, as shown in reaction 3 of figure 1.2. SAM synthetase activity has been detected in *E. coli* as well as a large variety of diverse pro- and eukaryotic species (reviewed by Tabor and Tabor, 1984). In general all SAM synthetase preparations have an absolute requirement for a divalent cation [Mg²⁺] and are markedly stimulated by a non-covalent cation [K+]. The first SAM synthetase gene isolated from a higher eukaryote, was from *Arabidopsis thaliana* (Peleman *et al.*, 1989a). This study proved *Arabidopsis*

thaliana contained two SAM synthetase genes per haploid genome, which has also been observed in yeast and mammals (reviewed by Tabor and Tabor, 1984). Northern hybridisations demonstrated SAM synthetase to have a 10-20 fold higher expression in stems, roots and callus compared to leaves, inflorescences and seed pods. This variance in SAM synthetase mRNA distribution was reflected within enzyme activity studies, suggesting SAM synthetase enzyme expression to be primarily controlled at the mRNA level. Transgenic A. thaliana containing a SAM synthetase promoter/ß-glucuronidase [gus] gene fusion exhibited 10 to 140 times more gus activity in the stems and roots compared with leaves, therefore it was concluded that the 5' end of the SAM synthetase gene was a major determinant for the differential expression pattern of the gene. Histochemical analysis proved that highest levels of gus expression occurred in the xylem and phloem throughout the whole plant, the sclerenchyma tissue between vascular bundles in older parts of the stems, and in the parenchyma cells of the root cortex. This strong cellular preference was suggested to partly correlate to lignification, a process known to require methylation (Peleman et al., 1989a).

The same group of workers also isolated the second SAM synthetase gene of *A. thaliana* (Peleman *et al.*, 1989b). Using 3' specific probes it was proven that the mRNA expression profiles of both genes were identical within *A. thaliana* organs. The SAM synthetase-gus fusion construct has also been introduced into *N. tabacum* and poplar [*Populus tremula x P. alba*], and the gus expression profile mirrors that observed within transgenic *A. thaliana*. (Peleman *et al.*, 1989b; van Doorsselaere *et al.*, 1993b).

Two separate SAM synthetase truncated cDNAs have been isolated from a parsley cDNA library prepared using mRNA isolated from cell cultures treated with fungal elicitor. An SHH cDNA was also isolated from this library (see section 1.8). Northern hybridisations detected large amounts of SAM synthetase within floral buds and roots, with lower levels in stems and only trace amounts in leaves. SAM synthetase mRNA levels were markedly increased by forced infiltration of fungal elicitor solution into leaves, as well as a 3-4 fold increase by elicitor induced expression within parsley cell cultures (Kawalleck *et al.*, 1992). SAM synthetase has also been cloned from other plant species including; carnation (Larsen and Woodson, 1991), poplar (Van Doorsselaere *et al.*, 1993a) and tomato (Espartero *et al.*, 1994). Three separate SAM synthetase cDNAs of the four possible genes in tomato have been isolated and each has been detected with a different expression profile in response to salt stress.

Within germinating embryos of wheat, SAM synthetase activity has been observed to undergo a 2-3 fold stimulation, which requires *de novo* protein synthesis but not transcription. Therefore it was concluded that this increase in activity occurred due to protein synthesis from stored mRNA. This is therefore an example of post-transcriptional regulation of SAM synthetase activity (Mathur *et al.*, 1991).

Gibberellic acid has been found to stimulate SAM synthetase activity 3 fold within the aleurone layers of de-embryonated half seeds of wheat. Whereas untreated controls contain one isozyme of SAM synthetase, treated controls contain three isozymes. This is interpreted as being due to the induction of a second SAM synthetase gene product to allow dimerization to form three isozymes (Mathur *et al.*, 1992). This has also been observed in pea epicotyls (Mathur and Sachar, 1991) and therefore is an example of differential expression of SAM synthetase genes.

This short overview describes a complex situation. Within those plant species in which SAM synthetase has been studied, a minimum of two coding genes appear to occur. The spatial organ-dependent patterns of SAM synthetase mRNA in *A. thaliana* and parsley are similar except for the levels within inflorescences and floral buds. It was implied that the inflorescences from the two species are incomparable or that SAM synthetase gene activity varies greatly with respect to floral development (Kawalleck *et al.*, 1992). Within *A. thaliana*, both SAM synthetase gene transcripts appear to be expressed within different organs, at the same levels. However in wheat and tomato, differential expression appears to occur in the specialised circumstances of gibberellic acid stimulation and salt stress, respectively. In *A. thaliana* SAM synthetase expression has been concluded to be mainly controlled at the transcriptional level, whereas within wheat embryos, post-transcriptional control of expression from stored mRNAs occurs. Therefore all of this data taken together

suggests SAM synthetase genes to be potentially regulated at several levels, but it is unknown if this regulation occurs at all sites of expression or only within specialised situations. However it is of no surprise that the gene encoding the only known enzyme for the synthesis of SAM, the second most abundant cofactor in metabolic reactions, is regulated in a complex manner.

1.4 Pathways utilising S-adenosyl-L-methionine

Although the first two pathways described in the following sections do not produce SAH as a by-product, they both represent pathways utilising SAM. As such these two pathways represent an additional drain on the SAM pool and therefore may affect SAM availability for the transmethylation reactions described in section 1.4.3.

1.4.1 Polyamine synthesis and activities

The term polyamine is used in this short overview in its restricted sense, meaning only those polyamines with more than two amine groups, such as spermidine [a triamine] and spermine [a tetraamine].

S-adenosyl-L-methionine decarboxylase [EC 4.1.1.50, SAMDC] is the key enzyme in the biosynthesis of polyamines (reviewed by Tabor and Tabor, 1984; first cloned from a plant species by Mad Arif *et al.*, 1994). The substrate for this enzyme is SAM and the product is decarboxylated SAM (reaction 9 in figure 1.2). SAMDC has been shown to be rate limiting for the biosynthesis of polyamines, and its activity can be fully repressed by the addition of spermidine and spermine to cell cultures (Hiatt *et al.*, 1986). The decarboxylated SAM can be utilised by spermidine synthase [EC 2.5.1.16] to donate its aminopropyl moiety to putrescine to synthesise spermidine, or alternatively it can be utilised by spermine synthase [EC 2.5.1.22] to donate its aminopropyl moiety to spermidine to synthesise spermine (reactions 10 and 11 respectively in figure 1.2). As previously described, the by-product of both of these reactions is MTA.

Numerous studies have correlated an increase in polyamine levels with cell division and a drop in polyamines during any subsequent lessening of metabolic activity (reviewed by Evans and Malmberg, 1989). Polyamines also bind to membranes and therefore alter their stability and permeability, as well as modifying DNA structures (reviewed by Slocum *et al.*, 1984). Several other activities of polyamines have been noted. These include, their involvement in root initiation and early growth, ovary and fruit development, differentiation of the floral meristem, and also the fact that exogenous polyamines have the ability to retard senescence (all reviewed by Evans and Malmberg, 1989).

1.4.2 Ethylene synthesis and activities

1-aminocyclopropane-1-carboxylic acid synthase [EC 4.4.1.14, ACC synthase] catalyses the elimination of the MTA group of SAM, leading to the formation of 1-aminocyclopropane-1-carboxylic acid [ACC] (Adams and Yang, 1977; reaction 7 in figure 1.2). This is the rate limiting step in the synthesis of ethylene which is produced from ACC due to the action of ACC oxidase (reaction 8 in figure 1.2). The induction of ethylene production by a variety of means is due to *de novo* synthesis of ACC synthase (Kende, 1989). ACC synthase is encoded by a highly divergent multigene family in which individual genes are differentially expressed in response to developmental, hormonal and environmental stimuli (Liang *et al.*, 1992).

Ethylene production is induced during several developmental stages including fruit ripening, seed germination, leaf and flower senescence and abscission. It is also induced by external factors such as wounding, anaerobiosis, viral infection, auxin treatment, chilling injury, drought and Cd²⁺ and Li⁺ ions (reviewed by Yang and Hoffman, 1984).

1.4.3 SAM-dependent transmethylation reactions

Of the 3,196 enzymes described in the latest version of *Enzyme* Nomenclature, 98 are SAM-dependent methyltransferases (Webb, 1992). SAM-dependent transmethylase enzymes catalyse a diverse range of methylation reactions resulting in the formation of methyl ester, methyl ether, methyl thioether, methyl amine, methyl amide and other derivatives on proteins, nucleic acids, polysaccharides, lipids, and various small molecules (Kagan and Clarke, 1994; reaction 4 in figure 1.2). This transfer of a methyl group from

SAM results in the concomitant production of SAH as a by-product. Several examples of biochemically proven plant transmethylase enzymes are known to have their enzymatic activity inhibited by SAH. The following are a few examples: SAM:isoliquiritigenin 2'-O-methyltransferase (Maxwell *et al.*, 1992); SAM:L-methionine *S*-methyltransferase [EC 2.1.1.12] (James and Hanson, 1995); SAM:quercetin-3'-O-methyltransferase [EC 2.1.1.42] (Tobias and Larson, 1991). Therefore all these examples require SHH enzyme activity to remove inhibitory levels of SAH accumulation. The following sections (1.4.3.1-7) highlight some specific examples of biosynthetic pathways requiring transmethylase enzyme activities.

1.4.3.1 DNA methylation

The most common modification of DNA in plant cells is the methylation of cytosine residues at carbon 5 to produce 5-methylcytosine (5meC) which was first observed in plants by Wyatt in 1951. In contrast to mammals in which 3-8% of cytosine residues are methylated (Shapiro, 1975), in plants up to 30% of cytosine residues are modified (Adams and Burdon, 1985). This difference is due to two factors: the CG dinucleotide, which is methylated to about the same extent (70-80%) in plants and animals, occurs more frequently in plants; and in addition plant DNA is methylated in CNG trinucleotides where N can be any base (Gruenbaum et al., 1981). There is a considerable inter-species variation in the level of cytosine methylation, ranging from 4.6% in Arabidopsis thaliana (Leutwiler et al., 1984), which has a small genome with relatively little highly repeated DNA, to 33% in rye, Secale cereale (Thomas and Sherratt, 1956). The distribution of 5-meC in the plant genome is not random, for example unique sequences which comprise approximately 38% of the cotton genome contain only 4% of the methylated cytosine residues (Guseinov et al., 1975). Also, although 70-80% of CG dinucleotides are modified, a number of plants have a fraction of unmethylated DNA with closely spaced potential methylation sites. These areas of DNA appear similar to CpG islands which are often associated with the 5' end of vertebrate housekeeping genes and have been found in the 5' region of a maize alcohol dehydrogenase gene (Antequera and Bird, 1988).
These patterns of DNA methylation are crucial as there are several lines of evidence to suggest that DNA methylation is important in the regulation of plant gene expression.

1) Plant DNA binding proteins (DBP) are affected by 5-meC and these DNA protein interactions constitute a crucial level in the regulation of gene expression. The binding of several sequence specific DNA binding proteins is inhibited by the methylation of CpG or CpNpG at their recognition sites (Staiger *et al.*, 1989; Inamdar *et al.*, 1991) and conversely, DBP-m (a non-sequence specific DBP) extracted from pea, preferentially recognises any methylated DNA (Zhang *et al.*, 1989). This would affect gene expression if the DBP unable to bind was a transcription factor, or if the DBP able to bind to methylated DNA, blocked the binding of a transcription factor.

2) The presence of 5-meC affects the secondary and tertiary structure of DNA (reviewed by Zacharias, 1993). Unorthodox DNA structures which are not present in right-handed B-form helixes can provide new and unique interaction sites with structure specific proteins. Cytosine methylation can influence these unusual structures by either enhancing or inhibiting their formation, therefore allowing an indirect effect on gene regulatory events *in vivo* by altering the equilibrium between unusual DNA structures and regular B-helix regions. Also the non B-form structures affect supercoiling, which is therefore an indirect result of cytosine methylation, and this can affect supercoil-dependent DNA-protein interactions and hence chromatin structure.

3) Methylation of DNA *in vitro* inhibits transcription, both transiently in protoplasts (Weber and Graessmann, 1989) and when the DNA is stably integrated in regenerated plants (Weber *et al.*, 1990).

4) The methylation pattern of tissue-specific genes has been found to be different in tissues where the gene is expressed in comparison to tissues where the gene is silent (reviewed by Finnegan *et al.*, 1993).

5) Phenotypic alteration has been noted in plants exposed to extreme environmental stress such as regeneration from culture. Some of these stable and reversible genetic alterations can be linked to changes in methylation. (reviewed by Finnegan *et al.*, 1993).

6) Transposable elements activities are regulated by methylation. A

correlation was found between activity of the maize Ac element and a lack of methylation in its 5' region but not in its gene (Kunze *et al.*, 1988). Reactivation of Ac elements were observed in plants regenerated from cultures and it has already been noted that extreme environmental stress can cause an alteration in methylation.

7) The activity of introduced genes in transgenic plants can be affected by *in vivo* methylation (reviewed by Finnegan *et al.*, 1993). This may arise due to the methylation of the whole T-DNA or alternatively specific methylation of the transgene promoter. The methylation of entire T-DNAs often correlates to the presence of more than one copy of the T-DNA and is thought to be one potential mechanism for the co-suppression phenomena (Matzke and Matzke, 1995). This is discussed further in chapter 8.

This evidence clearly suggests that the maintenance and control of DNA methylation has an important regulatory role in gene expression. It has been demonstrated that a DNA methyltransferase preferentially methylates hemimethylated DNA (Pradhan and Adams, 1995). This situation would occur during semiconservative DNA replication, leading to the conclusion that the enzymes required for this process would be present in areas of elevated cell division such as plant meristems; hence the use of pea shoot tips to isolate the pea DNA methyltransferase in the series of papers by Adams *et al.* (e.g. Pradhan and Adams, 1995). The first plant DNA methyltransferase was isolated from *Arabidopsis thaliana* using degenerate PCR primers (Finnegan and Dennis, 1993).

1.4.3.2 Protein methylation

A large group of post-translational modification reactions involve the addition of a methyl group to the polypeptide chain (reviewed by Clarke, 1993). These reactions can occur during or immediately after synthesis or at later points in the lifetime of a protein molecule. The enzymes catalysing such reactions are known as protein methyltransferases and can modify a variety of nucleophilic oxygen, nitrogen and sulphur atoms on the polypeptide chain. These processes result in methyl esters, methyl amines, methyl amides and other derivatives on the side chains of nine of the twenty common amino acids, as well as on several additional residues when present at the amino or carboxyl terminus.

Protein methyltransferases can be divided into two groups. The first group modifies carboxyl groups to form methyl esters and is generally a reversible reaction hence allowing its function of possibly regulating the activity of the protein. A specialisation of this group allows the recognition of damaged proteins to occur. The second group results in generally irreversible methyl transfer to sulphur and nitrogen atoms to produce a variety of new amino acids. These new amino acids are often found in structural proteins and may play a role in the stabilisation of polypeptides in complex structural structures (see Clarke, 1993).

Protein methylation has been specifically studied within fungal elicitor treated alfalfa cells, due to the known induction of SAM synthetase and SHH in response to elicitors (Daniell and Edwards, 1995). These studies showed protein carboxyl methylation to increase in alfalfa cell cultures as a late response to elicitor treatment.

1.4.3.3 Methylated phospholipid biosynthesis

The previously described studies of methyl flux in *L. paucicostata* suggested that the methyl groups of choline, phosphatidycholine and phosphocholine, were the major products of SAM-dependent transmethylations in this and possibly the majority of plant species (Giovanelli *et al.*, 1985; see section 1.2.3).

The pathway for phosphatidycholine synthesis within plants has been proposed to differ from those found in animals (Datko and Mudd, 1988). However several SAM-dependent transmethylase enzymes are known to occur within the biosynthetic pathway (reviewed by Browse and Somerville, 1991). The three methyl groups required to convert ethanolamine to choline originate from SAM. In certain plant species phosphoethanolamine is methylated sequentially to form phospho *N*-methylethanolamine, phospho N,N-dimethylethanolamine and phosphocholine, whereas in other species the phoshatidyl moiety may be utilised for the final two methylation reactions (Mudd and Datko, 1989).

1.4.3.4 Lignin precursor biosynthesis

Three monolignols differing only in the methoxyl groups on the aromatic ring can be polymerised into lignin. The relative abundance of the different monolignol residues in lignin varies between species and within species, as does the total lignin content. This methoxyl pattern of the aromatic rings is dependent on the action of *o*-methyltransferase [OMT] enzymes (reviewed by Whetten and Sederoff, 1995). Caffeic acid is methylated to form ferulic acid by caffeic acid 3-*O*-methyltransferase [C-OMT; EC 2.1.1.68]. This enzyme also catalyses the methylation of 5-hydroxyferulate to sinapate. These methylations limit the reactivity of the hydroxy groups, therefore reducing the number of sites on the aromatic ring that can form bonds to other monolignols during polymerisation.

A known maize mutant with altered lignin content and composition has recently been shown to correspond to the gene encoding C-OMT (Vignols *et al.*, 1995). High-level antisense expression of aspen OMT in tobacco resulted in depressed C-OMT activity and a reduced ratio of syringyl to guaiacyl lignin subunits (Dwivedi *et al.*, 1994)

A second OMT, caffeoyl-CoA-3-*O*-methyltransferase [CCoA-OMT; EC 2.1.1.104] was originally identified as a defense-related transcript within dicot. species, with a cDNA clone being isolated from parsley cell culture (Schmitt *et al.*, 1991). However CCoA-OMT has now been proposed to have a role in lignin synthesis due to its proposed ability to methylate both caffeoyl-CoA and 5-hydroxyferuloyl-CoA during monolignol biosynthesis (Ye *et al.*, 1994).

1.4.3.5 Phenylpropanoid metabolism

All phenylpropanoids are derived from cinnamic acid, with several simple examples produced from cinnamate via a series of hydroxylation, methylation and dehydration reactions. These include caffeic, ferulic and sinapic acids and simple coumarins (reviewed by Dixon and Paiva, 1995). Therefore the production of lignin precursors should strictly be included in this section, but due to the extensive research within this field, this data is summarised in the section above. However the impregnation of the cell wall with ferulate is a known early response of infected parsley leaves, and not the production of lignin (Hahlbrock and Scheel, 1989).

The phenylpropanoid subunits of suberin require the methylation of caffeic acid to ferulic acid in their biosynthesis, as occurs in lignin precursor biosynthesis. As only one distinct methylation is required it has been proposed that a separate OMT found only in the sites of suberin biosynthesis catalyses this methylation. Due to sequence homology and spatial and temporal expression, a clone from maize has been proposed to encode an OMT involved in suberin synthesis (Held *et al.*, 1993).

Many stress-induced phenylpropanoids are classified as phytoalexins which are antimicrobial compounds synthesised in response to pathogen attack. Several known OMT activities are involved in the pathways for the synthesis of several of these species-dependent compounds. In parsley, the furanocoumarin, psoralen, has methoxylated derivatives xanthotoxin, bergapten and isopimpinellin. The OMT activities required for the synthesis of these compounds have been reported to be induced by elicitor treatment of cultured parsley cells (Hauffe *et al.*, 1986).

In maize, a flavonoid 3'-O-methyltransferase enzyme has been purified from pollen and was found to utilise quercetin as its major substrate (Tobias and Larson, 1991). This is an example of the many flavonoid Omethyltransferases which have been isolated and studied due to methylation playing an important role in flavonoid biosynthesis, as it reduces the reactivity of the phenolic groups while enhancing lipid solubility and volatility of the flavonoids (see Tobias and Larson, 1991 and references therein).

In *Mesembryanthemum crystallinum*, the accumulation of *myo*-inositol and its methylated derivatives are correlated with tolerance to drought and/or salinity (reviewed by Bohnert *et al.*, 1995). In petunia, anthocyanins are known to be methylated by *o*-methyltransferases (see review by Holton and Cornish, 1995).

1.4.3.6 Chlorophyll biosynthesis

In chlorophyll, four pyrrole rings are ligated into a tetrapyrrole ring with

a magnesium atom in the centre. Ring II has a methyl group in position 3 in chlorophyll a, whereas position 3 is a formyl group in chlorophyll b. Chlorophyll b, found only in higher plants and algae, is derived from chlorophyll a, due to the action of an oxygenase (reviewed by von Wettstein *et al.*, 1995). The chlorophyll- and bacteriochlorophyll specific pathway from protoporphyrin IX to chlorophyll contains a single step catalysed by a methyltransferase enzyme. This methyltransferase has been cloned from *Rhodobacter capsulatus* and *R. sphaeroides*, and both were expressed in *E. coli* to identify the gene products as the methyltransferase for Mg-protoporphyrin IX (Bollivar *et al.*, 1994; Gibson and Hunter, 1994).

1.4.3.7 Alkaloid biosynthesis

Tropane and nicotine are a category of the secondary metabolite alkaloids derived from putrescine, which are found mainly in the Solanaceae. The first committed step within the biosynthesis of nicotine and tropane alkaloids is the methylation of putrescine to form *N*-methyl-putrescine. This reaction is catalysed by putrescine *N*-methyltransferase [EC 2.1.1.53] (reviewed by Kutchan, 1995). A cDNA has been isolated from tobacco and its activity confirmed by expression in *E. coli*. The transcripts of this gene accumulate predominantly, if not exclusively, in root tissues of wild-type tobacco, suggesting this organ to be the major site of nicotine biosynthesis (Hibi *et al.*, 1994). This corresponds to the site of tropane alkaloid biosynthesis in other members of the Solanaceae (Hashimoto *et al.*, 1991).

1.5 S-adenosyl-L-homocysteine metabolism in diverse species

SHH activity is utilised to metabolise SAH in the majority but not all living organisms. In some Gram-negative bacteria, SAH has been found to undergo cleavage of the glycosyl linkage, yielding adenine and ribosylhomocysteine (Duerre, 1962). This reaction is non-reversible, and catalysed by the enzyme S-adenosylhomocysteine nucleosidase [EC 3.2.2.9]. The same enzyme also cleaves 5'-deoxymethylthioadenosine, yielding methylribose and adenine. *E. coli* contains S-ribosylhomocysteine hydrolase [EC 3.3.1.3]

which cleaves the thioether linkage of S-ribosylhomocysteine, yielding free homocysteine and a pentose (Miller and Duerre, 1968). The cleavage of the glycosidic bond of SAH is necessary prior to the cleavage of the thioether linkage of S-ribosylhomocysteine (Miller and Duerre, 1968). In a study by Walker and Duerre (1975) bacteria, yeasts, plants and several organs from warm-blooded vertebrates were assayed for the presence of SHH, Sadenosylhomocysteine nucleosidase, and S-ribosylhomocysteine hydrolase. In all species in which SHH activity was detected, none were found to possess detectable S-adenosylhomocysteine nucleosidase or S-ribosylhomocysteine hydrolase activity. Those species tested included: the bacterial species; E. coli and Proteus vulgaris; the yeast species; Saccharomyces cerevisiae, Candida albicans and Candida utilis: the plant species; green bean, spinach, field corn and barley: the fish species; yellow perch and rainbow trout: the bird species; y morning dove and chicken: and the animal species; white rat, dog, and rabbit. However none of the small sample of prokaryotic species analysed in this study were found to contain SHH activity. A larger study of prokaryotic species was performed by Shimizu et al. (1984), in which 149 strains of bacteria and actinomycetes were analysed for SHH activity. SHH enzyme activity was widely distributed in a variety of both Gram-positive and Gram-negative bacteria and actinomycetes. However none of the 22 strains of Enterobacteriaceae and 9 strains of Bacillaceae investigated were found to exhibit detectable SHH activity. Within those strains exhibiting SHH activity, no Sadenosylhomocysteine nucleosidase nor S-ribosylhomocysteine hydrolase activity was detected. Conversely, those strains which did not exhibit detectable SHH activity, abundantly contained both S-adenosylhomocysteine nucleosidase and S-ribosylhomocysteine hydrolase activity.

Therefore, this summarised data clearly demonstrates the occurrence of SHH throughout evolutionary diverse organisms including pro- and eukaryotes. In those prokaryotes in which SHH is not active, a two-step enzymatic process requiring *S*-adenosylhomocysteine nucleosidase and *S*ribosylhomocysteine hydrolase activity is present to metabolise SAH. These two pathways for SAH metabolism have, to date, proven to be mutually exclusive. Further evidence of this mutually exclusive relationship has been provided by a study of the distribution of methylthioadenosine phosphorylase in eubacteria (Shimizu *et al.*, 1988). *S*-adenosylhomocysteine nucleosidase also hydrolytically cleaves 5'-deoxymethylthioadenosine, the nucleoside endproduct following SAM donating an aminopropyl group in polyamine synthesis. However, the previously described study of Shimizu *et al.* (1984) proved this enzyme activity was absent in eubacteria in which SHH activity was present. As SHH itself cannot hydrolyse 5'-deoxymethylthioadenosine, a further enzyme must be involved in its metabolism. This enzyme proved to be methylthioadenosine phosphorylase [EC 2.4.2.28] and is only present within SHH containing prokaryotic species in which *S*-adenosylhomocysteine nucleosidase is known not to occur.

1.6 SHH Enzymology

Significant structural similarities are found between SHH enzymes isolated from several sources. The enzyme always consists of a number of subunits; the mammalian enzyme is a tetramer with a M_r of ~190,000 and a subunit M_r of 47,000 (Richards *et al.*, 1978; Døskeland and Ueland, 1982; Fujioka and Takata 1981), the plant enzymes are dimers or tetramers with a subunit of Mr 55,000 (Guranowski and Pawelkiewicz, 1977; Sebestova *et al.*, 1984), and the bacterial enzyme from *Alcaligenes faecalis* is composed of six subunits of Mr 48,000 (Shimizu *et al.*, 1984). Each subunit contains 1 mol of tightly bound NAD⁺, which is required for activity (Richards *et al.*, 1978; Palmer and Abeles, 1976).

1.6.1 Studies on SHH enzymes isolated from animals

The role of the enzyme-bound NAD⁺ in the catalytic cycle was elucidated by Palmer and Abeles (1979), who showed that NAD⁺ participates as a coenzyme in the reaction, with the reduction of NAD⁺ to NADH and concomitant oxidation of the 3' hydroxyl of SAH to a keto group. After elimination of homocysteine, the enzyme bound NADH is then used to reduce the 3' keto group back to a hydroxyl before the release of adenosine.

Conflicting evidence exists as to whether or not SHH subunits are

structurally identical. Døskeland and Ueland (1982) assigned the mouse and bovine SHH tetramer enzymes as $\alpha_2\beta_2$. In contrast Gomi *et al.* (1985) concluded from analysis of the peptide map of SHH and from the stoichiometry of inactivation of the enzyme by adenosine that the four subunits of the rat liver enzyme are structurally identical and functionally equivalent. The structure proposed by Døskeland and Ueland (1982) may be supported by Abeles *et al.* (1982), who concluded from the partial reduction of NAD⁺ during "suicide" inactivation of the bovine liver enzyme that two of the four subunits are involved in enzyme catalysis and the other two subunits serve a regulatory function. This conflicting data may have arisen due to the mechanism of enzyme isolation. Gomi *et al.* (1985) proved that 'suicide' inactivation of SHH activity by adenosine [or its analogs] could only occur in the simultaneous presence of a thiol. This inactivation may therefore have occurred due to the presence of dithiothreitol in the reaction mixtures of previous workers.

Research has also been published which states SHH is a major adenosine and cyclic adenosine monophosphate [cAMP] binding protein (Hershfield and Kredich, 1978; Saebø and Ueland, 1978). It was demonstrated by de la Haba *et al.* (1986) that tightly bound NAD, which is essential for enzyme catalysis, can be made to dissociate reversibly from SHH. In the presence of ATP, Mg²⁺ and KCl, rat liver SHH is converted from an active enzyme to an inactive form with the release of all bound NAD. Incubation of the inactive enzyme form with NAD, leads to complete recovery of enzyme activity. The active enzyme does not bind cAMP, but in contrast the inactive form will bind cAMP. Therefore in earlier reports of SHH binding cAMP, this binding activity may be attributed to the inactive enzyme form.

This short review of SHH enzymology from non-plant sources, highlights the conflicting evidence found within the literature, which in several, if not all cases, may simply have arisen through the use of differing protocols to isolate SHH enzyme from several different species and organs.

1.6.2 Studies on SHH enzymes isolated from plants

Unlike the vast data available for animal SHH enzyme activities and

purification, relatively few studies have been performed using plant sources of SHH activity. Following the proven existence of SHH activity within plants (Walker and Duerre, 1975), Poulton and Butt (1976) were the first to purify a plant SHH enzyme. As previously observed (de la Haba and Cantoni, 1959) the hydrolysis of SAH is not easy to investigate in vitro because the reaction equilibrium favours the reverse direction in which SAH is synthesised from adenosine and L-homocysteine. Therefore in this study, in vitro hydrolysis was assisted by destruction of adenosine by added adenosine deaminase. Although Poulton and Butt (1976) purified SHH 100 fold from spinach leaves, no test of homogeneity was performed and therefore contamination of the preparation with enzymes which may act on any reaction products may have occurred. However the equilibrium constant is almost identical to that observed in rat liver enzyme (de la Haba and Cantoni, 1959). Spinach leaf SHH appeared to be specific for L-homocysteine although the presence of D-isomer does not inhibit activity of the enzyme. Despite a low Km for adenosine, the high Km for homocysteine suggests that SAH synthesis may only proceed in vivo at a significant rate when homocysteine accumulates (Poulton and Butt, 1976). Due to the reaction equilibrium, both adenosine and homocysteine must be removed for the hydrolysis of SAH to proceed. Therefore they postulated that L-homocysteine is methylated by N5-methyltetrahydrofolate to methionine (see section 1.2), and adenosine is converted to ADP through successive action of adenosine kinase and adenylate kinase (Poulton and Butt, 1976). All of these enzyme activities have been demonstrated in plants.

SHH has been purified to homogeneity from yellow lupin seeds [*Lupinus luteus*] (Guranowski and Pawelkiewicz, 1977). The active enzyme of yellow lupin consists of two non-covalently bound identical subunits with $M_r \approx 55,000$. This enzyme was only assayed in the technically simpler direction of SAH synthesis and was found to contain a strict requirement for L-homocysteine whereas some structural modifications of the nucleoside were tolerated (Guranowski and Pawelkiewicz, 1977).

SHH has also been purified to homogeneity from tobacco where the enzyme is also composed of identical subunits with $M_r \approx 55,000$, but the active enzyme is comprised of four of these subunits (Sebestova *et al.*, 1984).

This overview of studies using purified SHH enzymes, highlights the difficulties in comparing data from different workers due to the use of varied species and differing buffer systems. However, it has been noted that unlike animal SHHs, plant SHH enzymes are not inhibited by adenosine (Sebestova *et al.*, 1984). This has now been proven to be an erroneous statement, as adenosine inhibition only occurs upon animal SHH enzymes in the presence of a thiol (Gomi *et al.*, 1985). Therefore taking this finding into consideration the only significant difference between plant and animal SHH enzymes appears to be the subunit size of ~55,000 and ~47,000 respectively.

1.7 Possible further roles for SHH

1.7.1 SHH: a cytokinin-binding protein ?

A cytokinin-binding protein [CBP] complex was isolated from tobacco leaves (Mitsui and Sugiura, 1993) and found to consist of at least two subunits of 57 and 36kD (Mitsui *et al.*, 1993). A cDNA putatively encoding the 57kD subunit was isolated and found to share extensive homology with previously cloned SHH cDNAs. The purified CBP complex had SHH activity and this gave a first, although not direct, indication of a possible function of CBPs in the hormone signal transduction, as SHH determines levels of SAH and therefore influences nucleic acid and protein methylation (Mitsui *et al.*, 1993).

However later studies using maize have proven that SHH activity *in vitro* is not affected by cytokinins and that maize SHH enzymes and maize CBP activities showed a different elution pattern during all chromatographic fractionation (Romanov and Dietrich, 1995).

Although several CBPs have been isolated with no relationships defined between them (reviewed by Palme, 1993), it appears unlikely that SHH, a conserved protein, with a conserved biochemical function, may be part of a CBP complex within one plant species and not within another. Further work needs to be performed to assess whether SHH is part of a CBP complex in plants. If this is demonstrated, it then should be assessed whether SHH enzyme activity has any role within this complex.

1.7.2 SHH: a copper-binding protein ?

It has recently been proposed that mouse SHH is a bifunctional protein which not only catalyses SAH hydrolysis but also acts as a copper-binding protein (Bethin et al., 1995a). A mouse cDNA which was isolated following amino acid sequencing of a peptide fragment of purified copper-binding protein [CuBP] was >95% identical at the amino acid level, to the rat (Ogawa et al., 1987) and human SHH cDNAs (Coulter-Karis and Hershfield, 1989). This result was proven by a number of methods, not to be an artefact from copurification of SHH with a CuBP. It was also ruled out that these two proteins could share significant homology in the amino acid sequenced domain, leading to the isolation of the wrong cDNA (Bethin et al., 1995a). A single high affinity copper-binding site per 48kD subunit was determined for mouse liver SHH (Bethin et al., 1995b). SHH purified from mouse liver was found to have copper bound to it which suggests in vivo copper binding, but this binding has no effect on SHH enzyme activity. The higher levels of SHH observed in liver in several studies (for example, Walker and Duerre, 1975) may reflect this organ's ability to accumulate high concentrations of copper, and it has also been noted that decreased levels of copper lead to decreased levels of SHH in mouse liver (Bethin et al., 1995b). Although copper is tightly bound to mouse liver SHH, stable copper bound to SHH will freely exchange with radioactive copper, which is atypical for enzymes with copper found at their active sites, and has therefore been proposed to suggest an intracellular trafficking role (Bethin et al., 1995b).

This work suggests that the role of SHH in copper metabolism could involve both its enzymatic and copper binding properties. The homocysteine produced by SHH-catalysed hydrolysis of SAH can be used to synthesise cysteine in animals. This is the only pathway for cysteine synthesis in animals (Giovanelli, 1987). Cysteine is a major constituent of metallothionein, a metalbinding protein and also glutathione, a proposed copper trafficking factor. Therefore this may link SHH activity to copper metabolism in animals. In plants, *de novo* synthesis of homocysteine occurs from cysteine in a unidirectional manner (Giovanelli, 1987), and therefore this link between SHH activity and copper metabolism may not be necessarily true. However the proposed copper binding activity of SHH may have a role within plants. In animals it is proposed that copper binding by SHH may influence the distribution of intracellular copper (Bethin *et al.*, 1995a). SHH bound copper may influence copper distribution by equilibrating with various copper pools or by direct transfer of SHH copper to copper-binding enzymes or other proteins involved in copper metabolism (Bethin *et al.*, 1995a). To date the copper binding properties of plant SHH enzymes have not been studied.

1.8 SHH gene structure and regulation

When this research was initiated in autumn 1992, the following SHH clones had been published or were available on the current EMBL database: *Rattus norvegicus* [rat] (Ogawa *et al.*, 1987); *Dictyostelium discoideum* (Kasir *et al.*, 1988); *Homo sapiens* [human] (Coulter-Karis and Hershfield, 1989); *Caenorhabditis elegans* (Prasad *et al.*, 1993); *Leishmania donovani* (Henderson *et al.*, 1992); *Petroselinum crispum* [parsley] (Kawalleck *et al.*, 1992); *Rhodobacter capsulatus* (Sganga *et al.*, 1992).

1.8.1 Conservation of gene structure

A rat cDNA was the first SHH clone isolated, using antibodies produced following the isolation of purified rat liver SHH enzyme. Sequence analysis revealed one open reading frame of 432 amino acids and the deduced M_r of 47,300 was in excellent agreement with the reported M_r of 47,000. Experiments showed two post-translational modifications to occur at the NH₂-terminus with the initial methionine being removed and the new NH₂-terminal alanine being blocked (Ogawa *et al.*, 1987).

The Dictyostelium discoideum cDNA was isolated using the rat clone as a heterologous probe (Kasir *et al.*, 1988). On sequence analysis of this cDNA the predicted amino acid translation of 430 amino acids was found to be 74% identical to the rat cDNA's predicted amino acid sequence, and if conservative changes were taken into account this homology rose to 84%. The codon usage of rat and *D. discoideum* mRNA is very different with a preferential use by *D. discoideum* of codons with A or T in the third position, which favours weaker

codon-anticodon interactions. This codon usage difference could be clearly observed on comparison of the 33 leucine residues occurring in identical positions within the *D. discoideum* and rat predicted amino acid sequences. Of these 33 conserved leucines, 22 of the codons are changed in two bases [TTA in *D. discoideum* to CTG, CTT or CTC in rat] (Kasir *et al.*, 1988).

Initial analysis of these SHH cDNAs predicted amino acid structure identified sequence 'motifs'. In proteins in which the NAD⁺ -binding site has been determined, a 'fingerprint' characteristic for the dinucleotide-binding domains has been defined. This domain consists of a region of β -sheet, followed by a region of α -helix, and then another region of β -sheet, therefore making a $\beta \alpha \beta$ unit (Wierenga and Hol, 1983). This unit structure has also been shown to have the following features: the sequence Gly-Xaa-Gly-Xaa-Caly in the region joining the first β -sheet and the amino terminus of the α -helix; the presence of a hydrophilic residue at the beginning of the first β -sheet; the presence of an acidic residue at the carboxyl terminus of the second β -sheet; and finally several neutral or hydrophobic residues located at specific positions in the hydrophobic core of the $\beta \alpha \beta$ unit (Wierenga and Hol, 1983). Such a motif was observed in the rat and *D. discoideum* SHH predicted amino acid sequences (Ogawa *et al.*, 1987; Kasir *et al.*, 1988).

Both the isolated rat and *D. discoideum* cDNAs were expressed in *E. coli* and SHH activity was detected in the recombinant bacteria (Gomi *et al.*, 1989; Kasir *et al.*, 1988). The bacterial expressed rat SHH enzyme was purified from the *E. coli* as a tetramer, with NAD⁺ bound to each subunit. The NH₂-terminal alanine was no longer blocked although the initial methionine was removed. These results clearly indicated that the recombinant enzyme was structurally identical to the rat liver enzyme except for the absence of the NH₂-terminal blocking group (Gomi *et al.*, 1989). These findings also support earlier findings that SHH is composed of only one type of polypeptide chain (Gomi *et al.*, 1985; see section 1.6.1). Site directed mutants were created in which each of the three glycine residues of the putative NAD⁺ -binding site were converted to valine. Each of these mutant proteins were still immunoreactive but had no catalytic

activity, contained no bound NAD⁺ and did not form the same quaternary structure as the wild type protein expressed in *E. coli* (Gomi *et al.*, 1989). These findings add further support to the proposal that this specific region of the SHH protein binds the cofactor NAD⁺.

The extreme amino acid sequence homology was further highlighted following the isolation of SHH clones from several evolutionary divergent species; human (Coulter-Karis and Hershfield, 1989); *Caenorhabditis elegans* (Prasad *et al.*, 1993); *Leishmania donovani* (Henderson *et al.*, 1992); parsley (Kawalleck *et al.*, 1992); and *Rhodobacter capsulatus* (Sganga *et al.*, 1992). The SHH amino acid sequence from the purple non-sulphur photosynthetic bacterium *Rhodobacter capsulatus* shows a remarkable degree of amino acid sequence homology to the previously cloned cDNAs. This conservation is approximately 65% identity and 77% similarity to the previously isolated SHHs from rat (Ogawa *et al.*, 1987), *D. discoideum* (Kasir *et al.*, 1988), human (Coulter-Karis and Hershfield, 1989), and *C. elegans* (Prasad *et al.*, 1993). This is one of the highest levels of sequence conservation ever reported between proteins having a similar function in prokaryotic and human cells (Sganga *et al.*, 1992). This lack of sequence divergence may suggest a stringent requirement for SHH to retain its primary structure for function (Kasir *et al.*, 1988).

Several features of the *Rhodobacter capsulatus* SHH predicted amino acid sequence are notable. In rat, 5'-*p*-fluorosulfonylbenzoyladenosine (FSBA) inactivates SHH by promoting disulphide bond formation between Cys⁷⁸ and Cys¹¹² (Gomi *et al.*, 1986; numbering based on the rat sequence). However, when the *Rhodobacter capsulatus* SHH cDNA was cloned , it was found not to have a conserved Cys¹¹² and that site directed mutants of the rat SHH at these points do not inactivate the enzyme (Sganga *et al.*, 1992). Therefore, these two 'conserved' [or as first thought] amino acids may not be as important as earlier literature emphasised in the catalytic role of the enzyme.

Both the R. *capsulatus* and the parsley amino acid sequences (Kawalleck *et al.*, 1992) have an additional amino acid motif in comparison to the rat, *D. discoideum*, human, *C. elegans* and *L. donovani* sequences. *R. capsulatus* has an additional 36 amino acid region whereas parsley has an additional 41 amino

acids. These two additional stretches are found in exactly the same position in the predicted protein sequence but do not show significant homology.

An insertional mutation of the bacterial SHH gene was constructed and is the only example of an SHH null mutation (Sganga et al., 1992). As the methyl cycle cannot operate in these mutant strains due to the lack of SHH activity, the homocysteine required for methionine biosynthesis must be supplied by *de novo* synthesis from aspartate and sulphate. Although the mutant strain can not grow on minimal media, it can grow on minimal media supplemented with methionine or homocysteine. The SAM/SAH ratio is much lower in null mutants where SAH is not hydrolysed. This alteration in SAM/SAH ratio affects the synthesis of the photopigment bacteriochlorophyll which requires methyltransferase activity during its biosynthesis (see section 1.4.3.6). The methyltransferase involved in the biosynthesis has been demonstrated to be non-competitively inhibited by SAH, hence it is susceptible to a change in the SAM/SAH ratio. The bacterial SHH transcription is repressed by high light intensity, mimicking the repression of bacteriochlorophyll biosynthesis by high light intensity, therefore indicating photosynthetic bacteria may control pigment biosynthesis in response to light intensity, at least in part by altering the intracellular SAH/SAM ratio (Sganga et al., 1992).

The first and only genomic SHH clone from a higher eukaryote has been recently identified from rat (Merta *et al.*, 1995). This gene has a complex arrangement comprised of ten exons found within a 15kb span of DNA. Several pseudogenes are also present within this genome.

The only plant SHH cDNA published prior to the onset of this research, was isolated from cultured parsley cells, which had been treated with fungal elicitor that rapidly activates the transcription of many genes encoding specific steps in pathogen-defence related pathways (Kawalleck *et al.*, 1992). This cDNA is 64% identical to the rat cDNA and 79% similar at the amino acid level, reiterating the high levels of sequence conservation observed between diverse species. The extra stretch of amino acids present within the predicted parsley SHH sequence which is not found in the rat or *D. discoideum* sequences, may help to explain the estimated larger subunit size of plant SHH enzymes

compared to mammalian and bacterial (Kawalleck *et al.*, 1992; see section 1.6). The polyclonal antibody produced against the rat liver SHH purified protein (Ogawa *et al.*, 1987), cross reacted to the parsley cDNA transcription/translation product. Southern data showed a small gene family of SHH genes within parsley.

1.8.2 Regulation of SHH gene expression in plants

Northern analysis proved forced infiltration of fungal elicitor solution into parsley leaves markedly increased SHH transcript levels, and elicitortreated parsley cultured cells displayed a slow, but long lasting, increase in SHH mRNA abundance with a peak between 6-10 hours after addition. These increases in mRNA levels were proved to coincide with increased SHH enzymatic activity (Kawalleck *et al.*, 1992).

This summarised data on SHH transcript inducibility was postulated to suggest a close metabolic link between SHH activity and pathogen defence in leaf tissue, as several methylation reactions are known to be associated with pathogen defence in parsley leaves (Kawalleck *et al.*, 1992). Therefore high levels of SAM synthesis and of activated methyl group turnover may be required for these reactions to proceed at elevated rates in stressed areas of the plant, with lower constitutive levels occurring elsewhere in the plant serving a housekeeping function (Kawalleck *et al.*, 1992). Research on the concentrations of SAM and SAH within elicitor treated alfalfa cells suggest that, both increase in concentration after treatment. The ratio of SAM and SAH is also reduced from 10:1 to 4:1 after elicitation, suggesting increased methylation (Edwards, 1995).

1.9 SHH as a wound-induced gene in asparagus

As will be explained in chapter 3, the work described in this thesis originated from a clone isolated from a cDNA library prepared from RNA derived from mechanically separated asparagus cells. This model system was developed to study the wound response and therefore the asparagus SHH cDNA putatively represents a wound induced clone.

The response to wounding in higher plants involves a complex

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interplay of signals, which induce a large number of coordinated events involved in wound perception and subsequent protection of the plant. Not only are there local responses in the same organ but also many higher plants are capable of inducing systemic responses in undamaged distant tissues (For reviews see Bowles, 1990 and 1994; also see chapter 3). Many studies on wounding have used slices of storage tissue such as tubers (Logemann *et al.*, 1988) but analysis has revealed very few wound inducible transcripts, which possibly results from the fact that the viable cells in the local wound response zone occupy only a relatively small volume of the total explant. Therefore it was desirable to develop a system to study the local wound response in which viable cells adjacent to the wound surface and in the proximal wound zone were enriched. These requirements led to the study of mechanically isolated asparagus cells as a possible model system for wounding.

Viable single cells can be isolated mechanically in very large numbers from mesophyll tissues of asparagus (Paul *et al.*, 1989) and represent a population of physiologically and cytologically similar cells that are all uniformly wounded (further described in chapter 3).

1.10 Scope of this thesis

This thesis concerns the cloning and analysis of an asparagus cDNA, isolated from a library putatively containing a large number of wound-inducible clones. Prior to the onset of the research described in this thesis, partial sequence data of this clone suggested significant homology to previously cloned *S*-adenosyl-L-homocysteine hydrolase cDNAs.

The initial objective of the project was to characterise the asparagus cDNA with respect to its expression pattern within mechanically separated asparagus cells and other wounded asparagus tissue, to elucidate if this clone represented a wound inducible transcript. This cDNA also needed to be sequenced in full to confirm its putative identity as an asparagus *S*-adenosyl-L-homocysteine hydrolase clone.

As this asparagus cDNA putatively represented only the second plant SHH clone to be isolated, at the onset of this project, studies of gene sequence and structure conservation between plant SHH gene sequences were initiated. This utilised a PCR-based approach to attempt to amplify stretches of SHH genes from diverse plant species.

As well as this work on the conservation of plant SHH genes, a study of the regulation of SHH enzyme activity was also embarked upon within this project. To aid in this analysis an antibody had to be produced to help monitor SHH enzyme abundance. The study of SHH gene regulation would be aided by the use of reporter gene technology to monitor SHH promoter activity in transgenic plants, and so a further objective of the project was the isolation of such a promoter. These combined analyses would be used to assess if SHH was a constitutive housekeeping gene, whose temporal and spatial expression pattern could be upregulated by stimuli such as wounding.

A final objective of this work was to try and further analyse the role of SHH enzyme activity, by the perturbation of endogenous SHH gene expression, in transgenic plants.

To summarise the objectives of this work, this thesis aims to answer the following questions:

- Is the asparagus cDNA a wound-inducible transcript?
- Are SHH gene sequences conserved in evolutionary divergent plant species?
- Is the presence of an extra polypeptide motif conserved in diverse plant species?
- Are plant SHH genes conserved in their gene structure?
- Does the regulation of SHH enzyme activity occur at transcriptional, translational or post-translational levels?
- Do the locations of SHH promoter-driven transcription correlate to known sites of transmethylation reactions?
- Are any distinct phenotypic effects observed if endogenous SHH expression is inhibited?

Chapter 2

Materials and Methods

The constituents of solutions named within this chapter can be found in Appendix I. Bacteriological media preparations are described in Appendix II.

2.1 Sources of Molecular Biological Reagents, Enzymes, and Plant Tissue Culture Chemicals

Molecular biology grade chemicals and reagents were purchased from BDH Ltd., or from Sigma Chemical Company Ltd. Enzymes were obtained from BRL, Boehringer Mannheim, Stratagene or Pharmacia. Radio-labelled compounds were acquired from Amersham ([^{32}P] α -dCTP with a specific activity of 110TBq/mol and a concentration of 370MBq/ml, [^{32}P] γ -dATP with a specific activity of 110TBq/mol and a concentration of 370MBq/ml, [^{32}P] γ -dATP with a specific activity of 110TBq/mol and a concentration of 370MBq/ml, [^{35}S] α -dATP with a specific activity of greater than 22TBq/mol and a concentration of 370MBq/ml, [^{35}S] α -dATP with a specific activity of greater than 22TBq/mol and a concentration of 370MBq/ml, [^{32}P] γ -dATP with a specific activity of greater than 22TBq/mol and a concentration of 370MBq/ml, [^{14}C]-adenosine with a specific activity 53Ci/mol) Tissue culture salts and hormones were purchased from Flow Laboratories or Sigma. Agar and agar-based media were from Difco Laboratories.

2.2 Bacterial Culture and Storage

2.2.1 Strains and Genotypes

Escherichia coli:

XL1-Blue : recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F' proAB, lacIqZ Δ M15, Tn10 (tetr)], (Bullock *et al.*, 1987).

LE392 : e14⁻(mcrA), hsdR514, supE44, supF58, lacY1 or Δ (lacIZY)6, galK2, galT22, metB1, trpR55, (Sambrook *et al.*, 1989).

Agrobacterium tumefaciens

LBA4404, rifr binary construct host strain, (Hoekema et al., 1983).

2.2.2 Growth of Bacterial Cultures

Liquid cultures were inoculated from bacterial colonies or from glycerol stocks (bacterial growth media are described in appendix II). Single colonies were picked into the appropriate volume of liquid bacterial media containing selective antibiotics, using a sterile pipette tip. From glycerol stocks, a small piece of the frozen stock was removed using a flame-sterilized inoculating loop and placed into culture medium. Cultures were mainly grown overnight, or sometimes until the required optical density had been achieved, in a shaking incubator at 37 °C for *Escherichia coli*, or 28 °C for *Agrobacterium tumefaciens*. Single colonies were produced by using a flamed glass spreader to distribute 50-500 μ l of cell suspension onto agar-solidified selective media; or alternatively a flamed inoculating loop was used to streak overnight cultures onto similar plates of media. Media containing agar was allowed to cool to 50 °C before adding antibiotics and pouring into petri dishes. To decrease the risk of contamination, cultures were handled only within the confines of a laminar air flow cabinet.

2.2.3 Storage of Bacterial Cultures

Glycerol stocks were made from overnight cultures by mixing 1:1 with a solution of 60% glycerol in NB. The glycerol stock was then flash frozen in liquid nitrogen and stored at -80°C.

2.2.4 Antibiotics

The following antibiotics were commonly used for the selection of plasmids, episomes or bacterial strains carrying resistance genes, at the given final concentrations.

E. coli	Agrobacterium
100µg/ml	N/A
100µg/ml	50µg/ml
12.5µg/ml	N/A
N/A	50µg/ml
N/A	400µg/ml
N/A	850µg/ml
	E. coli 100μg/ml 100μg/ml 12.5μg/ml N/A N/A N/A

2.3 Cloning and Manipulation of Plasmids in E. coli

2.3.1 Large Scale Preparation of High Purity Plasmid DNA

Based on the method of Sambrook et al. (1989). Cells were harvested in a GSA bottle from 500ml of an overnight culture (grown as described in section 2.2.2), by centrifugation at 3000rpm using a Sorvall centrifuge. After removal of the spent broth the bacterial pellet was resuspended in 50ml of solution 1 (50mM Tris-HCl pH8.0, 10mM EDTA pH8.0). To this, 50ml of solution 2 (0.2M NaOH, 1% SDS) was added and the tube rolled several times until the solution cleared. Finally 50ml of solution 3 (3M Potassium Acetate pH5.2) was added and after gentle mixing, the glutinous precipitate was pelleted by centrifugation at 10,000rpm. The supernatant was filtered through poly-allomer wool prior to the precipitation of the total nucleic acid content by the addition of an equal volume of propan-2-ol. Following centrifugation at 10,000rpm the resultant pellet was resuspended in 10ml of dH2O and 1g of CsCl added /ml of dH2O (warmed to 30°C to dissolve CsCl if necessary). On addition of 800ul of 10mg/ml ethidium bromide the total volume of approximately 13.5ml was transferred to a Beckman polypropylene tube of this capacity. After heat sealing the tube, it was spun at 15°C in an ultracentrifuge under vacuum, at 55,000rpm, overnight. The following day the lower plasmid DNA band was visualised using a UV lamp and removed in a small volume (1ml) using a

needle and syringe. The EtBr was removed by mixing with an equal volume of $dH_2O/CsCl$ saturated propan-2-ol. Following centrifugation the EtBr was present in the upper layer while the DNA was present in the lower aqueous layer. After several of the above extractions, to remove all EtBr, the plasmid DNA was precipitated from the aqueous layer. To allow plasmid precipitation without precipitation of the CsCl, 3 volumes of dH₂O were added to dilute the salt concentration before adding 2.5 volumes of room temperature ethanol. The resultant pure DNA plasmid was pelleted by centrifugation, washed with 70% ethanol and vacuum dried before resuspension in 300µl of dH₂O.

2.3.2 Small Scale Preparation of Plasmid DNA

The cells from 1.5ml of an overnight culture (grown as described in section 2.2.2) were pelleted using a minicentrifuge at 13,000rpm for three minutes. After removal of the spent broth the bacterial pellet was resuspended in 200µl of solution 1 (50mM Tris-HCl pH8.0, 10mM EDTA pH8.0). To this, 200µl of solution 2 (0.2M NaOH, 1% SDS) were added and the tube inverted gently several times until the solution cleared. Following the addition of 200µl of solution 3 (3M Potassium Acetate pH5.2) and inversion of the tube, the glutinous precipitate was pelleted by centrifugation. Total nucleic acid was precipitated from the removed supernatant by the addition of an equal volume of propan-2-ol. The resultant nucleic acid pellet was resuspended in 200µl of dH₂O. An equal volume of 5M LiCl was added, to precipitate high molecular weight RNA. After centrifugation the DNA containing supernatant was removed and a phenol/chloroform extraction performed. The plasmid DNA was precipitated from the removed upper aqueous phase by the addition of 2.5 volumes of ice-cold ethanol. The resultant crude DNA plasmid pellet was washed with 70% ethanol and vacuum dried before resuspension in 20µl of dH₂O.

2.3.3 Restriction Digests

These were performed according to the manufacturers recommendations for each individual enzyme, using restriction enzyme buffers supplied with the enzymes.

2.3.4 Purification of DNA from Agarose Gels

Several methods of extraction were utilised during this work, each of which are outlined below:

2.3.4.1 Geneclean

This method utilises a kit containing all the named solutions, which is available from Bio-101 Inc. The desired DNA fragment was excised from a low melting point agarose gel and weighed in an 1.5ml microcentrifuge tube (the previously calculated weight of the tube was deducted from the above weight to give the weight of the gel slice). Three volumes of NaI were then added to the slice and this mixture incubated at 55°C for 5 minutes. Meanwhile the glass milk suspension was vortexed vigorously. When the agarose slice had dissolved, 5µl of glass milk suspension was added and the tube left on ice for 10 minutes, with regular inversion. After a short pulse in a microcentrifuge, the supernatant was removed and the pellet washed 3 times with 700µl of cold (-20°C) N.E.W. Wash, with resuspension occurring each time before a short spin. After the final wash all the buffer was removed and the pellet resuspended in 10µl of dH₂O, followed by incubation at 55°C for 5 minutes. The tube was spun for 30 seconds in a microcentrifuge and the DNA containing supernatant carefully removed to a fresh tube. This elution procedure was repeated and the second 10µl added to the first.

2.3.4.2 Electroelution

The desired DNA fragment was excised from an agarose gel and placed in the chamber of an electroelution tank which already contained 1X TAE to the correct working level. The collection chamber of the tank was then filled with High Salt Buffer to retain the DNA when a 100 volt charge was passed through the apparatus. After a one hour electroelution the High Salt Buffer containing the required DNA was removed from the collection chamber and an ethanol precipitation performed. The resultant DNA pellet was washed with 70% ethanol to remove excess salt before vacuum desiccation and final resuspension in an appropriate volume for the subsequent use of the DNA.

2.3.4.3 Gel Slot

Once the desired DNA fragment was separated from other DNA on an agarose gel, a narrow slot was cut in the gel just in front of the required DNA band. The gel was then placed back in the gel tank with enough 1X TAE to reach the top of the gel but not to cover it. The subsequent empty slot was filled with PEG/Salt solution and the gel ran for sufficient length of time to allow the band to run into the slot but not beyond. The PEG/Salt solution was pipetted from the slot into a microcentrifuge tube and visualised on a UV transilluminator to confirm the presence of ethidium bromide containing DNA. The DNA was precipitated from this solution using standard ethanol precipitation. The resultant DNA pellet was washed with 70% ethanol to remove excess PEG/Salt before vacuum desiccation and final resuspension in an appropriate volume for the subsequent use of the DNA.

2.3.4.4 Centrifugal elution

Although the method about to be described results in a relatively poor yield of DNA, with possible agarose contamination, it was found to be suitable for the isolation of DNA to be used subsequently in plasmid subcloning. It has the advantage of being very simple and quick. The gel slice was placed in a 0.5ml microcentrifuge tube which had previously been pierced at the bottom using a fine needle and had a small plug of poly-allomer wool placed in it. This tube was placed in a 1.5ml microcentrifuge tube and then underwent a short 20 second pulse in a microcentrifuge. The liquid content of the gel slice is spun into the lower tube and contains a portion of the DNA. The DNA was precipitated from this solution using standard ethanol precipitation. The resultant DNA pellet was washed with 70% ethanol to remove excess salt before vacuum desiccation and resuspension in an appropriate volume for the subsequent use of the DNA.

2.3.5 End-filling of Restriction Enzyme Generated Overhangs

To produce a blunt end fragment from one with 5' overhangs the cohesive ends were end filled by the addition of a tenth volume of 2mM dNTP

mix and 10 units of Klenow DNA polymerase directly following restriction enzyme digestion. This reaction was incubated at room temperature for 30 minutes before fragment purification from an agarose gel.

2.3.6 Dephosphorylation of DNA

Cloning, involving single restriction enzyme digested vector, had its efficiency improved by the dephosphorylation of the vector to prevent self ligation. Following digestion with the relevant enzyme, the DNA was extracted with phenol/chloroform, ethanol precipitated, vacuum dried and redissolved in a small volume of 1XCIP buffer. The relevant number of Calf Intestinal Alkaline Phosphatase units were added (0.1 unit/pmol 5' overhangs or 1.0 unit/pmol 3' overhangs or blunt ends) and the incubation allowed to proceed at 37°C for 30 minutes. The dephosphorylated vector was then extracted with phenol/chloroform, ethanol precipitated, dried and redissolved in an appropriate volume for subsequent cloning.

2.3.7 Ligation of DNA fragments

Purified DNA was mixed in an $\approx 3:1$ molar ratio of insert to vector before 5X ligation buffer and 1 unit of T₄ DNA ligase were added into as small a volume as possible. If the ligation involved a blunt ended fragment then 10X blunt end ligation buffer was used instead of 5X ligation buffer, to improve the efficiency of the reaction (Rusche and Howard-Flanders, 1985). Ligations were generally left at 12°C overnight before transformation into competent *E. coli*.

2.3.8 Transformation of *E. coli* with Plasmid DNA

2.3.8.1 Preparation of Competent Cells

A litre of 2X YT containing the appropriate antibiotic(s) was inoculated with 1/100th volume of an overnight bacterial culture. This was grown for 2-2.5 hours at 37°C, until the OD₆₀₀=0.5-0.7, upon which the culture was chilled on ice for 30 minutes. The following steps were all performed aseptically using previously autoclaved equipment. The culture was spun at 4000X gmax for 20 minutes in a prechilled rotor. The pelleted cells were resuspended in their original volume of ice cold 1mM HEPES, pH7.0. The culture was respun and

the cells resuspended in half of the original volume of the same HEPES buffer. Following centrifugation the pelleted cells were washed in 20ml of 10% glycerol plus 1mM HEPES, pH7.0. Finally, the recentrifuged cells were resuspended in 2.5ml of 10% glycerol and flash frozen in 50µl aliquots.

2.3.8.2 Transformation Procedure

A vial of electroporation-competent bacteria were thawed on ice, and 50 μ l of cells were transferred to each prechilled 0.2cm cuvette containing 1 μ l of ligation mix. The cuvette contents were shaken to the bottom and left on ice for 1 minute. The Gene Pulser apparatus (Bio-Rad) was set to give 25 μ F, 2.5kV with the pulse controller set to 200 ohms and the dried cuvette placed in the electroporation chamber. Immediately following the pulse (yielding a time constant of 4.6msec optimum) 1ml of 2X YT media was added to the cuvette and the cells resuspended. This was transferred to a 1.5ml microcentrifuge tube and incubated at 37°C for 1 hour while shaking to allow cell recovery, before plating.

2.3.9 Identification of Recombinant Plasmids in Transformed Colonies

Certain vectors used within this thesis allow a colour assay to identify recombinant clones, due to the presence of the multiple cloning site within the lacZ α gene. This gene's product is the α -subunit of β -galactosidase which complements a mutant gene in the host and facilitates the metabolism of the substrate X-GAL (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) when induced by IPTG (isopropyl- β -D-thiogalactopyranoside), forming a blue product within colonies. Therefore when this gene is interrupted by the presence of an insert the recombinant colonies are white and not blue. To allow this colour selection 20 μ l of 40mg/ml IPTG in water, and 20 μ l of 40mg/ml X-GAL in dimethylformamide were spread onto plates before spreading cells.

2.3.9.1 Amplification from Bacterial Colonies by the Polymerase Chain Reaction

If colour selection was not available, as in the later stages of multiple cloning steps, and only a small number of colonies were available to be

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screened, then PCR (Polymerase Chain Reaction) was utilised. A 100µl reaction mix (9.9µl 11X PCR Buffer, 10µl of each primer [100ng/µl], 1µl of Taq DNA polymerase [1 unit/µl], and sterile distilled water) was assembled and subsequently aliquoted. Each colony was picked with a sterile tip and dipped in a separate 10µl PCR reaction mixes before streaking on a fresh selection plate. The DNA was amplified using a Perkin Elmer Cetus DNA thermal cycling machine using the following programme: Denaturing step = 94°C for 1 minute; Annealing step = 50°C to 65°C for 1 minute (the annealing temperature is dependent on the specific primers utilised); and an Extension step = 72°C for 2 minutes. This was performed for 25 cycles and the products analysed by agarose gel electrophoresis.

2.3.9.2 Colony Hybridisation

If colour selection was not available, a large number of colonies were screened by regrowth on a nylon membrane. Following bacterial denaturation for 7 minutes in denaturing solution, 7 minutes neutralisation in neutralising solution, and a brief wash in 2X SSC to remove bacterial debris, the DNA was fixed to the membrane using a Stratalinker[®] UV crosslinker (Stratagene). The filter was prehybridised at 65°C for at least 1 hour in DNA hybridisation solution to block non-specific binding of the radio-labelled probe. After addition of the freshly boiled probe (prepared as described in section 2.9.4) the hybridisation was allowed to continue at 65°C overnight. Filters were washed at 65°C for 20 minutes in wash A, and if higher stringency was required a further 20 minute wash in wash B was performed. Following washing the membrane was wrapped in 'Saran Wrap' and exposed to X-ray film at -80°C in a cassette containing an intensifying screen. The hybridising clones of interest were then grown in liquid culture from the reciprocal bacterial streak on a duplicate plate, for later restriction enzyme analysis.

2.4 Cloning and Manipulation of Plasmids in Agrobacterium tumefaciens

2.4.1 Transformation of *Agrobacterium tumefaciens* with Plasmid DNA **2.4.1.1** Preparation of Competent Cells

This procedure is identical to the preparation of *E. coli* competent cells (section 2.3.8.1) except that the required optical density of cells requires an extended period of growth in comparison to *E. coli*. Therefore the 500ml culture inoculated with a 1/100th volume of a two day old starter culture, was left to grow at 28°C overnight before reaching the required OD.

2.4.1.2 Transformation Procedure

This procedure is identical to the transformation of *E. coli* competent cells (section 2.3.8.2) except that the Gene Pulser machine (Bio-Rad) was set to give 25μ F, 2.5kV with the pulse controller set to 600 ohms (yielding a time constant of 13.0msec optimum). The cells were left to recover for several hours at 28 °C before plating.

2.4.2 Small Scale Isolation of Agrobacterium tumefaciens Total Nucleic Acids

As described in Draper *et al.* (1988). A 5ml culture of *Agrobacterium tumefaciens* was grown overnight from a single colony. Cells were pelleted from 1.5ml of the culture by microcentrifugation for 5 minutes, and resuspended in 300 μ l of dH₂O, to which 100 μ l of 5% Sarkosyl was then added. After mixing, 150 μ l of 5mg/ml pronase was added and the mixture incubated at 37°C for 1 hour. The pronase treated cell suspension was mixed with 500 μ l of phenol/chloroform by vortexing and after centrifugation, the aqueous solution was removed and phenol/chloroform extractions performed in a similar manner for a further 3 times. Total nucleic acid was precipitated from the final aqueous layer by the addition of one twentieth volume of 5M NaCl and 3 volumes of -20°C ethanol. The nucleic acid was collected by microcentrifugation for 10 minutes, rinsed in 70% ethanol, dried, and redissolved in 50 μ l of dH₂O.

2.5 Small Scale Isolation of Total Nucleic Acids from Other Bacterial Species

A 10ml aliquot of culture was spun down and after discarding the supernatant, the resultant pellet was washed with 1ml of STE buffer to remove

residual media. After recentrifugation and removal of the wash buffer solution the pellet was resuspended in 50µl of freshly prepared lysozyme (50µg/µl in TE) and transferred to a microcentrifuge tube. This was incubated at 37°C for 15 minutes to allow cleavage of bonds between sugar residues in the polysaccharide components of the bacterial cell wall. To lyse the bacterial cells 450µl of GE reagent (5M Guanidium Thiocyanate, 0.1M EDTA) was added and the solution vortexed. To this, 250µl of ice cold 7.5M Ammonium Acetate was added and the solution left on ice for 15 minutes. Several chloroform/isoamylalcohol (24:1) extractions were then performed until the protein layer at the interphase was eliminated. The total nucleic acid was then precipitated with the addition of an equal volume of ice cold propan-2-ol. After centrifugation the nucleic acid pellet was washed with 70% ethanol and air dried, before resuspension of the nucleic acids in 50µl of dH₂O.

2.6 DNA Sequencing

2.6.1 Single-Stranded Template Production

Sequencing of single stranded plasmid relies on three factors. Firstly, the plasmid of interest must contain the F1 origin of replication to allow the production of single stranded DNA. Secondly, the plasmid must be in a male cell line, as these contain pili which allow M13 phage to infect the cells. Thirdly, the cells must be superinfected with defective M13 phage to rescue single stranded DNA from the plasmid of interest and extrude it into the medium.

At the same time as 5ml of 2X YT was inoculated with a single colony of the cell line containing the plasmid to be sequenced, 10μ l of VCSM13 helper phage was also added. This was left to grow, shaking at 37°C, for 1 hour before the addition of kanamycin to a final concentration of 100μ g/ml. After overnight growth in similar conditions the resulting culture should contain phagemid DNA packaged as single stranded filamentous phage which had been extruded from the cells. A 1.5ml aliquot of the overnight culture had the cells pelleted by centrifugation and the upper 1ml of the supernatant was removed to a fresh microcentrifuge tube. To precipitate the phage particles 200µl of 20% PEG8000/2.5M NaCl was added to the supernatant and the mixture left at room temperature for 5 minutes, before pelleting the phage by microcentrifugation for 10 minutes. All the supernatant was carefully removed and the pellet resuspended in 200 μ l of dH₂O. To remove the proteinaceous phage coats, phenol/chloroform extractions were performed until a clean interface was achieved. The aqueous single stranded DNA was precipitated with 15 μ l of 4M sodium acetate pH5.2 and 500 μ l of ethanol from the upper aqueous layer. Following a 10 minute microcentrifugation the pelleted DNA was rinsed with 70% ethanol, dried and resuspended in 25 μ l of water, of which 5 μ l was analysed by agarose gel electrophoresis.

2.6.2 Sequencing Using T7 DNA Polymerase

A T7 DNA polymerase sequencing kit (USB), using chain terminating ddNTPs (Sanger *et al.*, 1977) was utilised and the manufacturers instructions followed. The kit allows the same protocol for both single and double stranded templates, once double stranded DNA has been denatured. To denature $\approx 2\mu g$ of double stranded DNA, it was resuspended in 18 μ l of dH₂O, and 2 μ l of 2M NaOH added. After 5 minutes at room temperature, 2 μ l of 5M Ammonium Acetate pH4.8 were added and the DNA precipitated by the addition of 100 μ l of ethanol. After centrifugation, 70% ethanol washing, and drying of the pellet it could be utilised in future steps in a similar manner to single stranded template. To anneal the primer to the template, the following were mixed and the final volume made to 10 μ l with dH₂O:-

1µg template

2µl of 5X Sequencing Annealing Buffer

80 nanograms of primer

This mixture was warmed to 65° C for 2 minutes and then cooled slowly to room temperature. Once the Sequenase[®] enzyme had been diluted 1:8 with enzyme dilution buffer and the labelling mix diluted 1:5 with water, the labelling reaction was performed. To the now cold 10µl annealing reaction the following were added:-

1μl 0.1M DTT2μl of 1:5 dilute labelling mix

0.5μl [α-³⁵S] dATP

2µl of 1:8 diluted Sequenase® enzyme

This was mixed thoroughly and incubated at room temperature for 2-5 minutes. To terminate the reaction 3.5μ l of the labelling reaction was added to 2.5μ l of each of the four prewarmed dideoxy termination mixture. After 2-5 minutes at 37° C, 4μ l of stop solution was added to each of the four tubes. To load these reactions on a prepoured gel, each was heated to 90° C for 5 minutes and 2-3 μ l loaded per lane. Gels were made by polymerising 60ml of Sequencing gel mix by the addition of 70 μ l of TEMED and 170 μ l of 25% Ammonium Persulphate. This mixture was poured into sealed Bio-Rad sequencing plates and left to polymerise for 1 hour. The gels were run at an optimum temperature of 50° C in 1XTBE. Gels were dried onto Whatman 3MM filter paper and exposed to X-ray film at room temperature, overnight.

2.6.3 Automated Taq DyeDeoxy™ Terminator Cycle Sequencing

The Taq DyeDeoxy[™] Terminator Cycle Sequencing was performed on an Applied Biosystems Model 373A DNA sequencing system using ABI dyelabelled dideoxy-nucleotides as terminators and AmpliTag® DNA polymerase. In all cases the template plasmid DNA was double stranded and prepared by alkaline lysis (see section 2.3.2). To perform the reaction, $\approx 1 \mu g$ of template was mixed with 9.5µl of reaction premix, 3.2pmol of primer and the final volume made to 20µl with dH2O. After overlaying the above mixture with one drop of mineral oil the following cycling reactions were performed in a Perkin Elmer Cetus Model 480 thermal cycling machine: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, for a total of 25 cycles. To remove unincorporated excess DyeDeoxyTM terminators the reaction mix was increased to 100µl by the addition of dH₂O and then 100µl of chloroform added to dissolve the mineral oil. On addition of 100µl of phenol:H2O:chloroform (68:18:14) the mixture was vortexed and centrifuged, before removal of the upper aqueous phase from the lower organic dye terminator containing phase. This extraction process was repeated before precipitation of the extension products using 15µl of 2M sodium acetate, pH4.5 and 300µl of ethanol. The dried pellet was then handed to the automated DNA sequencing service operated at Leicester University where samples were run, and processed onto floppy disks for computer analysis.

2.6.4 Data Handling

Sequence data was analysed using the Wisconsin Genetics Computer Group (GCG) programmes (Devereux *et al.*, 1984). Amino acid alignments were performed using the CLUSTAL programme with default parameters (Higgins and Sharp, 1989).

2.7 Transcript Mapping

A suitable DNA restriction fragment was isolated and dephosphorylated as previously described in section 2.3.6. A further restriction enzyme digestion was then performed to internally cleave the fragment. The appropriate probe fragment, which would only be dephosphorylated at the end protected from S1nuclease due to hybridising to RNA, was then isolated from an agarose gel.

2.7.1 Kinase labelling

The isolated probe fragment was end labelled using T4 polynucleotide kinase in the following reaction:-

- 5µl Probe fragment (≈2pmoles)
- 5µl 10X T4 polynucleotide kinase buffer
- 3µl [γ-32P] dATP which equals ≈10pmoles
- 2µl T4 polynucleotide kinase (20units)
- 35µl dH2O

Following incubation at 37 °C for 30 minutes, 2μ l of 0.5M EDTA was added and the solution extracted once with phenol/chloroform before ethanol precipitation. The DNA was resuspended in 50µl of dH₂O and the unincorporated nucleotides removed by Sephadex G-50 spun column chromatography. Following ethanol precipitation the probe was dried and the incorporated counts measured by Cerenkov counting.

2.7.2 Nuclease-S1 Mapping

A 10µg aliquot of total RNA was mixed with 3µl of labelled probe (\approx 60,000 cpm) and precipitated using ethanol. The resultant dried nucleic acid pellet was resuspended in 30µl of nuclease-S1 hybridisation buffer and incubated at 85°C for 10 minutes to denature the nucleic acids followed by overnight incubation at the desired hybridisation temperature (40-65°C). The next day 300µl of ice-cold nuclease-S1 mapping buffer was then added and digestion performed at 37°C for 30 minutes. The reaction was cooled to 0°C and 80µl of ice-cold nuclease-S1 stop mixture added. Following a phenol/chloroform extraction, two volumes of ethanol were added and the nucleic acid pelleted by centrifugation. The pellet was washed with 70% ethanol, dried and resuspended in 4µl of dH₂O. Following the addition of 6µl of sequencing stop solution, the mixture was boiled for 2 minutes prior to loading on a denaturing polyacrylamide sequencing gel. Known DNA sequence ladder was run on the same gel to assay the size of any protected fragments.

2.8 Asparagus officinalis Growth and Culture

Asparagus officinalis (cv. Connover's colossal) seed was purchased from Nickerson Seeds Ltd.

2.8.1 Growth of Asparagus for Wounding Studies

For studies on the wound response in intact asparagus tissue, seeds were sown on sterile vermiculite and then grown in the dark at 26 °C. Etiolated seedlings were harvested 2 weeks after germination and mesocotyls were cut into 5mm lengths. Incubation proceeded on damp filter paper in Petri dishes at 26° C in the dark for the specified time periods.

2.8.2 Asparagus Cell Isolation

The mechanical isolation and culture of asparagus cells were carried out essentially as described by Paul *et al.* (1989). Six-week-old greenhouse grown plants had their cladodes stripped from the stems. The cladodes were surface sterilised in 10% bleach for 20 minutes, then the bleach was rinsed off the sterile cladodes with 4 washes of 400ml of sterile H₂O. Small amounts of sterile

cladodes were gently ground in a small quantity of sterile H2O using a mortar and pestle. The resulting cell suspension was filtered through a 64µm sieve to remove large debris from the resulting cell suspension. This grinding process was repeated on the large debris using fresh H2O to increase yields. After all sterilised cladodes had been ground twice, the total cell suspension volume was transferred to a GSA bottle and the intact cells pelleted by centrifugation at 800rpm in a benchtop Sorvall for 5 minutes. The resultant supernatant, containing debris from damaged cells, was removed and the pellet resuspended in 100ml of sterile H₂O. After identical centrifugation the supernatant was again removed and the pelleted cells resuspended in 80ml of asparagus medium. The intact cell concentration was calculated with the aid of a haemocytometer, and after similar centrifugation as described previously, the pelleted cells were resuspended to a final concentration of 4x10⁵ intact cells/ml of asparagus medium. The cell suspensions were placed in 10ml aliquots in 9cm petri dishes and after sealing with Nescofilm were incubated in the dark at 25°C on a rotating platform (40rpm). To harvest the cells, they were collected by centrifugation and stored at -80°C if not for immediate use.

2.9 cDNA and Genomic Library Screening

All libraries screened within this thesis had previously been constructed at Leicester. Credit must therefore be given to Dr. Simon Warner and Mr. Rob Darby for construction of *Asparagus officinalis* cDNA libraries and Dr. Michael Roberts for the construction of an *Arabidopsis thaliana* genomic library (Warner *et al.*, 1992; Roberts *et al.*, 1993). Both asparagus cDNA libraries were constructed in the same manner using the insertion vector Lambda Zap®II from Stratagene. This vector allows *in vivo* excision of a plasmid containing the cloned fragment. In both cases *Eco* RI/Not I adapters were used to allow removal of whole cDNAs from the vector. The *Arabidopsis* genomic library was constructed in Lambda Dash II (Stratagene). All libraries screened had been amplified.

2.9.1 Plating of Lambda Phage

cDNA libraries were plated on competent *E. coli*, strain XL1-Blue, while genomic libraries were plated on *E. coli*, strain LE392. Both strains of competent *E. coli* were produced by inoculating 50ml of LB medium containing 0.2%maltose and 10mM MgSO4 with 100µl of an overnight culture. Upon the culture reaching an OD₆₀₀=0.5, the cells were collected by centrifugation and resuspended in 0.5 volumes of ice cold 10mM MgSO4, and stored for up to a week at 4°C. These cells were utilised to obtain plaques of lambda clones within a bacterial lawn in the following manner. 800µl of competent cells were mixed with the desired number of p.f.u. (plaque forming units) from the recently titred library and incubated at 37°C for 15 minutes to permit adsorption of the phage to the cells. To this suspension, 8ml of molten top agar (appendix II) at 48°C was added, and the resulting mixture poured onto a 14cm petri dish containing solid NA medium. The plates were inverted for 8-16 hours at 37°C, dependent upon the size of plaque required. For 9cm petri dishes, 200µl of cells and 3ml of top agar was used.

2.9.2 Plaque Lifts and Library Screening

Petri dishes were cooled to 4° C to harden the top agar to help prevent the agar sticking to the nitrocellulose. Circles of Hybond-N (Amersham) were cut to the size of the plates in use, and the plaques transferred by laying the membrane onto the plates for 2 minutes. During this time orientation marks were made with a needle and a marker pen to allow realignment of the filter to the plate after hybridisation. If duplicate filters were taken from the same plate the second membrane was left on the plate for 4 minutes. Membranes were placed plaque side up on filter paper soaked in denaturing solution for 7 minutes, then transferred to filter paper soaked in neutralising solution for 7 minutes. After rinsing the filters in 2X SSC, the DNA was bound to the membrane using a StratalinkerTM UV Crosslinker (Stratagene). The membranes were then probed in exactly the same manner as described in section 2.3.9.2.

2.9.3 Storage of Isolated Phage Clones

Clones of interest were isolated by coring plaques from plates using a
Pasteur pipette and eluting the phage from the core into 500 μ l SM. If after several rounds of screening an individual plaque of interest could be isolated without contamination from any other plaques, it was eluted as described with the addition of 20 μ l of chloroform, to prevent bacterial growth, for long term storage at 4°C.

2.9.4 Preparation of Radio-Labelled DNA Probes

DNA probes were made using the random hexamer priming method of Feinberg and Vogelstein (1984). DNA probe stocks were isolated from agarose gels as low melting point agarose gel slices, or purified from the gel (section 2.3.4). After dilution to $1ng/\mu l$, $10\mu l$ of probe stock was removed and boiled for 5 minutes before addition to the following labelling reaction:-

10µl probe stock (10ng) 3µl Oligolabelling buffer 0.6µl of 10mg/ml BSA (DNase free) 1.5µl [³²P] α-dCTP

0.6µl DNA polymerase I Klenow fragment

The labelling reaction was allowed to proceed for 1 hour at 37° C before incorporation was measured.

2.9.5 Measurement of Isotope Incorporation into DNA

To stop the reaction, 85μ l of dH₂O was added to the 15µl labelling reaction to dilute the enzyme and inhibit its activity. To calculate the total number of radioactive counts in the reaction, 2µl was spotted onto a GF/C disk. A further 2µl was added to 500µl of 500µg/ml herring sperm which acts as a carrier. To precipitate the DNA, 125µl of 50% TCA was added and then the mixture spotted onto a fresh GF/C disk in a vacuum funnel. The GF/C disk in the funnel was washed twice with 10% TCA and once with IMS and therefore only incorporated radioactive nucleotides are found on this disk. The two disks were then placed in separate scintillation vials and counted on a LKB Wallac liquid scintillation counter. Incorporation was calculated as a percentage of the precipitated (incorporated) counts against the total counts in the reaction. To remove unincorporated nucleotides the probe was then spun down a G50

Sephadex (pre-autoclaved in STE to fully expand the Sephadex) column which had been made in a 1ml syringe barrel. The probe was boiled for 5 minutes to denature the DNA before addition to the hybridisation reaction.

2.9.6 Rescue of pBluescript Carrying cDNA Inserts from Lambda Zap II via an *In Vivo* Excision Protocol

Two hundred microlitres of positive phage stock were mixed with 200µl of *E. coli* (XL1-Blue) at OD₆₀₀=1.0, 1µl of R408 helper phage (>7.5X10¹⁰ p.f.u./ml) and incubated at 37 °C for 15 minutes. 5ml of 2X YT media were added and the culture shaken at 37 °C for 3 hours. The tube was then heated to 70 °C for 20 minutes to kill the host XL1-Blues before removing cell debris by centrifugation at 4000rpm for 10 minutes. The supernatant contains the pBluescript phagemid packaged as filamentous phage particles. Therefore to plate the rescued phagemid, 10µl of supernatant and separately 10µl of a 1:100 dilution of supernatant, were mixed with 200µl of *E. coli* (XL1-Blue) at OD₆₀₀=1.0 and incubated at 37 °C for 15 minutes before plating 1-100µl onto NA plates containing 100µg/ml ampicillin. Overnight incubation at 37 °C produced colonies of cells containing pBluescript in plasmid form, from which cDNA inserts could be isolated by restriction digests of purified plasmid DNA.

2.9.7 Preparation of Phage Lambda DNA

This method was essentially performed as described by Sambrook *et al.* (1989), with the addition of some extra centrifugation steps to remove insoluble matter which escaped earlier attempted removal. To 500ml of prewarmed NZCYM media, 1ml of an overnight culture of *E. coli* strain LE392 was added, and grown at 37°C for 3-4 hours until OD₆₀₀=0.5. This culture was then inoculated with 10¹⁰ p.f.u. of a pre-titred phage stock and incubation continued until lysis occurred after 3-5 hours. On lysis, 10ml of chloroform was added and incubation continued for 10 minutes. Upon removal of the chloroform 1µg/ml of DNase I and RNase were added to digest released bacterial nucleic acid. Once digestion had occurred for 30 minutes at room temperature, solid NaCl was added to a final concentration of 1M and dissolved by stirring. Following centrifugation to pellet bacterial debris, the supernatant was removed and solid

PEG8000 added to a final concentration of 10% and dissolved by stirring. To allow the phage particles to precipitate the solution was then left in iced water for an hour. The phage were collected by centrifugation and after removal of all the supernatant the pellet was resuspended in 8ml of SM. To remove particulate matter which could not be resuspended the sample was quickly spun again and the supernatant transferred to a fresh tube. 1µg/ml of DNase I and RNase was added to digest any remaining bacterial nucleic acid during a 30 minute incubation at room temperature. An equal volume of phage precipitation buffer was then added to precipitate the now pure phage particles. The phage were collected by centrifugation and after the removal of all the supernatant, the pellet was resuspended in 1ml of SM. EDTA pH8.0 was added to a final concentration of 20mM followed by addition of proteinase K to a final concentration of 50µg/ml. Prior to an hour incubation at 56°C, 50µl of 10% SDS was added. The solution was extracted twice with phenol/chloroform and the DNA precipitated by the addition of 1/20th volume of 3M sodium acetate pH7.0 and 2.5 volumes of ethanol. Following a 70% ethanol wash and drying of the DNA pellet, the phage DNA was resuspended in an appropriate volume of distilled water.

2.10 Analysis of Gene Expression by Northern Blotting 2.10.1 Extraction of Plant RNA

Total RNA was extracted from plant tissues essentially as described in Draper *et al.* (1988). Tissue was flash frozen in liquid nitrogen and ground with acid washed sand in a mortar and pestle. More liquid nitrogen was added and 2ml/g fresh weight of RNA grinding buffer was added whilst further grinding occurred. The ground mixture was transferred to a 50ml polypropylene tube and left on ice until the mixture was thawed. An equal volume of phenol/chloroform was added and the solution emulsified by inversion. The protein precipitate was collected at the interface by a 5 minute centrifugation at 3700rpm (4°C). The aqueous supernatant was removed and a further three phenol/chloroform extractions performed. The final aqueous phase was removed to a siliconised Corex tube and 1/20th volume of 4M sodium acetate and 2.5 volumes of ice cold ethanol added to precipitate total nucleic acids,

which were collected by centrifugation at 10,000rpm for 10 minutes at 4°C. The pellet was resuspended in 2ml of dH₂O and a half volume of 8M LiCl added. This was left on ice for 2 hours to allow the differential precipitation of RNA. The RNA was collected by centrifugation at 10,000rpm for 10 minutes at 4°C, the pellet resuspended in a small volume of sterile distilled water and the concentration measured by scanning spectrophotometry.

2.10.2 Scanning Spectrophotometry of Nucleic Acids

Nucleic acids have an absorption peak at 260nm and therefore OD_{260} is used to determine nucleic acid concentration. Nucleic acid solutions were diluted 1 in 200 and transferred to a quartz cuvette to be scanned across the range 200-300nm using a Hewlett Packard spectrophotometer. A peak at 260nm with a trough at 220-240nm indicates a pure nucleic acid preparation. An OD_{260} of 1.0 is equivalent to a 40µg/ml RNA solution, or a 50µg/ml double stranded DNA solution.

2.10.3 Northern Blotting

Based on a method by Fourney *et al.* (1988). 10µg of total RNA was denatured by heating to 65°C for 5 minutes in 19µl of RNA denaturing solution. After cooling, 3µl of RNA loading buffer were added and the samples loaded on a 0.8% agarose RNA formaldehyde gel. This was run at 100 volts in 1X MOPS. To visualise total RNA loadings the gel was placed in an ethidium bromide solution for 20 minutes. After photographing the gel, it was washed in 0.05M NaOH for 20 minutes prior to washing in 20X SSC for equilibration to allow capillary transfer of RNA onto a nylon membrane. Capillary transfer was performed as described in section 2.11.3 and after transfer the RNA was crosslinked to the membrane using a Stratalinker[™] UV Crosslinker (Stratagene).

2.10.4 Hybridisation of DNA Probes to RNA bound to a Nylon Support

Filters were prehybridised in RNA hybridisation solution for two hours at 42 °C before the addition of freshly boiled DNA radio-labelled probe. The hybridisation was performed overnight at 42 °C whereupon washes were performed using wash A (and wash B if necessary) at 42°C. Hybridising mRNAs were detected by autoradiography.

2.11 Southern Blot Analysis of DNA

2.11.1 Extraction of Plant DNA

DNA was extracted from plant tissues using a CTAB extraction method, essentially as described by Murray and Thompson (1980). Leaf tissue was flash frozen in liquid nitrogen and ground with the aid of acid washed sand and a mortar and pestle. This powder was transferred to a 50ml centrifuge tube and an equal volume of 2X CTAB extraction buffer added. After a 30 minute incubation at 60 $^{\circ}$ C a chloroform extraction was performed, and to the resultant removed aqueous phase 1/10th volume of 10% CTAB (prewarmed to 60°C) was added. Following a further chloroform extraction to remove proteinaceous material the DNA/CTAB complex was precipitated with the addition of 2 volumes of CTAB precipitation buffer. Following a hour incubation at room temperature, the precipitated DNA was pelleted by centrifugation and resuspended in 450µl of 1M NaCl. The DNA was precipitated from this by the addition of 900µl of room temperature ethanol, leaving the CTAB in solution. The pellet was resuspended in a 20µg/ml solution of RNase A and incubated at 37°C for 10 minutes. A final phenol/chloroform extraction was performed before an ethanol precipitation of the DNA. On resuspension the DNA was scanned on a spectrophotometer, as described in section 2.10.2, to allow dilution to a particular concentration.

2.11.2 Agarose Gel Electrophoretic Separation of DNA

Agarose gels were made by dissolving agarose in TAE buffer at 95° C and then adding ethidium bromide to a final concentration of 5μ g/ml. The agarose concentrations within gels varied from 0.6%-3% dependent upon the size of fragments requiring resolution. The required amount of 6X DNA loading buffer was added to each sample before pipetting into the well of the gel. The gels were run submerged in TAE buffer containing 1μ g/ml ethidium bromide at 40-150 Volts. The ethidium bromide in the gel and TAE buffer intercalates nucleic acids allowing their visualisation and photography under U.V light.

2.11.3 Southern Blotting

Based on the method of Southern (1975). To prepare gels for Southern blot transfer, each was soaked in 3 volumes of; depurinating solution for 7 minutes, denaturing solution for 30 minutes, neutralising solution for 30 minutes and finally a brief rinse in 20X SSC. To transfer the DNA, a capillary blot was set up in the following manner. The treated gel was placed on Whatman 3MM filter paper on a sponge standing in a tray of 20X SSC. The remaining surface of the paper and sponge was covered in cling film to prevent the 20X SSC from passing around the edges of the gel. Hybond-N (Amersham) was placed on top of the gel and marked with a pencil to allow later orientation to the original gel. Three pieces of 3MM soaked in 20X SSC were placed on top of the membrane followed by a further three pieces of dry 3MM. A wad of paper towels was placed over the 3MM with the final addition of a 0.5kg weight on the top. This was left overnight to allow capillary transfer. The transferred DNA was bound to the Hybond-N using a Stratalinker[™] UV Crosslinker (Stratagene). The membrane was then hybridised and probed as described in section 2.3.9.2.

2.11.4 Reuse of Blots

Probes were removed from filters by incubation at 45° C for 30 minutes in 100ml of 0.4M NaOH prior to washing in 100ml of Blot Strip Solution for 30 minutes at 45° C. The blots could then be prehybridised and hybridised with a new probe.

2.12 Plant Transformation Procedures

2.12.1 Tobacco (Nicotiana tabacum) Leaf Disk Transformation

Nicotiana tabacum (Petite Havana SR1) plants were transformed using the leaf disk transformation method as described in Draper *et al.* (1988). Young, nearly fully expanded leaves were removed from greenhouse grown plants and surface sterilised in 10% bleach for 15 minutes. Once sterilised all further manipulations were performed with sterile equipment in a laminar flow hood. To remove the bleach from the leaves, they were washed in four changes of 400ml of sterile tap water. Alternatively, 1cm square leaf disks were cut using a scalpel, or 1.5cm diameter circular disks were cut with a cork borer, in both cases midribs and major leaf veins were avoided. The cut leaf disks were inoculated by a 30 minute immersion in a suspension (an overnight culture diluted 1:50 with liquid MSD4X2) of Agrobacterium containing the binary construct of interest. As each disk was placed on solid MSD4X2 plates it was shaken to remove excess Agrobacterium suspension. These plates were incubated for 2-4 days, depending on when Agrobacterium growth could be visualised, in growth rooms at 24-26°C. Explants were then transferred to MSD4X2 plates containing selective antibiotics (400µg/ml augmentin and 100µg/ml kanamycin) and incubation in the same growth room at low lightintensity continued until antibiotic resistant shoots arose from the callus around the leaf tissue. Once large enough these shoots were aseptically removed and transferred to MSO medium containing the same concentration of antibiotics. In this medium the shoots produce roots and then the plantlets can be transferred to soil.

2.12.2 Axillary Bud Propagation of Tobacco

To allow continued investigation of reporter gene expression in primary tobacco transformants, axillary bud propagation (Draper *et al.*, 1988) was performed to produce large numbers of genetic clones of the original transformants. Mature stems were cut into nodal sections with short 6-8mm petiole stumps and sterilised in 10% bleach for 15 minutes before rinsing in four changes of distilled water. The bleach damaged areas were then removed using a sterile scalpel and the nodal sections placed in MSO containing $400\mu g/ml$ augmentin and $100\mu g/ml$ kanamycin. Following 2-3 weeks incubation at 24-26°C under moderate illumination an apically dominant axillary shoot developed which could then be rooted as described in section 2.12.1.

2.12.3 In planta Arabidopsis thaliana Transformation using Vacuum Infiltration

This was essentially performed as described by Bechtold et. al. (1993).

LBA4404 Agrobacterium containing the Bin19 derived binary vector of interest were grown with rifampicin (50µg/ml) and kanamycin (50µg/ml) for ≈14 h at 28°C in 1.5l of NB medium (final OD₆₀₀=0.8). After centrifugation, the bacterial pellet was resuspended in 500ml of infiltration medium. Meanwhile, ~50 Arabidopsis thaliana (Var. Columbia) plants which had been greenhouse grown in compost until the appearance of the first silique, were removed from the soil and rinsed carefully in tap water to wash off the soil. The plants were placed in a plastic beaker and the bacterial suspension added. To ensure the plants remain under the surface of the suspension, a fresh smaller plastic beaker with several holes punched in the bottom was placed on top of the plants and secured in position. The apparatus was then transferred to a vacuum dissector and left under vacuum for 20 minutes with occasional swirling. The plants were replanted in fresh compost and the seed tray placed in a sealed plastic bag to allow recovery for 2 days. The bag was removed and the plants left to complete their life cycle in a greenhouse. The resultant seed set was harvested and the transgenic progeny selected as described in section 2.12.5.

2.12.4 Arabidopsis thaliana Transformation using Root Culture

This was performed as described by Clarke *et al.* (1992). A. thaliana ecotype C24 and Columbia seed was vernalised at 4°C for one week, prior to surface sterilisation in 5% bleach for 20 minutes. Following extensive washing with sterile water to remove bleach, the seeds were plated onto germination media (≈40 seeds/9cm plate). The seeds were left to germinate and provide root material, for 3-4 weeks in a standard light regime. After this time period whole seedlings were removed from the agar and the root system excised. The intact roots were placed on callus inducing media and incubation continued for 3 days. Meanwhile the required Agrobacterium strain was grown up in liquid culture (as described in section 2.2.2) for 48 hours prior to dilution with Agrobacterium culture dilution media to a final OD₆₀₀=0.1. The intact roots were cut into 0.5cm lengths and suspended in 20ml of the diluted Agrobacterium. After 2 minutes the roots were filtered on a 100µm nylon mesh and finally blotted on filter paper to remove all excess bacterial suspension. The roots were placed back onto the callus inducing media plates for cocultivation with the *Agrobacterium* for 2-3 days. Bacterial growth was washed off the root explants using *Agrobacterium* culture dilution medium and once again the roots were blotted dry on sterile filter paper. The roots were suspended in molten shoot overlay medium (at 30 °C) and poured onto plates already containing 30ml of shoot inducing medium. Following several weeks of incubation, green callus with regenerating shoots developed from the root explants. Those shoots with expanded leaves were transferred to shoot elongation media in polypot containers. After incubation for 4 weeks, large stems were excised and placed in boiling tubes containing ~5ml of shoot elongation media to allow greater seed sets to occur.

2.12.5 Growing Plants from Seed

Any transgenic seed would be kanamycin resistant due to the binary vectors utilised. Therefore tobacco seeds were sown on 1/2MSO plates containing; 1% sucrose, $400\mu g/ml$ augmentin and $100\mu g/ml$ kanamycin, while *Arabidopsis* seeds were sown in identical conditions except $35\mu g/ml$ kanamycin was used. To sterilise the seed prior to sowing, it was wrapped in 'Miracloth' and placed in 10% bleach for 10 minutes followed by several rinses in sterile tap water. Any kanamycin resistant seedlings were transferred to soil for further analysis, once selection occurred.

2.13 Polymerase Chain Reaction (PCR) Based Analysis of Plant Material

2.13.1 Small Scale Plant DNA Extraction

Based on the method of Edwards *et al.* (1991). A microcentrifuge tube cap full of tobacco leaf tissue (or equivalent quantity of *Arabidopsis* leaves) was homogenised in 400 μ l of PCR Extraction Buffer. Following centrifugation at 13,000rpm for 5 minutes, 300 μ l of supernatant was removed to a fresh tube and an equal volume of propan-2-ol added to precipitate the total nucleic acids. Following a 5 minute 13,000rpm centrifugation to pellet the nucleic acids, the supernatant was removed and the pellet washed in 70% ethanol prior to drying. The dry pellet was resuspended in 20μ l of dH₂O.

2.13.2 Plant Genomic PCR

A large reaction mix was assembled prior to aliquoting into 18μ l volumes such that each aliquot contained 1.8μ l of 11X PCR Buffer, 100ng of each primer and 1 unit of Taq Polymerase. A 2μ l portion of the extraction outlined in section 2.13.1 was added as template DNA to bring the total volume to 20μ l. The DNA was amplified using the cycles described in section 2.3.9.1 but 30 cycles were normally performed.

2.13.3 Small Scale Plant RNA Extraction

Based on the method of Guerineau *et al.* (1991). Two whole 3 week old *Arabidopsis* seedlings, or an equivalent volume of material, were homogenised in 400 μ l of RNA extraction buffer, followed immediately by the addition of an equal volume of phenol/chloroform. A 300 μ l aliquot of the upper aqueous layer was removed after centrifugation and a further phenol/chloroform extraction performed. The final 200 μ l of removed upper aqueous layer was subjected to a standard ethanol precipitation to pellet the total nucleic acid fraction. This pellet was dried and then resuspended in 50 μ l of dH₂O prior to the addition of 50 μ l of 4M LiCl to differentially precipitate the RNA over a half an hour incubation period on ice. The resultant centrifugally produced pellet was washed with 70% ethanol and all the liquid removed prior to air drying. The RNA pellet was finally resuspended in 20 μ l of dH₂O and 4 μ l electrophoresed on an agarose gel to visually quantify the amount of RNA extracted.

2.13.4 Reverse Transcriptase mediated PCR on Plant Derived Templates

1µg of RNA extracted in the manner described in section 2.13.3 was added to a Reverse Transcription Mix and incubated at 42 °C for one hour, followed by boiling for 5 minutes to inactivate the reverse transcriptase before placing the reactions on ice. A 2µl portion of this reaction was used as a template in a PCR reaction performed exactly as outlined in section 2.3.9.1.

2.14 Analysis of Gene Expression in Transgenic Plant Tissues All GUS protocols are as described in Draper *et al.* (1988).

2.14.1 Histochemical Localisation of β-Glucuronidase (GUS) Enzyme

Fresh tissues were suspended in GUS histochemical buffer and vacuum infiltrated for 15 minutes prior to incubation at 37° C for 1-24 hours, depending on the intensity of the staining. The blue precipitate formed due to the presence of active GUS enzyme was more clearly visualised following the clearing of samples in 70% ethanol.

2.14.2 Quantification of GUS Activity Using a Fluorometric Assay

The tissue of interest was ground up in an appropriate amount of GUS extraction buffer using a mortar and pestle or a micro-homogeniser and microcentrifuge tube. Following pelleting of debris by a 5 minute centrifugation at 13,000rpm, the supernatant was transferred to a fresh tube and stored on ice until assaying. Sufficient wells of a microtitre plate were filled with 200 μ l of 200mM Na₂CO₃ for the time points of the assays being performed. The reaction was started by adding 10 μ l of plant extract supernatant to 500 μ l of GUS fluorometric assay buffer (prewarmed to 37°C) and immediately pipetting 20 μ l of the mixed reaction into one of the filled wells in the microtitre plate. This acts as a zero time point. During incubation at 37°C this procedure was repeated at regular intervals to achieve a time course. The fluorescence from each individual well in the microtitre plate was then measured using a Perkin-Elmer fluorimeter. Dilutions of 1mM 4-methyl umbelliferone were used to allow a standard concentration against fluorescence curve to be calculated.

2.14.3 Luciferase Assays

These assays were performed essentially as described by Ow *et al.*, (1986). The tissue was ground up in 500μ l of luciferase extraction buffer using a pestle and mortar. Following pelleting of debris by a 5 minute centrifugation at 13,000rpm the supernatant was transferred to a fresh tube and stored on ice

until assayed. To assay samples, 25μ l of protein extract was added to a bioluminescence photometer reaction cuvette and placed in the luminometer. The machine injects measured aliquots of ATP buffer and 0.025mM luciferin (made up in 100mM sodium citrate) and measures the light emitted.

2.14.4 Protein Quantification using Bradfords' Assay

The assay (Bradford 1976) was performed in a microtitre plate with 20µl of protein sample, 100µl of water and 100µl of Bradford solution. Bovine serum albumin standards were prepared to allow the direct calculation of protein concentration using a custom written programme on a Dynatech MR5000 microtitre plate reader.

2.15 SHH Enzyme Assay

The following method to assay SHH enzyme activity was kindly donated by Dr. Robert Edwards of Durham University where most of this work was performed. The assay relies on the ability of the enzyme SHH to catalyse the synthesis of *S*-adenosyl-L-homocysteine from homocysteine and adenosine, which is the reverse reaction to the normal *in vivo* activity. This method can be divided into two separate protocols; the first for the crude isolation of SHH enzyme from plant tissues, and the second for the assaying of SHH enzyme activity within these crude extracts.

2.15.1 Crude Enzyme Extraction

Plant tissue (\approx 1g) was ground in a pestle and mortar with 2v/w of SHH enzyme extraction buffer, a pinch of acid washed sand and a spatula of PVPP. The liquid portion was decanted into a microcentrifuge tube and following centrifugation at 17,000g for 15 minutes at 4°C, the supernatant was removed to a small beaker noting the volume. To this, 0.56g of solid ammonium sulphate was added per ml and stirred at 4°C for 30 minutes. The precipitated protein was pelleted by centrifugation and resuspended in 2.5ml of SHH enzyme assay buffer. After clarification by centrifugation the protein extract was desalted using a Pharmacia PD-10 Sephadex G-25 column which had been preequilibrated with SHH enzyme assay buffer according to the manufacturers instructions. The resultant solution was used in the assay step following the estimation of protein content using Bradford's method as described in section 2.14.4.

2.15.2 Enzyme Assay

To perform the assay the following reagents were sequentially added to a microcentrifuge tube:-

10µl DL-Homocysteine (omitted in controls)

Appropriate amount of crude enzyme extract

10µl of ¹⁴C-adenosine/adenosine (100µl of 20mM adenosine added to 100µl of ¹⁴C-adenosine.)

Make up to 100µl with SHH enzyme assay buffer.

After mixing, the contents of the tube were incubated for 30 minutes at 30° C. The reaction was terminated by the addition of 10μ l of 50% trichloroacetic acid and left to stand on ice for 10 minutes. Following centrifugation, to pellet precipitated protein, 20μ l of reaction was spotted in a 1.5cm strip on a silica TLC plate containing fluorescent indicator (F254). To ease visualisation of the SAH product, the origin was preloaded with 5μ l of a 0.5mg/ml solution of SAH. The plate was developed for 10cm in butan-1-ol:acetic acid:water (12:3:5) and after the plate dried the SAH was visualised by viewing the plate with a UV lamp at 254nm. The SAH product was cut from the plate and placed in 0.5ml of methanol in a scintillation vial to elute SAH from the silica. After 10 minutes, scintillant was added and the sample counted.

2.16 Analysis of Gene Expression by Antibody Detection

2.16.1 Peptide Design and Synthesis

The design of the three peptides will be discussed in chapter 5. The three peptides synthesised at the University of Leicester were:

SHH-PEP1	CTSSGREYKVKD
SHH-PEP2	TGQVPDPTSTDN
SHH-PEP3	QEYWWCTERALD
An aliquot of each	peptide was sent to ZENECA Pharmaceuticals where

the following procedures were all performed, so that the test bleed sera and final bleed sera were eventually received at Leicester for analysis.

Each peptide was conjugated to an immunogenic carrier molecule Bovine Serum Albumin [B.S.A.] prior to immunisation. Peptides 2 and 3 were coupled to the B.S.A using glutaraldehyde which couples the peptide at the free amine group. Peptide 1 was coupled to the B.S.A using MBS (mmaleimidobenzoic acid N-hydroxysuccinimide ester) which couples to the free sulfhydryl group on the N-terminal cysteine residue. It should be noted that on immunisation, an immune response is usually generated against the carrier (B.S.A), sometimes the crosslinker (glutaraldehyde or MBS) and hopefully the peptide.

2.16.2 Antibody Production

For each of the three conjugated peptides, three individual New Zealand White rabbits were immunised. The following list outlines the immunisation time course, which is the normal procedure performed at ZENECA.

1) 500µl of prebleed taken from each rabbit.

2) 200 μ g of conjugated peptide was subcutaneously injected into each rabbit in a total volume of 1ml of Freund's complete adjuvant.

3) Four weeks later 100 μ g of conjugated peptide was subcutaneously injected into each rabbit in a total volume of 1ml of Freund's incomplete adjuvant.

4) Four weeks later 100µg of conjugated peptide was subcutaneously injected into each rabbit in a total volume of 1ml of Freund's incomplete adjuvant.5) Two weeks later a 5ml test bleed was performed.

6) Two weeks after the test bleed, and therefore four weeks after the last immunisation, 100µg of conjugated peptide was subcutaneously injected into each rabbit in a total volume of 1ml of Freund's incomplete adjuvant.7) Two weeks later a 5ml test bleed was performed.

8) Five weeks after the test bleed, and therefore seven weeks after the last immunisation, $100\mu g$ of conjugated peptide was subcutaneously injected into each rabbit in a total volume of 1ml of PBS.

9) Eleven days later the final bleeds were taken.

Prior to any test bleeds or the final bleed being sent to Leicester for

analysis the serum was separated from the whole blood. Therefore each bleed was allowed to clot at room temperature and then left at 4° C overnight. The serum was decanted off from the clot and spun at 1500g at 4° C for 20 minutes. The cleared serum was then frozen and packaged for storage.

2.16.3 Screening for Antipeptide Antibodies

Each test bleed and final bleed serum received from ZENECA throughout the immunisation procedure, was screened for antipeptide antibodies using peptide dot blots. The peptides used on these blots were non-conjugated and therefore any immunodetection was caused by antipeptide antibodies rather than anticarrier or anticrosslinker antibodies. Specific quantities of peptide were aliquoted onto Hybond Super-C membrane (Amersham) which unlike Immobilon does not require pre-wetting and therefore allows the loading of the peptide solution in tight dots. These strips of Hybond were then used in the manner described in section 2.16.7.

2.16.4 Extraction of Plant Proteins

To extract a crude protein sample, ten individual 3 week old *Arabidopsis* seedlings were homogenised using a mortar and pestle in 1ml of Anti-Proteinase Extraction Buffer (alternatively equivalent weight/volume ratios were utilised for differing numbers of seedlings or other tissue samples). All samples were stored on ice throughout the extraction. Following homogenisation, the extract was transferred to a microcentrifuge tube and spun at 13,000rpm for 5 minutes at 4°C. The resultant supernatant was removed to a fresh tube and a Bradford assay performed to quantify the protein concentration (as detailed in section 2.14.4). If the sample needed diluting to achieve a workable concentration, dilution was achieved with further quantities of Anti-Proteinase Extraction Buffer. To prepare the protein for separation by SDS-PAGE an equal volume of 2X SDS Gel Loading Buffer was added to an aliquot of the quantified extraction and boiled for 5 minutes to ensure SDS binding to the whole protein.

2.16.5 SDS-PAGE Gel Electrophoresis and Staining of Proteins on these Gels

Laemmli mini Bio-Rad II protein gel kits were used and assembled according to the manufacturer's instructions. Prior to pouring the gel, a comb was inserted between the empty plates to mark the position of the resolving gel and stacking gel interface at 1cm below the teeth of the comb. The percentage of gel used was dependent on the size of proteins to be separated. After pouring the desired percentage resolving gel, a layer of butanol:dH2O (1:1) was overlaid on the acrylamide mix to exclude oxygen from the polymerisation reaction. Following a 30 minute polymerisation this overlay was poured off and the stacker gel solution added, into which the combs were placed. Once polymerised the combs were removed and the wells rinsed with dH2O. The gels were placed in the electrophoresis apparatus and glycine running buffer added to the inner and outer chambers insuring no leakage from the inner to the outer chamber was occurring. The appropriate volume of boiled protein sample was loaded per well and the gels run at 125V until the sample dye front reached the stacker gel/resolving gel interface, at which point it was increased to 150V until the dye front reached the base of the gel. In all cases the gels were run in duplicate to allow blotting of one and Coomassie staining of the other. The gel to be stained was immersed in Coomassie stain and incubated on a rocking platform for one hour. The stain was then removed and the gel immersed in destain solution (10% acetic acid, 10% methanol), which was renewed frequently throughout the overnight destaining process. The destained gel was stored in 10% glycerol in an airtight plastic bag.

2.16.6 Western Blotting of SDS-PAGE Gels

To transfer the separated protein samples from the SDS-PAGE gel to Immobilon P (Millipore) membrane a Milliblot-SDE system was used. One piece of Immobilon P and six pieces of Whatman 3MM paper were cut to the size of the gel. Two pieces of Whatman were pre-wet in anode buffer 1 and placed on the blotter. One piece of Whatman pre-wet in anode buffer 2 was placed on top. The Immobilon P membrane was activated by pre-wetting in methanol, rinsed in dH₂O and then placed on the three pieces of Whatman. The SDS-PAGE gel was then added and the final three pieces of Whatman prewet in cathode buffer were placed on top of the gel. The blotter apparatus was run for one hour at 50mA for one gel or multiples of this for more than one gel.

To confirm protein transfer, the Immobilon P was submerged in Ponceau solution for 5 minutes and cleared by rinsing with dH₂O. The position of the markers were noted before all traces of stain were removed by washing in TBS-Tween.

2.16.7 Immunodetection of Proteins or Peptides

To block portions of the membrane with no proteins bound, it was incubated in TBS-Tween containing 5% Marvel for 1-2 hours at room temperature or overnight at 4°C. The membrane was then sealed in a plastic bag with 5ml of TBS-Tween containing 1% Marvel and the relevant dilution of primary antibody. After a one hour incubation at room temperature, the membrane was washed three times for 5 minutes in 50ml of TBS-Tween. The membrane was then sealed in a plastic bag with 5ml of TBS-Tween containing 1% Marvel and a 1:1000 dilution of secondary antibody (alkaline phosphatase conjugated goat anti-rabbit immunoglobulins; DAKO). This was incubated and washed in the same manner as the primary antibody. The membrane was rinsed in BCIP buffer to equilibrate it and then transferred to 5ml of BCIP buffer containing 50 μ l of 50mg/ml BCIP and 50 μ l of 30mg/ml NBT. The colour development was allowed to proceed until the position of the detected protein/peptide was clearly visible before stopping the reaction by washing the membrane with excess dH₂O.

The above procedure was utilised for both western blots of SDS-PAGE gels and peptide dot blot strips with the exception of the use of a multi slot tray for antibody incubation with peptide dot blots, rather than plastic bags.

Chapter 3

The isolation and sequence analysis of *Asparagus officinalis* SHH cDNAs

3.1 Introduction

The response to wounding in higher plants may not only involve local responses but also may induce systemic responses in undamaged distant tissues (reviewed by Bowles, 1990 and 1994). Within the local response there appears to be two distinct stages; the first, occurring within minutes generally does not involve transcription/translation; the second later phase, is the de novo synthesis of mRNA, proteins and organic molecules involved in a variety of defence mechanisms. Some of the events reported to occur during the first phase include an oxidative burst and cell wall strengthening (Bradley et al., 1992; Olsen and Varner, 1993; and Thompson et al., 1987), an increase in respiration (Bostock and Stermer, 1989 and Lu et al., 1993), the production of plant cell wall-derived elicitors (Darvill and Albersheim, 1984), an increase in jasmonic acid (Doares et al., 1995), the release of hydrolytic enzymes from the vacuole, electrolyte leakage, changes in ion flux, membrane depolarisation and production of callose (Bostock and Stermer, 1989), ethylene production (Yang and Hoffman, 1984) and changes in protein phosphorylation (Farmer et al., 1989). The more extensively studied events of the second phase require de Figure 3.1 Asparagus officinalis seedlings and cells

Figure 3.1A A three week old asparagus etiolated seedling which was utilised for wounding studies by cutting into 5mm sections.

Figure 3.1B A four week old asparagus frond from which the cladodes were stripped for mechanical separation of asparagus cells.

Figure 3.1C An initial crude preparation of mechanically separated asparagus cells after passing through a 64µm sieve. The arrows point to dead cells with disrupted chloroplasts, and the scale bar represents 50µm.



novo gene expression and include; the induction of phenylpropanoid biosynthesis (Jahnen and Hahlbrock, 1988), further modification of the cell wall proteins (Showalter, 1993), lignification (Smith *et al.*, 1994), production of hydrolytic enzymes such as chitinases and glucanases (Simmons *et al.*, 1992) and synthesis of polygalacturonase and proteinase inhibitor proteins (Bergmann *et al.*, 1994 and Peña-Cortés *et al.*, 1988). Wounding has also been reported to induce several novel genes whose function has not yet been assigned, such as AoPR1, the intracellular PR-protein from asparagus (Warner *et al.*, 1992).

The wound site is sealed in different manners depending on the plant species. Most monocotyledonous species have no meristematic activity associated with the wound site, therefore cell autolysis and cell death occurs at the wound site, with the cells immediately adjacent to the wound surface becoming infused with an extensive layer of lignin and other phenolic based polymers. In dicotyledonous plants and some monocots, including asparagus, parenchymal cells at the wound site redifferentiate to allow cell division to occur to form a suberized wound periderm (Bostock and Stermer, 1989).

A number of the particular processes involved in the wound response have already been discussed in chapter 1, with particular reference to the steps requiring transmethylation reactions in which SAH will be produced as a byproduct.

At Leicester, a model system to study the local wound response was developed using mechanically isolated asparagus cells. These cells will dedifferentiate, initiate DNA synthesis, expand and divide within 4-5 days with a high degree of reproducibility (Paul *et al.*, 1989). Over 60% of the single cells survive mechanical grinding and around 90% of the viable cell population enter into division in liquid medium (Paul *et al.*, 1989; see figure 3.1).

Studies of these cells have shown that, as well as cell expansion occurring 3 days post isolation, there is a large rise in respiration rate and a massive increase in RNA synthesis (Harikrishna *et al.*, 1991). Extensive qualitative changes in the protein content of the cells occurs during the first few days in culture (Harikrishna *et al.*, 1991 and 1992). Further work has provided evidence that mechanically isolated asparagus cells exhibit a typical,

but greatly amplified wound response during the first 3-4 days after cell separation in comparison to chopped asparagus seedling tissue (Darby et al., 1996). This conclusion was reached by assaying a range of molecular, biochemical and physiological parameters indicative of a normal wound response, in finely chopped seedling tissue and in the model system cells; for example the kinetics of phenylalanine ammonia lyase induction was identical in the two tissues but the amplitude within chopped seedlings was lower than in the mechanically isolated cells. This study also analysed changes within the high abundance class of mRNAs in the asparagus model system by performing in vitro translation of mRNA and separation of the synthesised proteins by 2-D gel electrophoresis. This revealed a doubling of the numbers of distinct proteins within the five day period post-isolation when the wound response and cellular dedifferentiation occur. These inducible proteins were grouped into three classes: class A were induced rapidly, but transiently, by cell separation, peaking by one day and then falling back to basal levels after 24-36 hours; class B were induced more slowly, peaking after 3 days and then slowly returning to the basal levels; class C showed a similar gradual rise in abundance but this was maintained as the cell population entered mitosis. On the basis of this 2D-gel analysis, cDNA libraries were constructed and differentially hybridised to cDNA populations synthesised from unwounded cladode tissue or mechanically separated cells. These experiments suggested that over 30% of the clones found in the libraries represented transcripts induced specifically by cell separation. Almost half of the clones in the libraries failed to hybridise to either probe. These 'cold plaques' (Hodge et al., 1992) were utilised in northern dot blots to determine that 20% of the non-hybridising clones represented medium-low abundance transcripts induced by cell separation or wounding. Northern blots using 150 differentially expressed cDNAs as probes, showed that most transcripts rapidly induced in mechanically separated cells, had a similar temporal expression profile in wounded seedlings. However, for nearly all genes, the transcript abundance was much lower in mRNA populations from wounded seedlings when compared to that observed in the model system cells. The temporal expression of transcripts from several genes fell into the broad categories outlined by the Figure 3.2 Northern analysis of AoSHH transcripts

Figure 3.2A Northern analysis of AoSHH transcripts following cell isolation. Total RNAs hybridised to the DB6 insert. RNAs were isolated from the following:

Lanes 1-5	asparagus cell population 0, 1, 3, 5 and 7 days
	post isolation respectively.
Lane 6	asparagus cell population 2 months post
	isolation.

Figure 3.28 Northern analysis of AoSHH transcripts following wounding. Total RNAs hybridised to the DB6 insert. RNAs were isolated from the following:

edlings
sterile

10µg of total RNA was loaded per lane.



2D analysis (Darby et al., 1996).

Once it was shown that the cDNA libraries potentially yielded a large number of wound induced clones [including AoPR1 (Warner *et al.*, 1992)], Simon Warner, at Leicester, randomly selected clones which appeared rapidly induced and each were partially sequenced. The predicted amino acid sequences were subjected to searches using Pearson and Lipman algorithms for homologous sequences within the EMBL database to try and elucidate putative clone identities. One such clone, named DB6, hybridised to a 1.8kb transcript, which was upregulated by the mechanical isolation of asparagus cells. The partial sequence of DB6 had a significant level of identity to a cloned rat cDNA present in the EMBL database which encoded the enzyme *S*-adenosyl-Lhomocysteine hydrolase. A few weeks into this work a second plant SHH cDNA from parsley was reported (Kawalleck *et al.*, 1992; as described in chapter 1). This partially sequenced asparagus cDNA was the starting point for the work described in this thesis.

The objectives for this section of work were, to fully sequence the asparagus cDNA to confirm or disprove its putative identity, and to study the transcript profiles of this clone in mechanically separated cells and other wounded asparagus tissue.

3.2 Northern analysis of DB6 transcript within the model system

Further northern analysis was performed using DB6 as a probe, to determine in detail the expression profile of the DB6 transcript in response to the mechanical isolation of asparagus cells and the wounding of etiolated asparagus seedlings. The northern utilised RNA extracted from asparagus cell populations at several time points post isolation (figure 3.2A), and confirmed that DB6 hybridises to a transcript approximately 1.8kb in length. The abundance of the hybridising mRNA increased in the asparagus cell population compared to the basal level found in the freshly separated cells. This transcriptional activation occurred within one day of cell separation and was maintained for up to seven days. The elevated level of the hybridising transcript was not maintained indefinitely, being much reduced in long term cultured cell total RNA (2 months post isolation). To test whether the clone Figure 3.3 Restriction site map of DB6 and comparison to DBF (AoSHH)

Figure 3.3A Schematic drawing of DB6 and its subclones.

The restriction enzyme sites used to derive the four subclones of DB6 (DB6.1-DB6.4) are shown. The relative position and orientation of the three primers (MSK1-3), constructed to complete the sequencing of DB6, are also shown.

Figure 3.38 Comparative restriction enzyme analysis of the two asparagus SHH cDNAs isolated from lambda ZAPII cDNA libraries, and rescued as pBluescript®II SK- clones, namely DB6 and DBF (AoSHH).

All odd numbered lanes contain DB6 restricted DNA and all even numbered lanes contain DBF restricted DNA. Due to the use of *Eco RI/Not* I adaptors during library construction, *Not* I was used in each digest to remove the whole cDNA. Unlike *Eco RI*, *Not* I has no internal recognition sites. Digests were as follows:

Lanes 1 and 2	Bam HI and Not I
Lanes 3 and 4	Eco RI and Not I
Lanes 5 and 6	Eco RV and Not I
Lanes 7 and 8	Hind III and Not I
Lanes 9 and 10	Not I
Lanes 11 and 12	Sac I and Not I
Lanes 13 and 14	Xho I and Not I
Lanes 15 and 16	Bam HI, Eco RI and Not I
Lanes 17 and 18	Hind III, Xho I and Not I
Lane M	1KB ladder





B

M 1 2 3 4 5 6 7 8 9 101112131415161718M



represented a wound-inducible transcript, total RNA was also isolated from etiolated asparagus seedlings which had been chopped into 5mm sections and incubated on sterile Whatman 3MM paper dampened with dH2O, for the specified time points post wounding. This RNA was utilised in the same northern blot and can be seen in figure 3.2B. These results prove wound induction of DB6 transcript to occur within one day post wounding, but transcript levels return to the basal level found in etiolated seedlings within three days. This wound induction therefore has a different induction profile to that observed with the hybridising transcript detected in the isolated cell RNA. As both the northerns presented in figure 3.2 were part of the same blot, which underwent identical treatments, including the same exposure period, the levels of hybridising transcript are directly comparable. Comparing both day zero time points, greater transcript levels are detected in etiolated seedlings in comparison to green cladode tissue. However as previously noted (Darby et al., 1996) the mechanically separated cell population exhibits an amplified wound response in comparison to chopped seedling tissue.

3.3 Sequence analysis of DB6

A crude restriction enzyme map of DB6 had previously been constructed by Karl Deacon at Leicester. To facilitate sequencing of DB6, subclones were generated using internal restriction sites, as shown in figure 3.3A. To allow the final unknown sequence to be elucidated, three primers were designed within the previously sequenced regions. The position and orientation of annealing of these primers can be seen in figure 3.3A. These primers were;

- MSK15' GGTAACAAAGATATCCGCC 3'MSK25' GCCTGGAAGGGTGAGACCC 3'
- MSK3 5' GGAAGAGAGTGCCGGCATCC 3'

The 1633bp contiguous sequence derived from the four subclones and the internal primers, illustrated that the whole DB6 clone was potentially derived from an SHH transcript but that it was not a full length cDNA. Not only was the poly A tract absent from the 3' terminus of the clone, but a putative translational start site was not present at the 5' end. On comparison of the predicted amino acid sequence of DB6 to that of the published parsley SHH

Figure 3.4 Sequence analysis of AoSHH

Nucleotide sequence and deduced amino acid sequence of the *Asparagus officinalis S*-Adenosyl-L-Homocysteine Hydrolase cDNA, AoSHH. The first nucleotide of the start codon is base 0 and the initial methionine is assigned number 1. The stop codon is represented by an asterisk. The position of the first nucleotide of the initial clone, DB6, is 34 and the position of the final nucleotide, 1666.

-25	CTCGTTTCAGATCCGATCTGAAGAAATGGCTCTCCTCGTTGAGAAGACTACCTCTGGCCG	34
	MALLVBKTTSGR	12
35 13	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	94 32
95 33	ACTOCAGCTCTACOGTCCCCGAGTACCOGACOGCACOGCCCCGCCCC	154 52
155 53	CAAGGGGGGAAAAATGACTGGATCCCTCCACATGACGATGCAAACTGCCGTCCTCATGGA K G A K I T G S L H M T I Q T A V L I B	214 72
215 73	AACCCTAACCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	274 92
275 93	GGACCATGCCGCTGCCATTGCCGTGGACGCCGCGCGGAGGGGGA D H A A A A I A R D S A S V F A W K G E	334 112
335 113	GACCETOCAGGAGTACTGGTGGGCGGGGGGGGGGGGGGGGGGGGG	394 132
395 133	CCCTGACCTCATCGTCGATGACGGCGGCGACACCACTCTCTTGATCCATGAGGGGGGGG	454 152
455 153	GCCCAGAGAAGAAGAAGAAGACGGGGAAGATGCCGGACCGGCGTCTACCGACAATGC A E E E Y E K T G K M P D P A S T D N A	514
516		574
173	B P Q I V L T I I R D G L K V D P T K Y	574 192
575 193	CAGGAAGATGAAGATAGGATTGTOGGTGTGTGGGAGGAGAGACGACGACGAGGGGGGGGGG	634 212
635 213	GCTTTACCAGATGCAGGCTAACAATTCOCTTCTTTTTOCCTGGGATCAATGCAGTGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTCAATGCAGTTGAGTCAGTC	694 232
695 233	CGTCACCAAGAGCAAGTTTGACAATCTGTATGGATGGCAGCACTCTCTTCCCGATGGTCT V T K S K P D N L Y G C R H S L P D G L	754 252
755 253	GATGAOGGCCACTGATGTTATGATTGCTGGCAAGGTTGCAGTTGTCTGGGGTATGGTGA M R A T D V M I A G K V A V V C G Y G D	814 272
815 273	TGTCOGAGAOGOCTGTGCTOCTOCTOCACTCAAGCAOOCTGGTOCCOGTGTTATTGTGACOGA V G E G C A A A L K Q A G A R V I V T E	874 292
875 293	GATCGACCCCATCTGTGCTCTTCAAGCCCTAATGGAGGGTCTTCAGGTCCTCACCCTCGA I D P I C A L Q A L M E G L Q V L T L E	934 312
935 313	GGATGTTGTCTCAGAGGCGGATATCTTTGTTACCACCACGGGTAACAAGGACATCATCAT D V V S E A D I F V T T T G N K D I I M	994 332
995 333	GCTGGACCACATGAGGAAGATGAAGAACAATGCCATTGTCTGCAACATTGGTCACTTTGA L D H M R K M K N N A I V C N I G H F D	1054 352
1055 353	CAACGAGATTGACATGCTAGGTTTGGAGACATACCCTGGCATCAAGAGAATCACCATCAA N E I D M L G L E T Y P G I K R I T I K	1114 372
1115 373	GCCCAGACTGACCGTGGGTCTTCCCTGAAACCAACACTGGTATAATTGTTCTTGCTGA PQTDRWVFPETNTGIIVLAE	1174 392
1175 393	GOOCOGACTCATGAACCTTGGGTGTGCCACTGGTCACCCCAGCTTTGTCATGTCCTGCTC G R L M N L G C A T G H P S F V M S C S	1234 412
1235 413	CTTCACCAACCAGGTGATTGCTCAGCTAGAGTTGTGGAATGAGAAGGCAAGCGGCAAGTA F T N Q V I A Q L E L W N E K A S G K Y	1294 432
1295 433	TGAGAAGAAGJTTTACGTGCTCCCCAAGCATCTTGATGAGAAAGTAGCAGGGCTTCACTT EKKVYVLPKHLDEKVAALHL	1354 452
1355 453	GGGCAAGCTCGGAGCCAAGCTTACAAAGCTCAGCCCTTCACAGGCGGACTACATCAGCGT G K L G A K L T K L S P S Q A D Y I S V	1414 472
1415 473	CCCCATCGAGGGTCCCTACAAGCCACCTCACTACAGGTACTAGACGCTGTTGTGCGGGG PIEGPYKPPHYRY*	1474 485
1475	AGAGATCATCGCAGCAAGAAAGTATTAAGATTGAAGAAGAGAGTTGTTATGGAGGA	1534
1535	GCTATATTTACTTTATTTOCTACCTATTTCTTGCTGTTTCTCTTTCCGAACTTTTAGACT	1594
1595	GATCCTCTTCTCTTTGATTTATTACGATATGAATTCTGTTTAAATTTTGCTTATTCT	1654
1655	CTAATGACGAGGCAGGCATATGTTCTGTGGTAGAATAACGAGGTTTTGAACTTTGTG	171
1715	Салалалалалалалалалалалал 1741	

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cDNA (Kawalleck *et al.*, 1992) it was discovered that DB6 was 11 amino acids shorter at the amino terminus than the parsley SHH clone. Therefore it was concluded that DB6 was a truncated cDNA, and so cDNA libraries were screened to identify a full length asparagus SHH cDNA (see figure 3.4).

3.4 Screening of cDNA libraries for full length cDNAs

DB6 had been previously isolated from a lambda ZAPII cDNA library constructed using mRNA extracted from mechanically isolated cells 1-3 days post separation. A library constructed in exactly this manner but containing cDNAs synthesised from RNA extracted from one day post mechanical isolation cells, also existed. Both libraries were screened to try and isolate a full length DB6 cDNA. Approximately 25,000 plaques were plated from each library for the primary screen. The resultant plaque lifts were probed with radiolabelled DB6.4 plasmid insert, the 5' end of the DB6 cDNA (see figure 3.3A).

Following three rounds of screening one positively hybridising clone was obtained which originated from the library constructed from cells one day post isolation, and therefore was from a different library to DB6. To assess if this new clone (designated DBF) was a full length version of DB6, initial studies utilised the presence of several restriction endonuclease sites, found by the sequencing of DB6, to ascertain if the same sites were present in the same locations within DBF. The resultant agarose gel of this analysis can be seen in figure 3.3B. This mapping project proved the presence of the known restriction enzyme sites within DB6 to also be present within DBF. It also showed DBF to - be a larger cDNA than DB6, with the extra sequence being present at both the 5' and 3' termini of the cDNA. To demonstrate that DBF probably originated from the same transcript as DB6, it was sequenced.

3.5 Sequence analysis of AoSHH (DBF) and its identity to DB6

Portions of DBF were sequenced using the external primer sites in the vector and using the primers MSK1-3 synthesised to sequence DB6. Due to the 100% identity found using computational analysis (Devereux *et al.*, 1984) between overlapping DBF and DB6 sequenced regions, DBF was not sequenced in its entirety as the initial sequence data suggested both clones corresponded to

Figure 3.5 Alignment of predicted amino acid sequences of AoSHH and SHH genes from diverse species

CLUSTAL V multiple sequence alignment of all cloned SHH proteins available upon the completion of the sequencing of AoSHH. SHH protein sequences were deduced from the following sources: a=A. officinalis, b=P. crispum (accession number=M81885), c=R. capsulatus (M80630), d=R. norvegicus (M15185) e=H. sapiens (M61831), f=D. discoideum (M19937), g=C. elegans (M64306), and h=L. donovani (M76556). The * represents non divergent amino acids and the . represents conservatively substituted amino acids.

MALSVERTAAGREYKKKDMSLADFG RLELELAEVEMFGLMSCRTEFGPSQ PFK-ARITGSLHWTIGTGVLLETUT ALGAEVRMCSCNIFSTQDHAAAIA RDSCAVFAMKGETLQEVMMCTERAL -----B-KLPYKVADIGLAAMG RKALDIAENEMPGLMRWRERYSASK PLKGARLAGCLHMTVETAVLLETUV TLGAEVQMSSCNIFSTQNHAAAAIA KAGIPVYAMKGETDEEYLMCIEQTL MALLVEKTISGREYKVKDMSQADFG RLEIELAEVEMPGLMACRAEFGPAQ PFKGAKITGSLHMTIGTAVLLETLT ALGPEVRMCSCNIFSTGDHAAAAIA RDSASVFAMKGETLQEYMMCTERAI ----AD----YIVKDIKIAEFG RKELDIAETEMPGLMACREEFGPSQ PLKGARIAGSLHMTIQTAVLIETLK ALGADVRWASCNIFSTQDHAAAAIA AGGTPVFAVKGETLEEY-WAYTDKI ----AD-KLPYKVADIGLAAMG RKALDIAENEMPGLARMREMYSASK PLKGARIAGCLHMTVETAVLIETUV ALGAEVAMSCNIFSTQDHAAAAIA KAGIPVFAMKGETDEEYLMCIEQTI M-----T--KLHYKKDISLAAMG RKEIEIAANEMPGIMTLRKKYGPAQ ILKGARIAGCHHMTIQTAVLIETUT ALGAQVQMSSCNIFSTQDQAAAIA ATGVPYYMKGFTEEEYMMYGPZUT ----AOSKPAYKVADIKLADFG RKEIILAENEMPGLMAMRSKYGPSQ PLKGARLAGCLHMTIQTAVLIETLT ALGAEVQMSSCNIFSTODHAAAAIA QTGVPVYAMKGETDEEYEMCIEOTI ---AD----YKVKDISLAEWG RKAIELAENEMPGIMELRREYGPSQ PLKGAKIAGCLHMTVQTAVLLETLK ALGADVRWSSCNIFSTQDNAAAAIA KAGVPVFAWKGETDEEYEWCIAQTV * ** **** * * * ************** * * * * * * ***** * * *** * * * * * * * * * * * * * * * * * * --W --W ż -W Ł

-DWGFGGGPDLIVDDGGDTTLLIHE GVKAEEEYEKTGKMPDPASTDNAEF QIVLTIIRDGLKVDPTKYRWKDRI VGVSEFTTTGVKRLYQMQANNSLLF PAINVNDSVTKSKFDNLYGCRHSLP GVKAEEEYKKSGAIPDPASTDNAEF QIVLSIIRDGLKSDPMKYHMKDRL VGVSEETTTGVKRLYQMQQNGTLLF PAINVNDSVIKSKFDNLYGCRHSLP FOFPEGTC-NMILDDGGDATLYILL GARVEAG--ETDLIATPTSEDEV-- --CLFNQIKKRWESFGWFTQQAAI KGVSEETTTGVHKLYDLHKKGLLFF PAINVNDSVTKSKFDNKYGCKESLV -----PPQYLAGI RELSEETTTGVHNLAKMLAKGDLKV PAINVNDSVTKSKFDNLYGIRESLP ----HPELVPKI FGISEETTTGVKNLYKRLSKGNLPM CAINVNDSVTKSKFDNLYGCRESLV ------HPQLLSGI RGISEFTTIGVHNLYKMAANGILKV PAINVNDSVTKSKFDNLYGCRESLJ -----YPQLLPGI RGISEETTTGVHNLYKMMANGILKV PAINVNDSVTKSKFDNLYGCRESL -----YPQFLAGI KGISEETTHGVHNLYKMFKEGKLKV PAINVNDSVTKSKFDNLYGCRESL ** ** *********** * ** ***** * ------TLVHEK------------NLVHAK---------XLHITN-----HUIVIN-----DWGPDGGPDLIVDDGGDATLLIHE H-FKDG-PLNMILDDGGDLT-V-FQDG-PLNMILDDGGDLT-Y-FKDG-PLANILDDGGDLT-V-FKDGQPLAMILDDGGDLT-KGFSGDGLPNMILDDGGDLT-***** * $(\mathbf{p}, \mathbf{q}, \mathbf{q}) = (\mathbf{q}, \mathbf{q})$

DAWERATDWILAGETCCVCCSFGIVG KGCAAALRAFGARUVUTEVDPINAL @ASMEGYQVALVEDVNADAHIFVIT TGNDDIITISEHFPHMEDDAIVCNIG HFDTEIQVGMLEA-NAKEHVEIKPQ DGIMRATDWIAGKVAVVCGYGDVG EGCAAALKQAGARVIVTEIDPICAL QAIMEGLQVLTLEDVVSEADIFVTT TGNKDIIMLDHMRAKANAIVCNIG HFDNEIDMLGLETYPGIKRITIKPQ TGNKDIIMVSDMRKMKNNAIVCNIG HFDNEIDMLGLETYPGVKRITIKPQ TGNKDVIRIEHMREMKDMAIVGNIG HFDNEIQVAALKN-H--KWTNIKDQ DGIKRATDVMLAGKUAVVAGYGDVG KGCAQALRGFGARVIITEIDPINAL QAAMEGYEVTIMDEACKEGNIFVTT TGCVDIILGRHFEQMKDDAIVCNIG HFDVEIDVKMLME-NAVEKVNIKPQ DGIKRATDAMIAGKUAUVAGYGDVG KGCAQALRGFGARVIITEIDPINAL QAAMEGYEVTTMDEACQEGNIFVTT TGCIDIILGRHFEQMKDDAIVCNIG HEDVEIDVKMLME-NAVEKPUKRQ DGIKRATDWIAGKVAVVAGYGDVG KGCAQSLSKMGARVLVTEIDPINAL QACMDGYQIVTMETAAPLSNIFVTT TGCRDIVRGEHFAVMKEDAIVCNIG HFDCEIDVAMLNA-NA-KKDTVKPQ DGIKRATDVMLAGKVAVVAGYGDVG KGSAASLKAFGSRVIVTEIDPINAL QAAMEGYEVTTLEEAAPKANIIVTT TGCKDIVTGKHFELLPNDAIVCAVG HFDCEIDVKMLNT-NATKKDTIKPQ DGIMRATDVMIAGKVALIAGYDGVG KGCAAAMKQAGARVIVTEIDPICAL QATMEGLQVLPLEDVVSEVDIFVTT DGIRRATDVMMAGKVAVVCGYGDVG KGSAASLRGAGARVKVTEVDPICAL QAAMDGFEVVVLEDVVADADIFITT (j) (g) (g) (g) (g) (g) (g)

TDRWFPDTGRGIIILABGRLMMLG CATGHPSFVMSCSFTNQVIAQLELM NE-KSSGKYEKK----VYVLPKHLD EKVAALHLGKLGAKLTKLSKDQADY ISVPVEGPYKPAHYRY VDRYLLKN-GHRIILLAEGRLVNLG CAMGHPSFVMSNSFTNQVMAQIELM TH---PDKYP----VGVHFLPKKLD EAVAEAHLGKLNVKLTKLTEKQAQY LGMPINGPFKPDHYRY VDRYRLKN-GRRIIILLAEGRLVNLG CAMGHPSFVMSNSFTNQVMAQIELM TH---PDKYP----VGVHFLPKKLD EAVAEAHLGKLAWKLITKLTEKQAQY LGMSCDGPFKPDHYRY VDRYTLAN-GVHIIILAEGRLVNLG CGTGHPSFVMSNSFCNGTLAQIALM TK---TEEYP----LGVHLLPKILD EEVARLHLDQLGAKLTTLTEKQSEY LSVPVAGPYKVDHYRY VDRYTLKN-GRHVIILAEGRLVNLG CATGHPSFVMSNSFTNØVLAQVELM TKFGTPQEYK----LGLYVLPKTLD EEVAYLHLAQLGVKLTKLSDEQASY LGVPVAGPYKPDHYRY TDRWVFPETNTGITVLAEGRLMNLG CATGHPSFVMSCSFTNQVIAQLELM NE-KASGKYEKK-----VYVLPKHLD EKVAALHLGKLGAKLTRLSPSQADY ISVPIEGPYKPPHYRY VDMIEMPS-GARIIILZEGRLINUG NATGHPSFVMSASFTNØVLAQIELM TK---GAEYQPG----VYILPKSLD EKVARLHLKKIGVKLTTLRPDQAEY IGVTVEGPFKSDHYRY . ** ** ** ** ******** *** *** *** $(\mathbf{f}) = (\mathbf{f}) = ($

transcripts from the same gene. This 100% nucleotide sequence identity was observed in the 3' untranslated regions as well as in the coding sequences.

DBF therefore became designated as *Asparagus officinalis S*-Adenosyl-L-Homocysteine Hydrolase (AoSHH). AoSHH was 1767bp in length, which is the approximate predicted size of the transcript detected on northern analysis. AoSHH encodes the entire asparagus SHH amino acid sequence of 485 residues, with 25bp of 5' untranslated leader. The 284bp of 3' untranslated sequence contains a putative polyadenylation signal (AATAA) 20 bases upstream of the 3' terminal poly A tract. The nucleotide sequence and predicted amino acid sequence of AoSHH can be seen in figure 3.4.

3.6 Amino acid sequence conservation between AoSHH and other cloned SHH cDNAs

Interestingly, predicted amino acid sequence comparisons to other SHH cDNAs, showed that AoSHH contained an extra stretch of amino acid residues (numbered 145-191 in figure 3.4) at the same position as observed in the predicted translation product of the *Petroselinum crispum* (Kawalleck *et al.*, 1992) and *Rhodobacter capsulatus* (Sganga *et al.*, 1992) cDNAs, which are not present in SHH cDNAs from non-photosynthetic species.

The multiple sequence alignment (Higgins and Sharp, 1989) shown in figure 3.5, highlights the high level of sequence conservation between the predicted amino acid sequence of AoSHH and other SHH clones available at the time. This amino acid sequence similarity can be seen to cross major evolutionary boundaries. For example the predicted amino acid sequence of the dicotyledonous plant *Petroselinum crispum* (Kawalleck *et al.*, 1992) is 90.1% identical to that from the monocotyledon *Asparagus officinalis*. This observed conservation even occurs across different kingdoms with the predicted amino acid sequence of SHH from the bacterium *Rhodobacter capsulatus* (Sganga *et al.*, 1992) being 58.7% identical to AoSHH.

The result of a similar CLUSTAL alignment, performed at a later date to include three further species [*Triticum aestivum* (Richards and Gardner, 1994), *Catharanthus roseus* (Schröder *et al.*, 1994) and *Plasmodium falciparum* (Creedon *et al.*, 1994)] found to contain the extra amino acid motif, are

Figure 3.6 Dendrogram of diverse SHH gene sequence alignment data

Dendrogram of a similar amino acid sequence multiple alignment to that shown in figure 3.5. As well as the eight species utilised in figure 3.5, three more recently cloned SHH cDNAs, found to contain the extra amino acid motif, are included [namely, *C. roseus* (Schröder *et al.*, 1994), *T. aestivum* (Richards and Gardner, 1994), and *P. falciparum* (Creedon *et al.*, 1994)].





% similarity of amino acids
displayed as a dendrogram in figure 3.6. This clearly shows a clustering of the four plant SHH sequences utilised, namely AoSHH, C. roseus (Creedon et al., 1994) P. crispum (Kawalleck et al., 1992) and T. aestivum (Richards and Gardner, 1994), with the highest percentage similarity of amino acids over the entire predicted amino acid sequence lengths. The two species with the greatest individual percentage similarity of 97%, are the two mammalian species, H. sapiens (Coulter-Karis and Hershfield, 1989) and R. norvegicus (Ogawa et al., 1987). Although the SHH cDNAs from both R. capsulatus (Sganga et al., 1992) and P. falciparum (Creedon et al., 1994) contain the extra amino acid motif, neither of these species is more closely related to the plant grouping than to the non-plant species. Particular note should be made of R. capsulatus whose sequence is more similar to the group containing H. sapiens (Coulter-Karis and Hershfield, 1989), R. norvegicus (Ogawa et al., 1987), C. elegans (Prasad et al., 1993), D. discoideum (Kasir et al., 1988) and L. donovani (Henderson et al., 1992) than that of the plant species, while the amino acid sequence of P. falciparum is the least conserved compared to any other species. Even the level of amino acid percentage similarity between P. falciparum and all the other diverse species is above 50% and represents a significant sequence conservation.

3.7 Discussion

Previous work by Simon Warner and Karl Deacon at Leicester had putatively identified an asparagus cDNA which originated from an SHH transcript, upregulated by the mechanical separation of mesophyll cells. This initial cDNA, DB6, was not full length, which led to the isolation of AoSHH (formerly called DBF). It was concluded that these two cDNAs arose from the same transcript due to their nucleotide sequence identity at least within the overlapping regions sequenced. The remarkable SHH amino acid and nucleotide sequence identity reported in this chapter and elsewhere (Kasir *et al.*, 1988, and Sganga *et al.*, 1992), suggests that if two genes encoding SHH exist within one species, then little or no nucleotide sequence diversity may exist. Therefore if DB6 and AoSHH originated from transcripts of separate asparagus SHH genes, their nucleotide sequence may be identical. However both cDNAs contained 3' untranslated portions of nucleotide sequence which were also found to be identical. Evidence presented in chapter 6 suggests the presence of a possible small SHH gene family within asparagus. This could allow certain gene family members to be expressed in a differential manner to other family members, as found with many other genes, for example, phenylalanine ammonia-lyase (Shufflebottom *et al.*, 1993). On screening libraries to isolate full length copies of DB6, a cDNA was isolated which originated from the same transcript and therefore the same gene. This finding may suggest that this is the asparagus SHH gene family member induced by the process of cell separation, a model for the study of wounding.

Northern analysis confirmed AoSHH transcript to be highly elevated by the mechanical separation of asparagus cells. This increase also occurred upon conventional wounding of asparagus seedlings. However, as noted previously, mechanically separated cell populations exhibit an amplified wound response, including increased gene expression, in comparison to chopped seedling tissue (Darby *et al.*, 1996). This amplified wound response may simply be due to the extreme nature of wounding found in the model system, or a portion of the increased levels of SHH transcript may be due to an extra requirement for SHH enzyme activity to allow the huge metabolic transition occurring within the cells. This transition is superimposed on the wound response as quiescent photosynthetic mesophyll cells dedifferentiate to form dividing heterotrophic cultured cells.

Studies by Paul *et al.* (1989) and Harikrishna *et al.* (1991) have shown that mechanically separated cells dedifferentiate to form dividing cultures in which the first cell divisions occur within four days post isolation, and therefore at this time point, DNA replication has already occurred. The increase in the DNA content of the cell cultures was measured and DNA synthesis estimated to start after two days of cell separation. This change from quiescent mesophyll cells to dividing culture cells requires the enzymes of DNA replication to be active within the cells. DNA methylation is an integral process of DNA synthesis if control of gene expression is to be maintained (reviewed by Finnegan *et al.*, 1993; discussed in chapter 1). Therefore within the rapidly dividing cell population, SHH enzyme activity will be required to

metabolise SAH produced by the DNA methyltransferases. This requirement may explain the slightly elevated levels of SHH transcript observed within long term cultures in comparison to freshly separated cells.

Within the wound response several phenylpropanoid branch pathways are known to be activated, as reviewed in chapter 1 (Hahlbrock and Scheel, 1989). Several known transmethylation reactions occur within these pathways and some of the induced methyltransferase enzymes responsible have been cloned. For example, a cDNA for CCoA-OMT (S-adenosyl-L-methionine:transcaffeoyl-coenzyme A 3-O-methyltransferase), was isolated from a Zinnia elegans model system (Ye et al., 1994). Like the asparagus model system, this system utilises separated mesophyll cells and therefore the two systems are very analogous. When the Zinnia cells are cultured in basal media with the addition of NAA (1-naphthaleneacetic acid) and BA (benzyladenine) hormones, the mesophyll cells differentiate into tracheary elements (TE), but in basal media alone, or with the addition of single hormones, no TE differentiation occurs. Northern analysis revealed CCoA-OMT mRNA levels to differ in a media-dependent manner. Although the greatest induction of CCoA-OMT mRNA occurred during TE differentiation, due to the lignin synthesis, it was noted that mRNA accumulates at lower levels within dedifferentiating cultured cells in basal media without hormones or with the addition of a single hormone. Recently a cDNA for CAOMT (caffeic acid Omethyltransferase) was isolated from this model system (Ye and Varner, 1995). Unlike CCoA-OMT, CAOMT transcription was greatly induced in cells in basal media, in comparison to hormone-containing media. These papers conclude that many enzymes required for lignin synthesis are induced as a result of the wound response during cell separation. Specifically, two proven methyltransferase enzymes have been shown to be induced by the separation of Zinnia mesophyll cells, an analogous system to that used for asparagus. With the induction of several transmethylation-requiring pathways, the basal levels of SHH gene expression required for various housekeeping functions of primary metabolism may not be sufficient under stress conditions, causing the observed transcriptional activation of SHH.

A similar model system, for the isolation of defence related transcripts,

was utilised for the isolation of the parsley SHH cDNA (Kawalleck et al., 1992). This cDNA was isolated from cell suspension cultures after treatment with a known elicitor from the fungus Phytophthora megasperma f. sp. glycinea. The parsley system utilises long established, dividing heterotrophic suspension cultures treated with elicitor, whereas the asparagus system uses freshly isolated mesophyll cells. In the long established parsley culture the initial wound response has faded and therefore any induction of PcSHH transcript was due to the addition of the elicitor. However AoSHH transcript was upregulated without the use of an elicitor and therefore is present in the library due to the mechanical separation of the cells. It has been noted that several genes are induced both by the process of wounding and pathogen attack (for example, Shufflebottom et al., 1993). This difference in the model systems used to isolate these two plant SHH cDNAs, may explain the slight variance in induction profile between the cDNAs. After elicitor treatment of the parsley cells, PcSHH mRNA levels peak between 6-10 hours, with levels above basal observed in the final time point of 31 hours; whereas in the asparagus system, the AoSHH transcript is expressed at its peak level up to 7 days post separation.

The comparison of the predicted amino acid sequence of AoSHH to other cloned SHH cDNAs highlights the high level of sequence conservation that has occurred within the SHH proteins primary structure. Similar studies using species separated by about one billion years in evolutionary history, led to the postulation that this conservation of the amino acid structure served an important function (Kasir *et al.*, 1988). This remarkable sequence conservation facilitated the study of SHH sequence structure in diverse plant species as described in the next chapter.

Chapter 4

Investigation of SHH gene conservation across several plant species

4.1 Introduction

Comparisons of the AoSHH predicted amino acid sequence to previously cloned SHH cDNAs suggested a high degree of amino acid sequence conservation (see chapter 3). The percentage sequence identity observed upon alignments of the AoSHH, parsley (Kawalleck et al., 1992), Rhodobacter capsulatus (Sganga et al., 1992) and human (Coulter-Karis and Hershfield, 1989) predicted amino acid sequences are 90.1%, 58.7% and 58.1% respectively. To fully appreciate this level of sequence conservation, these results need to be viewed in the context of other sequence identities for genes isolated from diverse species. SAM decarboxylase isolated from potato is only 34.9% identical to a human SAM decarboxylase predicted amino acid sequence and 30.7% identical to one from yeast (Mad Arif et al., 1994). SAM synthetase-1 from Arabidopsis thaliana is 49% identical to E. coli and 57% identical to yeast versions of this gene (Peleman et al., 1989a). By focussing on a specific gene found only in plant species, the percentage sequence identity between the monocot species (asparagus) and the dicot species (parsley) SHH clones of 90.1% can be seen to be greater than 81.55% or 81.45% identity for chalcone synthase

clones from Zea mays and Petunia hybrida, or Hordeum vulgare and Antirrhinum majus, respectively. These percentage identities observed in diverse plant species chalcone synthase genes, are considered to show very strong conservation through evolution (Niesbach-Klösgen et al., 1987).

Other workers (Kasir *et al.*, 1988; Sganga *et al.*, 1992) have also noted the high degree of primary amino acid sequence conservation within SHH cDNAs from diverse species, and both Kawalleck *et al.* (1992) and Sganga *et al.* (1992) remarked on the presence of an 'extra amino acid motif' found within SHH cDNAs isolated from species with a photosynthetic ability. Although this extra amino acid motif differed in size between parsley and *Rhodobacter capsulatus*, 41 amino acids and 36 amino acids respectively, it was present in the same relative position. This data related only to genes from a single plant and a single bacterial species and therefore was of uncertain significance.

However, when upon sequencing of AoSHH, it was also found to contain this extra amino acid motif (chapter 3) in the same position within the derived amino acid sequence, I decided to test the hypothesis that the presence of the extra amino acid motif was due to the photosynthetic nature of the species from which the clones were isolated. A PCR-based approach was utilised to amplify SHH gene portions from several diverse plant species to assess whether the extra amino acid motif was present. The study was extended to also include non-photosynthetic and photosynthetic bacterial species. A similar PCR approach was also utilised to study a further aspect of SHH gene conservation within several plant species, namely intron position.

4.2 SHH primer design

To allow amplification of a portion of the SHH gene(s) from plant species, two primers were designed to anneal to regions of DNA spanning the sequence encoding the extra amino acid motif. To design primers which could be utilised on a diverse range of plant species, the sequence of each primer was based upon alignment of three species SHH cDNA sequence. When the primers were originally designed the only plant SHH sequence in the databases was that of parsley cDNA (Kawalleck *et al.*, 1992). The SHH sequences from asparagus (this work), parsley (Kawalleck *et al.*, 1992), and that from rat (Ogawa *et al.*, 1987) were aligned using programmes from the GCG software package (Devereux *et al.*, 1984) and degenerate primers were designed on the basis of this alignment. By using two plant and one animal SHH cDNA sequences it was anticipated that the primers would allow the amplification of SHH gene portions from a diverse range of plant species. A conserved putative NAD binding site had been identified in both published SHH cDNA sequences (Ogawa *et al.*, 1987; Kawalleck *et al.*, 1992) so primers were not designed to this portion of the gene as this sequence would be present in other genes requiring this cofactor.

Suitable primer sites were found either side of the extra amino acid motif coding region, found within the plant cDNAs. The first primer, MSPCR1 has 8 fold degeneracy while the second MSPCR2 has 4 fold degeneracy. *Xba* I restriction sites were included at the 5' end of each primer together with 3 extra bases to aid in the cloning of any product. The position of annealing of these primers with respect to the AoSHH sequence shown in figure 3.4 are, MSPCR1 255 to 277 and MSPCR2 718 to 696.The sequence of these primers are:-

MSPCR1 5' GCG TCT AGA TGC AAC ATA/C TTC TCC/A ACC/T CAG GA 3' Xba I

MSPCR2 5' GCG TCT AGA TTA/G TCA AAC TTG CTC TTG GTA/G AC 3' Xba I

It was hoped that these primers would allow the specific amplification of SHH sequences from all plant species and that the size of the product would reveal whether the extra amino acid motif was present.

4.3 Amplification of SHH sequence from several diverse plant species

PCR amplifications were performed on genomic DNA from Arabidopsis thaliana cv Columbia, Asparagus officinalis cv Connover's Colossal, Nicotiana tabacum cv Petite Havana SR1 (tobacco), Brachypodium distachyon and Triticum aestivum (wheat). Asparagus was used as a control as AoSHH was utilised in the primer design and therefore a PCR product should be amplified Figure 4.1 Amplification of SHH gene fragments from diverse plant species

Figure 4.1A MSPCR1- and MSPCR2-primed amplified products ran on a 1% agarose gel, proving all five plant species to produce a \approx 470bp product containing the extra amino acid motif.

M=1kb ladder size marker 1=Arabidopsis thaliana 2=Asparagus officinalis 3=Nicotiana tabacum 4=-ve control (no template DNA) 5=Brachypodium distachyon 6=Triticum aestivum

Figure 4.18 Southern blot of the gel in figure 4.1A hybridised with the radio-labelled DB6, demonstrating DNA sequence conservation between amplification products from different species.



from a genomic DNA template from this species. Wheat and *Brachypodium* are both monocotyledonous species like asparagus. Unlike the hexaploid species wheat, *Brachypodium* has a small genome size (Shi, 1991) and therefore is potentially a model monocotyledonous species for gene isolation. *Arabidopsis* and tobacco are both well characterised model dicotyledonous species.

Figure 4.1A shows the products of this PCR amplification. The asparagus genomic DNA, positive control template, gave an expected \approx 470bp PCR product. All species tested demonstrated the same sized SHH PCR product which is consistent with the presence of an extra amino acid motif and the absence of introns within this sequence.

A single product was observed in all amplifications except from the wheat genomic DNA template which had a product of the predicted size and a smaller product. The size of the second smaller wheat product was consistent with the predicted product size if amplification had occurred from an SHH template, that has no extra amino acid motif.

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Southern blot hybridisation with the AoSHH probe demonstrated that all PCR products hybridised after high stringency washes (0.5X SSC and 0.5% SDS at 65 $^{\circ}$ C for 15 minutes) to the AoSHH probe confirming the PCR products were indeed amplified from SHH genes. This suggests conservation of the nucleotide sequence to have occurred between diverse plant species and the resultant autoradiograph of the experiment can be seen in figure 4.1B.

4.4 Cloning and sequence analysis of plant SHH amplification products

To analyse the PCR products further, each amplified fragment was cloned into pCR^{TM} vector (Invitrogen), following the manufacturer's instructions in the TA cloning kit. Both strands of the resultant plasmid inserts were sequenced from primers in the vector and using MSPCR1 and MSPCR2 as sequencing primers. The resultant nucleotide and predicted amino acid sequences were used in sequence alignments and this proved that each PCR product was a result of the amplification of an SHH gene. The smaller wheat PCR product was shown to be derived from an SHH gene sequence but, as

Figure 4.2 Alignment of the predicted amino acid sequences of the SHH PCR products

Figure 4.2A CLUSTAL V multiple sequence alignment of the sequenced PCR products, which demonstrates the presence of the extra amino acid motif in all plant species tested. The extra amino acid motif is shown in green, the * represents non divergent amino acids and the . represents conservatively substituted amino acids.

Figure 4.28 Identical to figure 4.2A, except for the removal of the non-plant SHH 370bp amplification product. This figure highlights the conserved nature of all the plant SHH amplification products.

A

A. officinalis T. aestivum N tabacum A. thaliana B. distachyon 370bp product A. officinalis T. aestivum N tabacum A. thaliana B. distachyon 370bp product A. officinalis T. aestivum N tabacum A. thaliana B. distachyon 370bp product

B

- A. officinalis
- T. aestivum
- N tabacum
- A. thaliana
- B. distachyon

A. officinalis

- T. aestivum
- N tabacum
- A. thaliana
- B. distachyon
- A. officinalis
- T. aestivum
- N tabacum
- A. thaliana
- B. distachyon

HAAAAIARDSASVFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDTTLLIHEGVKA RAAAAIARDSASVFAWKGETLQGYWWCTERALDWGPGGGLDLIVDDGGDTTLLIHEGVKA HAAAAIARDSRAVFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDATLLIHEGVKA HAAAAIARDSAAAFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDATLLIHEGVKA HAAAAIARDSAAVFAWKGETLEEYWWCTERCLDWGVGGGPDLIVDDGGDPTLLIHEGVKA .******** .************

YQMQANNSLLFPAINVNDS YQMQANNSLLFLTINVNDS YQMQANGTLLFPAINVNDS YQMQQNGTLLFPAINVNDS YQTQNPGTLLFPAINVNDS ** * ...*** .***** expected, did not contain the extra amino acid motif found in all the other amplification products. To investigate the origin of the smaller PCR product in wheat, a fresh DNA isolation was performed and used in PCR with the primers MSPCR1 and MSPCR2. The smaller SHH product was not amplified but the approximately 470bp fragment was still present. No further products could be radioactively detected upon Southern blotting (result not shown). Further experiments were performed using several small scale DNA extractions (Edwards *et al.*, 1991) but the smaller product was never observed. This will be discussed later, but it should be noted that the origin of this smaller amplification product was thought to be non-plant, contaminant DNA.

The predicted amino acid sequences of all six amplification products were aligned using CLUSTAL V (Higgins and Sharp, 1989) as shown in figures 4.2A and 4.2B. The protein sequence of the extra amino acid motif found in the 5 plant derived SHH amplification products is conserved, with levels slightly lower but similar to those found when comparisons are made using full length plant sequences. In addition, a high degree of conservation was observed in the sequences flanking the extra amino acid motif. A dendrogram of these PCR products predicted amino acid structure can be seen in figure 4.3.

4.5 Amplification of SHH sequence from diverse bacterial species

As it was concluded that the origin of the 370bp amplification product within the original wheat template reaction was due to non-plant contaminant DNA, this suggests that the primers MSPCR1 and MSPCR2 may allow amplification of DNA from evolutionary diverse species apart from plants. This was tested using bacterial species. As discussed in chapter 1, not all bacteria have SHH enzyme activity, and therefore do not contain the SHH gene. Studies by several groups (Walker and Duerre, 1975; Shimizu *et al.*, 1988) have shown which bacterial species contain SHH enzyme activity. Based upon these studies SHH gene amplification was attempted from three species shown to contain SHH activity, namely, *Halobacterium salinarum*, a *Rhodospirillum* species and *Agrobacterium tumefaciens* LBA4404. *Rhodospirillum* species are close relatives of *Rhodobacter* species, in which SHH was found to contain the extra amino acid motif (Sganga *et al.*, 1992). The choice of these three species Figure 4.3 SHH protein dendrogram of predicted amino acid sequence derived from PCR product sequence

A CLUSTAL V dendrogram alignment of the sequences shown in figure 4.2A.



% Similarity of amino acids

also facilitates the study of the correlation between extra amino acid motif presence and the photosynthetic nature of the species. *Halobacter* and *Rhodospirillum* species are photosynthetic, while *Agrobacterium* is a nonphotosynthetic bacterial species. Using template DNA, derived as described in section 2.5, amplification was performed on the listed bacterial species. Amplification products were observed in *Halobacter* and *Rhodospirillum* template reactions which were identical in size to the amplification product found in an asparagus positive control. However, no amplification product could be observed in samples with *Agrobacterium* template DNA (results not shown). Therefore, although the amplification products were not cloned and sequenced, their size proves the presence of the extra amino acid motif within two further photosynthetic bacterial species.

4.6 A further primer design to investigate conservation of plant SHH intron position

Upon the isolation and sequencing of a genomic *Arabidopsis* SHH gene (discussed in chapter 6), it was discovered that a single intron existed within the coding region. Thus a further primer was designed, which in conjunction with MSPCR1, would allow amplification of the *Arabidopsis* intron and surrounding DNA sequence. The use of two SHH-specific primers would allow the design of a simple assay to determine the presence or absence of an intron at or near the same position as that found in *Arabidopsis*, in several diverse plant species. Due to the high level of nucleotide sequence conservation found between diverse plant species in the original PCR experiments, the sequence of primer MSPCR3 was simply designed upon the nucleotide sequence of the *Arabidopsis* genomic clone. A GC rich region of the genomic clone which was close to the 3' end of the intron and within the coding sequence was utilised. Therefore the sequence of MSPCR3 is:-

MSPCR3 5' GCG TCT AGA ATC ATG ACA TCA GTG GCC CTC AT 3' Xba I

Figure 4.4 PCR of an SHH intron from diverse plant species

MSPCR1- and MSPCR3-primed amplified products ran on a 1% agarose gel, proving that 3 of the 5 species tested contain an intron between the primer annealing sites.

M=1kb ladder size marker 1=Arabidopsis thaliana 2=Asparagus officinalis 3=Nicotiana tabacum 4=-ve control (no template DNA) 5=Brachypodium distachyon 6=Triticum aestivum



4.7 Amplification of SHH intron/exon boundaries from plant species

The sequence data obtained from AoSHH (a cDNA) and AtSHH (a genomic clone) allow the size of amplification products to be predicted if no introns exist between MSPCR1 and MSPCR3. Using *Arabidopsis* as a positive control for a species known to contain an intron within this amplified region, PCR analysis was performed on the same species as previously described, using the same template DNA. The resultant amplified products were analysed by gel electrophoresis and can be seen in figure 4.4. This gel was Southern blotted and hybridised to a radio-labelled AoSHH probe, proving all amplification products cross hybridise to AoSHH (results not shown), and only one product is amplified from each species.

The size of the products (\approx 520bp) from amplification from an asparagus and tobacco genomic DNA template were consistent with the predicted size based on the AoSHH cDNA sequence for an SHH gene lacking an intron. However *Arabidopsis* produced an 820bp product as predicted from the genomic clone, while both *Brachypodium* and wheat produced larger amplified products of approximately 1.3kb and 1.1kb respectively. This result suggests both *Brachypodium* and wheat SHH genes contain introns at or near to the position of the intron in the *Arabidopsis* SHH gene, but that in both these species the introns are larger.

4.8 Cloning and sequence analysis of further plant SHH amplification products

To confirm that each of the amplified products were the correct portion of DNA, each was cloned into a suitable vector for sequencing. MSPCR3 primer contained an *Xba* I site similar to MSPCR1 and therefore following precipitation and digestion of the asparagus and tobacco PCR reactions, these amplification products were cloned into similarly cut pBluescriptTMSK-. Due to the presence of an *Xba* I site within the *Arabidopsis* amplification product, and the larger size of the *Brachypodium* and wheat products, with an increased chance of containing this restriction enzymes recognition sequence, these three products were cloned in pCRTM (Invitrogen). Both strands of the plasmid

Figure 4.5 Plant SHH intron/exon boundaries

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Schematic diagram of the intron/exon boundaries within the PCR products shown in figure 4.4. In those species containing an intron, the boundary was found to be at exactly the same position within the amino acid sequence. The intron splice site consensus sequences are shown.

INVNDSVTKSK NoIntron FDNLYGCR	INVNDSVTKSK No Intron FDNLYGCR	I N V N D S V T K S K ≈280 bp intron F D N L Y G C R aag agc aag / gtattg ttc gac aac	I N V N D S V T K S K ≈800bp intron F D N L Y G C R aag agc aag /gtcctt taacag/ ttc gac aat	INVNDSVTKSK ∞600bp intron FDNLYGCR
is	acum	liana	yon	mnai

В.

inserts were sequenced as well as several subclones. The resultant nucleotide sequence demonstrated the presence of a single intron in exactly the same position within the predicted amino acid sequence in three of the five plant species tested. This is schematically represented in figure 4.5. The predicted amino acid sequence of each product is not shown as it is identical to that of the MSPCR1 and MSPCR2 amplified products, apart from an extra 19 amino acids present at the MSPCR3 termini of the product.

The introns found within the Brachypodium and wheat SHH gene amplification products were not completely sequenced, therefore only approximate sizes are given on figure 4.5. The sequence data proved the presence of typical splice site junctions in all three introns [5' splice site consensus AG/GTAAG; 3' splice site consensus TGCAG/G (Simpson et al., 1992; Goodall and Filipowicz, 1991)], as shown in figure 4.5. The Arabidopsis intron is discussed in detail in chapter 6 and the splice site junctions and other sequence consensus elements are in agreement with the previous criteria for dicot introns (Goodall and Filipowicz, 1989); it is larger than the minimum length of 70 nucleotides, and greater than 59% AU rich, at 67.7%. The two other SHH genes found to contain an intron are from monocotyledonous species. The AU richness of monocotyledonous species introns varies to a greater degree than dicotyledonous species introns, with the optimal splice site sequences alleviating the need for a high AU content (Goodall and Filipowicz, 1989). As the entire introns were not sequenced, the 100 nucleotides next to each splice site were compared to the PCR products coding regions. In Brachypodium the coding region has only 40% AU content whereas the 200 sequenced nucleotides of the intron have a 63% AU content. In wheat, these figures are 38% and 56% respectively. This average monocotyledon species intron AU content may compensate for the lack of optimal splice site sequences.

4.9 Discussion

Each of the five plant species tested within this analysis were found to contain the extra amino acid motif postulated to exist within SHH genes from photosynthetic species, therefore the working hypothesis holds for all cases tested. The presence of an amplification product from the original wheat template of an SHH gene not containing the extra amino acid motif, was unexpected. This led to repeat experiments being performed, using freshly extracted wheat genomic DNA template where the 370bp fragment was not observed. It was therefore concluded that the initial 370bp product amplified in the wheat DNA template reaction, probably arose from a contaminant. Sequence comparisons of the predicted amino acid structure with SHH genes found on the EMBL database suggested the 370bp product was not from a plant origin, but was most similar to D. discoideum (Kasir et al., 1988), C. elegans (Prasad et al., 1993), H. sapiens (Coulter-Karis and Hershfield, 1989) and R. norvegicus (Ogawa et al., 1987) SHH sequences, with approximately 68% identity in all cases. This proves that the PCR contamination did not arise from a human source and that this 370bp SHH sequence was an artifact. This idea is also supported by the lack of a 370bp product in PCR reactions from several fresh DNA extractions from wheat. However, this data demonstrated that the MSPCR1 and MSPCR2 may be used to amplify SHH sequences from many species across kingdoms.

Repeating the analysis using bacterial species demonstrated the presence of the extra amino acid motif within two further photosynthetic bacterial species, but unfortunately amplification did not work in the nonphotosynthetic species utilised. Therefore although the correlation of the photosynthetic nature of the species and the presence of the extra amino acid motif was confirmed, the absence of the extra amino acid motif within nonphotosynthetic bacterial species could not be shown. However this study endorses the possible non-plant origin of the 370bp amplification product from the original wheat extract.

This work has confirmed the correlation of the presence of the extra amino acid motif within photosynthetic species. The isolation of further SHH cDNAs has also added further examples to this correlation. These include the dicotyledonous plant species, tobacco (Mitsui *et al.*, 1993), *Catharanthus roseus* (Schröder *et al.*, 1994) and *Medicago sativa* (Abrahams *et al.*, 1995); the monocotyledonous plant species wheat (Richards and Gardner, 1994); and perhaps more surprisingly the malarial parasite *Plasmodium falciparum* (Creedon *et al.*, 1994). The presence of the extra amino acid motif within the non-photosynthetic *Plasmodium* malarial parasite is unexpected, as prior to this clone, only photosynthetic species contained this region. However, ribosomal sequences and the presence of a 35-kb plastid-like cytoplasmic element in *Plasmodium* has led to the proposition of an algal-like origin to these parasites (Creedon *et al.*, 1994), which may help to explain the presence of this extra amino acid motif in a non-photosynthetic species. The presence of the extra amino acid motif in SHH sequences from photosynthetic species as well as its absence in non-photosynthetic species, has been suggested to indicate an early evolutionary deletion or insertion in the SHH gene (Aksamit *et al.*, 1995).

Despite the accumulation of data in support of this correlation as a result of these studies, no attempt has been made to explain the role of the motif within these species. It has been proposed by other workers that a photosynthetic specific role is performed by the extra amino acid motif within these species (Richards and Gardner, 1994; Creedon et al., 1994). However a recent paper by Aksamit et al., (1995) has demonstrated that the presence of the extra amino acid motif is not essential for a functional SHH gene within Rhodobacter capsulatus. A mutant strain of R. capsulatus called StLB1 has been identified which contains a disrupted SHH gene (Sganga et al., 1992). This strain has no detectable SHH activity causing reduced bacteriochlorophyll synthesis, due to the inhibition of a required methyltransferase reaction as a result of SAH accumulation. The rat SHH cDNA was expressed in strain StLB1, causing detectable SHH activity and increased levels of photopigments. Therefore the expression of the rat SHH can functionally complement the mutant strain R. capsulatus StLB1. This result can be interpreted in two ways: a photosynthetic specific function cannot be assigned to the extra amino acid motif present in *R. capsulatus*; or alternatively this motif may contribute to a different role such as regulation of enzyme activity or another unknown activity for the enzyme. Whether this conclusion can be extrapolated to include plant species has not been addressed. The dendrogram shown in chapter 3 and the multiple sequence alignments of Aksamit et al. (1995), highlight the higher level of conservation found within plant derived extra amino acid motifs

compared to the same gene sequence from other phyla. The percentage sequence identity of several extra amino acid sequence motifs are quoted by Aksamit *et al.* (1995) with all plant derived sequences scoring above 70%, while the *R. capsulatus* sequence scores only 22% (PcSHH was used as the reference sequence). This score is actually lower than the 27% sequence identity observed for *P. falciparum*, which is known to be a non-photosynthetic species. Therefore, for sequence diversity of this magnitude to occur within a portion of a gene with proven sequence conservation occurring elsewhere, suggests that, as with *P. falciparum*, this portion of the gene may not be utilised for a specific purpose such as photosynthesis. This may explain the lack of requirement of this portion of the SHH gene in the restored, rat cDNA containing, *R. capsulatus* StLB1. To test if a similar approach would restore SHH activity within plants, a mutant would be required with no or perhaps reduced SHH activity. This will be discussed in chapter 8.

On isolation of an Arabidopsis genomic SHH clone (see chapter 6) a single intron was found within the coding sequence. It is interesting to note that this intron occurred exactly at the position in which the PCR primer MSPCR2 annealed. Therefore in Arabidopsis only the 15 nucleotide bases at the 3' end of MSPCR2 could anneal to the genomic template to allow the cloned amplification product to have been produced. The 8 nucleotide bases complementary to the 5' end of MSPCR2 were present at the other side of the intron within the genomic DNA. This must also be true for Brachypodium and wheat which were shown to contain an intron at exactly the same position within the nucleotide sequence. The original wheat DNA extract was utilised for amplification using MSPCR1 and MSPCR3 and only a single product was detected. This result infers that MSPCR3 does not anneal to the contaminant DNA to allow amplification to proceed. Unlike MSPCR1 and MSPCR2, which were designed on comparison of diverse SHH sequences and therefore contained degeneracy, MSPCR3 was simply the nucleotide sequence taken from the Arabidopsis genomic clone. The lack of degeneracy and the simple design of MSPCR3 has potentially only allowed its use on the closely related plant SHH sequences. To test this, MSPCR1/MSPCR3 primed amplification was performed on the bacterial species previously utilised. No amplification was

observed from any bacterial species except a faint band of approximately 520bp from *Halobacterium salinarum*. This is the expected product size, as prokaryote species do not contain introns within their genes. This result suggests MSPCR3 is not a good primer for the amplification of SHH products from non-plant sources.

The presence of an intron in exactly the same position within three of the five plant species tested, suggests that the structure of the SHH gene has been conserved, in addition to the noted primary amino acid sequence conservation. Results presented in chapter 6 suggest both asparagus and tobacco contain small SHH gene families, therefore MSPCR1/MSPCR3 primed amplification may have generated more than one product, as intron size could vary between each copy of the particular species SHH genes. The results obtained can either be explained by the total absence of any introns in any copy of either species SHH genes, resulting in a single product; or the intron which may exist in certain copies within these species is too large to amplify using the conditions in this experiment. The presence of an intron in the same position within three diverse plant species may suggest that the ancestral copy of the gene contained this intron, as it is unlikely for an intron to arise through evolution in exactly the same position at least three times. An example of conservative intron position is found in chalcone synthase (CHS) genes which also have a conserved amino acid structure (see section 4.1). Sequence analysis detected an intron in exactly the same position within four species; Hordeum vulgare and Zea mays (monocots), Petunia hybrida and Antirrhinum majus (dicots). It was suggested that the conserved position of this intron may be due to its presence prior to the division of mono and dicotyledons (Niesbach-Klösgen et al., 1987).

Chapter 5

Characterisation of SHH gene expression

5.1 Introduction

Prior to the isolation of SHH cDNAs or the production of SHH antibodies, the only data characterising SHH gene expression within plant tissues came from studies of SHH enzyme activity. Although most studies concentrated on the enzymology of the purified protein rather than the comparative activity profile in separate tissues, they do provide evidence of SHH enzyme activity within particular tissues. Following the demonstrated presence of SHH enzyme activity within Phaseolus vulgaris and Spinacea oleracea leaf tissue, and Phaseolus vulgaris, Zea mays and Hordeum distichon etiolated seedlings (Walker and Duerre, 1975), several studies of plant SHH enzymology were initiated. These studies only used two extractions from the same species, which showed the SHH enzyme activity within one week-old etiolated Phaseolus vulgaris seedlings was greater than in leaf tissue (Walker and Duerre, 1975). SHH activity has also been detected within Beta vulgaris L. ssp vulgaris leaves (Poulton and Butt, 1976), Lupinus luteus seeds (Guranowski and Pawelkiewicz, 1977) and Nicotiana tabacum cell suspension culture (Sebestova et al., 1984).

SHH activity was studied in detail within extracts of Lupinus luteus

cotyledons at different stages of seed formation and seedling development. Constitutive SHH activity was present in yellow lupin cotyledons during seed formation and germination, with the lowest activity levels present during the dry dormant seed stage. This low level of activity is reached following a steady decrease during the maturation period. SHH activity levels increase to a maximum in seedlings four days post-germination (Guranowski and Pawelkiewicz, 1978).

The parsley SHH cDNA generated the first molecular probe available to study SHH transcript levels in plants (Kawalleck *et al.*, 1992). Northern analysis of parsley organ RNA, revealed the SHH transcript to be most abundant in floral buds and stems, with lower levels being detected in roots and leaves. The parsley SHH transcript was also upregulated upon pathogen attack of the plant tissue, as discussed in chapters 1 and 3. When this present study of SHH gene expression was initiated this was the only molecular data available for SHH transcript abundance within any plant species.

Over the duration of the work presented within this thesis, several SHH clones were isolated and published, including; *Nicotiana sylvestris* [tobacco] (Mitsui *et al.*, 1993), *Triticum aestivum* [wheat] (Richards and Gardner, 1994), *Plasmodium falciparum* (Creedon *et al.*, 1994), *Catharanthus roseus* (Schröder *et al.*, 1994), *Xenopus laevis* (Seery *et al.*, 1994), *Mus musculus* [mouse] (Petrovic *et al.*, 1994) and *Medicago sativa* (Abrahams *et al.*, 1995).

Most of the plant SHH cDNAs isolated were obtained as a result of differential library screens, to try and isolate transcripts involved in several diverse phenomena. The wheat cDNA was isolated from screens using aluminium tolerant and sensitive wheat cultivars (Richards and Gardner, 1994). The *Catharanthus roseus* cDNA was identified in a differential screen for transcripts induced/upregulated by the addition of 8% sucrose to cell suspension cultures (Schröder *et al.*, 1994). The *Medicago sativa* cDNA was isolated in a screen to identify transcripts predominantly expressed in the stem (Abrahams *et al.*, 1995). However the tobacco clone was isolated following purification of a cytokinin-binding protein (Mitsui *et al.*, 1993; see chapter 1). These identifications of SHH transcripts within several experimental approaches, for the isolation of transcripts expressed in specific patterns,

highlights the potential diverse situations in which SHH activity is required and might reflect complex control mechanisms governing it's expression.

This portion of work aims to identify the pattern of expression of SHH in plants and assess whether the regulation of SHH enzyme activity occurs at the level of transcription, translation or as a post-translational event.

5.2 Derivation of probes, antibodies and assays utilised in this study

5.2.1 DNA probes

The results presented in chapter 4 emphasise the amino acid sequence identity observed between SHH genes of diverse plant species, but also demonstrate cross species hybridisation of radio-labelled DNA probes to occur due to the nucleotide sequence identity. It is therefore possible to perform northern analysis using heterologous species SHH DNA probes. However once the SHH PCR products derived in this project had been cloned, probes from several species were available. Throughout this study a number of SHH DNA probes were utilised including DB6, AoSHH, AtSHH (see Chapter 6) and tobacco and *Arabidopsis* PCR products, so for each blot described, the probe utilised is named.

5.2.2 SHH antibodies

Kawalleck *et al.* (1992) utilised the SHH antibody raised against the purified rat SHH protein (Ogawa *et al.*, 1987), to detect an *in vitro* transcription/translation product of the parsley SHH cDNA. Dr. Cantoni (Natl. Inst. of Mental Health, Bethesda, MD) kindly sent an aliquot of this antibody but upon its use on western blots it was observed to bind to several plant proteins and therefore to be too non-specific for its required purpose (results not shown). An *Arabidopsis* SHH specific antibody was therefore raised within this project as described in chapter 2. The three peptides synthesised for antibody production were chosen from the AtSHH derived amino acid sequence (see chapter 6) because of several criteria, including; the presence of hydrophilic residues which are more likely to be found on the protein surface; and secondary structural predictions, as turns are often very immunogenic (See

Figure 5.1 SHH antibody derivation and analysis

Figure 5.1A Sequence of the three synthetic peptides used for antibody production, and comparison to the same regions of the AoSHH and PcSHH amino acid sequence.

Figure 5.18 Representative peptide dot blots probed with sera from the first bleed of rabbits injected with peptide 1. The numbers represent separate blots for each rabbit injected with the same peptide. The letters represent each protein/peptide loaded on each strip. These were as follows;

a=500ng of unconjugated peptide 1 b=100ng of unconjugated peptide 1 c=10ng of unconjugated peptide 1 d=100ng of unconjugated peptide 2 e=100ng of unconjugated peptide 3 f=100ng of BSA g=100ng of lysozyme Greek letters represent differing dilut

The Greek letters represent differing dilutions of each rabbits sera. These were as follows;

 α =1:10 dilution of sera

 β =1:100 dilution of sera

 γ =1:1000 dilution of sera

Therefore lanes d-g were utilised to assay the specificity of each immunogenic response. The lack of cross reaction to the other nonspecific peptides and lysozyme proved the antigen recognition to be specific. The BSA sample cross reacted due to anti-carrier antibodies. Similar experiments were performed with sera from each of the nine rabbits utilised, after each test bleed.

Figure 5.1C Western blot of $10\mu g$ of total protein/lane, isolated from three plant species leaf tissue, probed with anti-AtSHH antibody (peptide 3). Proteins were isolated from the following:

Lane 1	Arabidopsis leaf tissue
Lane 2	asparagus cladode tissue
Lane 3	tobacco leaf tissue



Gullick, 1994 for further discussion). As the SHH protein sequence is well conserved between plant species, all areas of amino acid sequence fulfilling the initial requirements were compared to known plant sequences to allow utilisation of the more conserved peptides (see figure 5.1A).

The resultant antibody was also needed to monitor SHH protein levels in SHH perturbation experiments, as described in chapter 8.

5.2.2.1 Antibody titre

As outlined in chapter 2, the serum extracted from each test bleed was analysed for the presence of SHH specific antibodies using non-conjugated peptide dot blots. These blots were utilised to assess the absence of peptide specific antibodies within the preimmune-bleeds (data not shown) and to quantify the amounts of pure peptide detected by each bleed (an example is shown in figure 5.1B). These results suggested that the serum from each of the nine rabbits displayed a specific response to the individual peptide injected. The rabbit serum able to detect the lowest concentration of peptide, with the greatest antibody dilution was defined as the serum containing the greatest immunogenic response. The serum with the greatest immunogenic response for each peptide was then used to probe a western blot of plant proteins to assess the specificity of each antibody. This experiment served a dual purpose as the blot contained protein extracts from the leaves of the three plant species utilised in this study; Arabidopsis thaliana, Nicotiana tabacum, and Asparagus officinalis. These blots therefore provided evidence for the use of the antibodies against SHH protein from further plant species. The results clearly showed that peptide 3 produced the most specific reaction against total plant proteins and this specificity to occur in the reaction against tobacco and asparagus SHH protein as well as against A. thaliana SHH protein (see figure 5.1C).

5.2.3 SHH enzyme analysis

Following unsuccessful attempts to perform SHH enzyme analysis at Leicester, this portion of work was performed at the University of Durham with the assistance of Dr. R. Edwards. The method of assaying SHH enzyme

Figure 5.2 Concentration dependent SHH enzyme analysis

SHH enzyme activity assays ran on a TLC plate, to demonstrate that the assay is protein concentration dependent. The following volumes of $2.6\mu g/\mu l$ mechanically separated asparagus cell protein extract was assayed:

Lane 1	no protein
Lane 2	10µl protein extract
Lane 3	20µl protein extract
Lane 4	40µl protein extract
Lane 5	80µl protein extract
Lane 6	80µl protein extract but no homocysteine added



activity (described in Chapter 2) was formulated by Dr. R. Edwards. Initial analysis was performed using protein extracted from mechanically separated asparagus cells as northern analysis had previously suggested these samples to contain the greatest quantities of SHH transcript (see Chapter 3). Therefore protein extracted from these samples was utilised in initial assays, as increased levels of SHH protein and perhaps enzyme activity were more likely to be found in these samples, to assess if the assay was protein concentration dependent.

The assay measures the synthesis of SAH from adenosine and homocysteine, which is the thermodynamically favourable SHH catalysed reaction. However, although unlikely, if sufficient SAH were synthesised within the assay time period, the SHH enzyme may perform the reverse reaction, the hydrolysis of SAH to adenosine and homocysteine. Therefore to ensure that this equilibrium point was not reached, several concentrations of protein were assayed in the initial experiment. The resultant autoradiograph of the TLC plate can be seen in figure 5.2. This demonstrates that with the samples used, the assay is linear with respect to protein concentration, up to the addition of 200μ g of total protein. This suggests that the equilibrium point of the SHH mediated reaction was not reached.

Two further control reactions can also be observed in figure 5.2. The omission of homocysteine inhibits the reaction completely, therefore the ¹⁴C label is only observed in the adenosine, proving that the other ¹⁴C labelled band is not a naturally occurring adenosine degradation product. The omission of any crude protein extract proves that the reaction is mediated by the plant protein extract and not by impurities in the chemicals. The rf values of both ¹⁴C labelled compounds compared favourably with those obtained for unlabelled sources that were run on the plates simultaneously and detected by UV fluorescence.

5.3 Analysis of Asparagus officinalis SHH protein

The northern analysis presented in chapter 3 (see figure 3.2) proved that SHH transcript abundance was increased by the mechanical separation of asparagus cells and by the wounding of etiolated asparagus seedlings. However Figure 5.3 SHH protein and enzyme activity analysis from mechanically separated asparagus cells

Figure 5.3A Western blot of 10µg of total protein/lane, isolated from mechanically separated asparagus cells, and probed with anti-AtSHH antibody (peptide 3). Proteins were isolated from the following:

Lanes 1-5asparagus cell population 0, 1, 3, 5 and 7 days
post isolation, respectively.

Protein size marker is indicated in kDa at the side of the blot.

Figure 5.3B SHH enzyme activity assays of protein isolated from mechanically separated asparagus cells. Proteins were assayed from cell populations 1, 3 and 5 days post-separation. (Points represent the mean and the vertical bars represent the standard deviation_{n-1} of data from three measurements.)


B

A





Figure 5.4 SHH protein and enzyme activity analysis from wounded, etiolated asparagus seedlings

Figure 5.4A Western blot of 10µg of total protein/lane, isolated from chopped, etiolated asparagus seedlings, and probed with anti-AtSHH antibody (peptide 3). Proteins were isolated from the following:

Lanes 1-4 asparagus seedlings 0, 1, 2 and 3 days post wounding, respectively.

Protein size marker is indicated in kDa at the side of the blot.

Figure 5.4B SHH enzyme activity assays of protein isolated from chopped, etiolated asparagus seedlings. Proteins were assayed from seedlings 0, 1, 2 and 3 days post-wounding. (Points represent the mean and the vertical bars represent the standard deviation_{n-1} of data from three measurements.)





B





nmol/min/mg protein

northern analysis only provides data regarding steady state levels of transcripts which does not necessarily correlate with protein abundance or enzyme activity.

5.3.1 Western analysis

Once a good antibody raised against the synthetic *A. thaliana* peptides was obtained, analysis of protein levels within the asparagus mechanically separated cell populations was performed. Figure 5.3A shows the result of such a western blot. An increase in SHIH protein abundance can be observed within one day post cell separation. The SHIH protein abundance continues to increase over the seven day time course. Figure 5.4A shows a western blot of proteins extracted from a time course post-wounding of etiolated asparagus seedlings. This result suggests that the SHIH protein concentration does not alter significantly by wounding in asparagus seedlings. A similar result was observed using tobacco leaf disk samples, wounded by stabbing (result not shown; method described in chapter 7).

5.3.2 Enzyme analysis

SHH assays were performed to test if SHH protein abundance correlates with SHH enzyme activity.

These analyses demonstrated that SHH enzyme activity was induced by wounding in asparagus. The results of the assays on samples from mechanically separated cells and finely chopped etiolated seedlings are represented graphically in figures 5.3 and 5.4. Figure 5.3B clearly shows that SHH enzyme activity increases over the five day period post asparagus cell separation. Figure 5.4B shows that SHH enzyme activity in etiolated seedlings is already present at higher levels than in light-grown seedlings and is further induced by wounding. This increase in activity is only observed for one day before starting to return to basal levels. The incorporation of ¹⁴C label into SAH, with respect to the total protein content within the assay, appears to be greater in the etiolated seedling time course compared to the mechanically separated asparagus cell population time course.

Figure 5.5 Northern analysis of SHH transcripts within tobacco organs

Figure 5.5A Initial northern analysis of SHH transcripts within tobacco organs. Total RNAs hybridised to the DB6 insert. RNAs were isolated from the following:

Lane 1	leaf
Lane 2	root
Lane 3	stems
Lane 4	petals
Lane 5	anthers

Figure 5.58 Northern analysis of SHH transcripts within tobacco organs, including a temporal division of anther maturation. Total RNAs hybridised to the DB6 insert. RNAs were isolated from the following:

Lanes 1-5	identical to figure 5.5A
Lane 6	pollen
Lane 7	immature anthers
Lane 8	1 day post-wounded asparagus etiolated seedlings

Lane 9 5 day post-separated asparagus cell populations It should be noted that similar results were obtained using a tobacco probe derived from PCR experiments.

Figure 5.5C Northern analysis of CAD transcripts within tobacco organs, including a temporal division of anther maturation. Total RNAs hybridised to a tobacco CAD probe. This blot is the same blot presented in figure 5.5B, following the removal of SHH probe.

 $10 \mu g$ of total RNA was loaded per lane.



5.4 Northern analysis in tobacco

SHH transcript analysis was performed on tobacco, as well as asparagus, for two reasons; firstly to provide an abundant source of RNAs isolated from specific organs, and secondly to provide transcript analysis for later comparisons with SHH promoter/reporter gene fusions in transgenic tobacco.

5.4.1 SHH transcript expression in tobacco organs

Northern analysis was performed using total RNA extracted from several tobacco organs. The results from this analysis can be seen in figure 5.5. Within tobacco, SHH transcript can be detected in all tissues tested. Tobacco SHH mRNA was most abundant in anthers, with smaller levels in petals and roots, and only trace amounts in leaves and stems (see figure 5.5A). The highest transcript levels observed in anthers, were further analysed, in a temporal context. The initial RNA extraction utilised anthers dissected from flowers at all stages of maturity, which often had pollen attached. Therefore a simple sub-division of anther maturation was utilised. Fresh RNA extractions were performed from pollen on dehydrated, dehisced anthers which are metabolically inactive and therefore this represented a pollen RNA population, while a further total RNA population was extracted from immature anthers which had been dissected from tobacco buds. These samples were used in a fresh northern blot with the original tobacco organ extractions and two asparagus RNA extractions used in figure 3.2, to assess comparative transcript levels (see figure 5.5B). This result indicates that although SHH transcript is present within both immature anthers and mature pollen, the greater transcript abundance occurs in the mature pollen rather than the developing anthers. The use of asparagus RNA samples highlights the relatively high transcript levels observed in mechanically isolated asparagus cells and wounded asparagus seedlings, compared to the lower transcript levels observed in tobacco organs.

It was predicted that SHH transcript might be present within stem tissues at a greater level than detected, due to the process of lignification occurring in this tissue, as transmethylation reactions are required to generate the lignin Figure 5.6 Northern analysis of SHH transcripts during tobacco stem and flower maturation

Figure 5.6A Northern analysis of SHH transcripts within tobacco stems. Total RNAs hybridised to the DB6 insert. RNAs were isolated from three separate tobacco plants. Lanes 1-3 were isolated from a 2 week old plant, lanes 4-6 were isolated from a 3 week old plant, and lanes 7-9 were isolated from a 4 week old plant. For each plant three stem sections were utilised. Therefore lanes 1, 4 and 7 contain RNA from the oldest section, nearest the base of the stem; lanes 2, 5 and 8 contain RNA from middle sections; and lanes 3, 6 and 9 contain RNA from the youngest section, nearest the apical meristem. Lane 10 contains RNA from 1 day postwounded asparagus etiolated seedlings, and lane 11 contains RNA from 5 day post-separated asparagus cell populations.

Figure 5.68 Northern analysis of CAD transcripts within tobacco stems. Total RNAs hybridised to a tobacco CAD probe. This blot uses the same stem RNAs as used in figure 5.6A and hence the labels are the same.

Figure 5.6C Northern analysis of SHH transcripts within tobacco buds and anthers. Total RNAs hybridised to the DB6 insert. RNAs were isolated from the following:

Anthers from A9 barnase transgenic 4-6mm
tobacco buds
Anthers from 8-12mm tobacco buds
Anthers from <8mm tobacco buds
>35mm tobacco buds
15-20mm tobacco buds
10-14mm tobacco buds
4-10mm tobacco buds

10µg of total RNA was loaded per lane.



precursors. To assess whether the RNAs did indeed represent those extracted from tissue active in lignification, the RNA blot was reprobed with a tobacco CAD [cinnamyl alcohol dehydrogenase] cDNA probe (kindly provided by ZENECA; Knight *et al.*, 1992). CAD is a good marker for the process of lignification as it is on the branch point of the phenylpropanoid pathway that leads to the production of lignin precursors. As can be observed in figure 5.5C, the largest accumulation of CAD transcript was found within immature anthers. No CAD transcript was detected within asparagus tissues, suggesting that the heterologous tobacco probe does not hybridise to asparagus transcripts. The lack of hybridising CAD transcript in mature pollen samples suggests that CAD expression during pollen maturation occurs at a specific time period during anther development, and not at other times. As with SHH, greater quantities of hybridising transcript are detected in roots and petals than in stems. This suggests that the RNA extracted from mature stem tissues does not represent the RNAs present during the process of lignification.

5.4.2 SHH expression in lignifying tobacco stems

The previous results obtained using an RNA extraction from tobacco stems, suggested that SHH and CAD transcripts were very low, if present at all. To assess if this situation occurred at all stages of tobacco stem maturation, further tobacco stem RNA extractions were performed. Therefore three tobacco plants of 2, 3 and 4 weeks old, had RNA extracted from their stems. Three sections of the stems of each plant were utilised for RNA extraction, to try and encompass the diverse stages of lignification within stem tissue. Northern analysis of these RNA samples can be seen in figure 5.6A. Once again the levels of SHH transcript were low in comparison to mechanically separated asparagus cells or wounded asparagus seedlings, but not to the same extent as that observed in the stem extract used in figures 5.5A and 5.5B. Although the transcript levels within tobacco stem sections do not vary to a large extent, a significant level of transcript abundance can be observed in this northern. For the two youngest plants the transcript abundance in the three stem sections is slightly higher within the most immature section [the section nearest the apical meristem], and the more immature the plant, the greater the transcript

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abundance overall.

A separate blot containing the same stem RNA extractions, probed with CAD can be observed in figure 5.6B. CAD transcript can be observed in every stem sample, as seen with SHH, but the levels of transcript detected are much lower. The only two samples in which CAD transcript abundance is elevated are the oldest stem sections from the two older tobacco plants, which are more lignified.

5.4.3 Temporal regulation of SHH transcript during tobacco bud maturation

Previous analysis had shown SHH transcript was present at higher levels within mature pollen than immature anthers RNA samples. This investigation of SHH transcript abundance within maturing tobacco buds/flowers was continued further using an RNA blot donated by Dr. M. Roberts (Leicester) following its use in other studies. The stage of development of the gametophyte can be predicted from the length of tobacco buds and/or anthers (Roberts *et al.*, 1993). This led to the use of the samples observed in figure 5.6C. It was of interest to analyse SHH transcript abundance within these contexts with respect to future experiments (see chapter 8). The abundance of SHH transcript can be observed to increase during bud maturation, with SHH transcript more abundant in anthers from larger rather than smaller buds. The relevance of the first lane will be discussed later.

5.5 Western analysis

When the SHH antibody was available to study SHH protein levels within tissues, an *Arabidopsis thaliana* SHH genomic clone (described in chapter 6) had been isolated. It was therefore known that both transgenic tobacco and *A. thaliana* plants were being produced to monitor reporter gene fusion constructs. Hence, the western analysis of endogenous SHH protein levels was performed on *A. thaliana* total protein extracts as well as tobacco, unlike the transcript analysis. These studies were undertaken to assess if post-transcriptional control of SHH expression occurred.

Figure 5.7 SHH protein and enzyme activity analysis from tobacco organs

Figure 5.7A Western blot of total protein isolated from equal fresh weight samples of tobacco organs, probed with anti-AtSHH antibody (peptide 3). Proteins were isolated from the following:

Lane 1	Stems
Lane 2	Roots
Lane 3	Leaves
Lane 4	Bud

The size of the detected protein is indicated at the side of the blot

Figure 5.78 SHH enzyme activity assays of protein isolated from tobacco organs. Proteins were assayed from buds, leaves, leaf midveins, and varying sections of stem tissue. Stem 1 represents protein extracted from a two week old whole tobacco plant stem, while stem 2-5 represents protein extracts from a single six week old tobacco plant stem, where stem 2 is the youngest portion of the stem and stem 5 is the oldest. (Points represent the mean and the vertical bars represent the standard deviation_{n-1} of data from three measurements.)



B

A



nmol/min/mg protein

5.5.1 SHH protein in tobacco organs

SHH protein was immunologically detected in all tobacco tissues analysed (see figure 5.7A) with the lowest levels occurring in mature roots. The four lanes of figure 5.7A represent SHH protein detected within stems, roots, leaves and buds of tobacco. The low level of protein detected in the root extract may reflect the mature nature of the roots utilised.

5.5.2 SHH protein in Arabidopsis thaliana organs

SHH protein was immunologically detected in all four *A. thaliana* tissues analysed (see figure 5.8A). However, the levels observed in each tissue were not identical, with buds, roots and stems containing significantly greater quantities of SHH protein than leaves. As found with tobacco samples, buds contain the greatest quantity of SHH protein.

5.6 SHH enzyme assays

Although both SHH transcript and protein levels have been analysed, further control mechanisms may operate to regulate SHH enzyme activity and therefore enzyme assays were performed.

5.6.1 SHH enzyme assays from tobacco tissues

The graphical representation of SHH enzyme activity assay results for tobacco tissues, shown in figure 5.7B, clearly shows enzyme activity to vary between tissues. The lowest activity was measured within tobacco leaf lamina tissue but if the midrib of such a leaf was analysed then an approximate 10 fold higher SHH activity is observed. As observed in the western analysis, SHH enzyme activities are greater in buds than in leaf tissue. The northern analysis of tobacco stem extracts suggested SHH transcript to be more abundant within younger portions of the stem and within the stems of younger plants. This is reflected in the enzyme assay results with the greatest activity in stems being observed in the one extract from two-week old tobacco stems. The other four extracts from the same six-week old tobacco plant, decrease in measured SHH enzyme activity as the stem matures. Figure 5.8 SHH protein and enzyme analysis from *Arabidopsis* organs

Figure 5.8A Western blot of total protein isolated from equal fresh weight samples of *Arabidopsis thaliana* organs, probed with anti-AtSHH antibody (peptide 3). Proteins were isolated from the following:

Lane 1	Buds
Lane 2	Leaves
Lane 3	Roots
Lane 4	Stems

The size of the detected protein is indicated at the side of the blot

Figure 5.8B SHH enzyme activity assays of protein isolated from *Arabidopsis thaliana* organs. Proteins were assayed from buds, leaves, stem meristem, roots and stems. (Points represent the mean and the vertical bars represent the standard deviation_{n-1} of data from three measurements.)



B

A



5.6.2 SHH enzyme assays from Arabidopsis thaliana tissues

The graphical representation of SHH enzyme activity assay results for *Arabidopsis* tissues, shown in figure 5.8B, clearly shows enzyme activity to vary between tissues. Each specified organ or tissue was dissected from 4 week old *A*. *thaliana* plants which were just starting to bolt. As previously observed in the tobacco western analysis, the *Arabidopsis* leaf tissue contains the lowest SHH enzyme activity. The levels of SHH protein within buds, roots and stems appeared quite similar upon western analysis, with buds appearing to contain the greatest SHH protein. However, the enzyme analysis suggests buds to contain slightly more enzyme activity than stems but less than root tissue.

5.7 Discussion

The use of synthetic peptides based on *Arabidopsis thaliana* SHH predicted amino acid sequence (see chapter 6) allowed the production of SHH specific antibodies. The serum from rabbit one which had been immunised with peptide three gave the best results and allows immunological detection of SHH in the three plant species tested.

The initial attempts to perform SHH enzyme assays at Leicester used tobacco tissue because of its size and availability. The use of this species may have caused the problems encountered in obtaining a positive assay result based on the method of Poulton and Butt (1976). Tobacco is known to contain many phenolics which are normally sequestered within specialised organelles, however upon grinding tissue to isolate protein samples, these phenolics are released (reviewed by Anderson, 1968). The phenolic compounds are oxidised upon their release and inhibit many enzyme activities. To try and circumvent this problem, Dr. R. Edwards (Durham) suggested the addition of PVPP, sodium metabisulphite and ascorbic acid to the extraction buffer, to try and prevent phenolic oxidation. These additions to the simplified assay method, communicated by Dr. R. Edwards, were used with success for all plant species.

Results presented in chapter 3 suggested that SHH transcript was upregulated by the mechanical separation of asparagus cells, and the wounding of etiolated asparagus seedlings. Data presented within this chapter confirm this induction, at both the protein and enzyme level. The SHH transcript within the asparagus cell population was upregulated within one day of cell separation and maintained for up to seven days. The protein, however, is upregulated within one day but continues accumulating to higher levels over the seven day period tested. This is reflected in the enzyme assay studies, with increasing SHH activity being detected over the five day period tested.

The northern analysis of SHH transcript presented in chapter 3, suggests a short temporal induction of SHH mRNA following wounding of etiolated asparagus seedlings, with a return to basal levels within 3 days post wounding. Although western data suggests that SHH protein levels remain constant throughout this wounding time course, the enzyme activity mirrors the transcript induction. SHH enzyme activity is increased within one day post wounding and then decreases towards the basal levels observed in etiolated seedlings. This suggests that the enzyme assay is a more sensitive method of assaying active enzyme accumulation than western analysis.

Quantitative comparisons of enzyme activity within the two experimental conditions tested, suggest that SHH activity is greater in wounded etiolated seedling experiments than in mechanically separated cells. This is contradictory to the northern analysis of SHH transcript presented in chapter 3. The comparative transcript levels within mechanically separated cells are much greater than those observed in etiolated seedlings. However, it has been noted that mechanically separated cells produce a large increase in total soluble protein content, unlike chopped seedlings (Darby *et al.*, 1996). The chopped seedlings are etiolated material and therefore do not contain the major proteins of light-grown material, namely the large and small subunits of Rubisco. Therefore although all data is consistent with the observed transcript abundance, the relative enzyme activities may not be directly comparable as they are expressed on a per protein basis.

This analysis of SHH protein levels and enzyme activity in asparagus has confirmed the wound inducible nature of SHH, with the possible roles for wound induced SHH activity having been already discussed in chapter 3. This study also suggests that SHH gene expression and enzyme activity is regulated mainly at the transcriptional level.

The northern analysis of tobacco organs confirms the greater abundance of SHH transcript in tobacco roots compared to leaves (Mitsui et al., 1993). However this study includes RNA from several other tobacco organs. This northern analysis (figure 5.5B) contained RNA from stem tissue. As this tissue sample produced a weak signal when hybridised to the SHH probe, the blot was reprobed with CAD (figure 5.5C), as a control probe for lignification. This also gave a lower than expected signal, so RNA was extracted from several stem samples. By comparing the SHH signals in any of the stem samples (figure 5.6A), to the stem sample on figure 5.5B, and using the common asparagus extracts as standards, the SHH transcript levels detected within the original stem RNA extraction can be observed to be much lower than all the other stem samples. This may reflect an incomplete RNA extraction from the initial stem source, with only RNA, present in the outer dermal layers, being extracted, rather than the RNA present in the lignifying/lignified tissue which is much more recalcitrant to grinding. Therefore the results observed within figure 5.6A are considered to be a true reflection of SHH transcript presence within tobacco stems.

The observation that SHH transcript is more abundant within younger portions of stems of varying aged plants, and that younger plants contain greater quantities of SHH transcript in their stems, correlates with SHH enzyme activity. However, CAD is predominantly transcribed in the older, more mature, portions of the stem of each plant. This pattern of transcript abundance, and observed SHH enzyme activity correlates to the process of lignification in tobacco stems. As reviewed in chapter 1 (see section 1.4.3.4), monolignol biosynthesis needs SAM-dependent OMT enzyme activity, hence requiring SHH enzyme activity to catabolise the inhibitory accumulation of SAH by-product. These OMT-mediated reactions occur earlier in monolignol biosynthetic pathways than CAD-mediated reactions, which reduce hydroxycinnamaldehydes to hydroxycinnamyl alcohols at the end of the pathway (reviewed by Whetten and Sederoff, 1995). However, it should be noted that CAD transcript has also been detected within cells not producing lignin, and therefore any hybridising CAD transcript may not only be present for monolignol biosynthesis (O'Malley et al., 1992). Despite this possibility, the

SHH and CAD transcript abundance within tobacco stems of varying age, correlates to the process of monolignol biosynthesis; SHH activity present prior to CAD, as found in the biosynthetic pathway.

Recently an alfalfa [Medicago sativa] SHH cDNA was isolated from a differential screen for stem abundant cDNAs (Abrahams et al., 1995). Although isolated due to its abundance in stems, this cDNA hybridised to transcripts in all other tissues tested, including leaves, roots and floral buds, as observed in the tobacco northern, presented in figure 5.5A or B. Northern analysis of alfalfa RNA isolated from individual stem internodes, indicated SHH transcript to be present equally in all samples, except for a decreased level in the very youngest stem tissue analysed. This data does not concur with the present analysis of tobacco stem tissue and may represent a species-dependent difference in the production of monolignol biosynthesis or other pathways occurring in stem tissue which require SHH enzyme activity. However, unlike this study, Abrahams et al. (1995) have not analysed SHH enzyme activity within these alfalfa stem sections to confirm its correlation to transcript levels. Therefore the transcript levels observed within alfalfa stems may remain constant, except in very young stems, but the translation from these transcripts may be regulated or alternatively the activity of the enzyme may be regulated.

This analysis of SHH transcript within alfalfa also concludes that SHH transcript decreases upon wounding of stems. This decrease can be observed within four hours of wounding, and within 24 hours post-wounding no SHH transcript was detected. This negative response to wounding is in direct disagreement with data presented from asparagus tissue. These apparently conflicting results may have arisen through the use of different plant species, experimental design or explant utilised. Apart from the obvious difference in species used, very different explants were analysed. In the case of alfalfa, stem explants were sliced into 2-5mm sections and then incubated in 0.01M phosphate buffer, pH 7.0, whereas etiolated asparagus seedlings were utilised in this study, which were similarly sliced but incubated over a longer time course in dH₂O in the dark. The relatively high transcript presence within alfalfa stems compared to other tissues, may provide the required SHH activity needed during the wound response, and therefore no transcript induction is

observed. The actual reduction in transcription may suggest the termination, upon wounding, of the processes previously requiring SHH activity within these stems.

The temporal abundance of SHH transcript within anther/pollen maturation suggests a greater transcript requirement within mature pollen rather than immature anthers, although both tissues contain relatively high levels of SHH transcript in comparison to tobacco leaves. This temporal regulation is also observed in figure 5.6C, in which greater SHH transcript is detected as buds mature, and older anthers contain more hybridising transcript than younger anthers. The first lane of this blot contains RNA from a transgenic tobacco line in which a tapetum-specific promoter has been used to express Bacillus amyloliquefaciens RNase [barnase] (Paul et al., 1992). Expression of this cytotoxic gene within any cell-type causes cell ablation, therefore by using a tapetum-specific promoter the tapetal cells are essentially all destroyed, resulting in male-sterile plants due to the lack of development of microsporogenous cells. The use of this sample in the northern analysis suggests that SHH expression may occur in the tapetal cells or the microsporogenous cells, due to the reduced signal observed in this extraction compared to similar wild-type extractions.

The western analysis of tobacco and *Arabidopsis thaliana* SHH protein confirmed that SHH protein could be detected in every plant tissue tested. As exemplified by the analysis of wounded, etiolated asparagus seedlings, the enzyme assay is a more sensitive tool to study gene expression and regulation than western analysis. However, simply because the transcript and enzyme activity follow identical profiles within the analysed asparagus samples, this does not exclude translational or enzyme activity control mechanisms within other species, tissues or environments.

Due to the absence of large and small subunits of Rubisco, the major proteins of light grown plant tissue, in root tissues the proportion of SHH protein relative to the total protein content of a root extract will be increased. To try and alleviate this potential problem, equal weight samples of tobacco and *Arabidopsis* organs were homogenised in the preparation of total protein extracts. Therefore the total protein loaded in each lane on these western blots was proportional on a fresh weight basis. However the enzyme assays are expressed on the basis of total protein extract and therefore the results observed for *Arabidopsis* roots may be non-comparable to other tissues.

A wheat SHH cDNA has been isolated from a root tip library and has only been studied within roots (Richards and Gardner, 1994). Aluminium is ' known to inhibit root growth due to the inhibition of cell division, under acidic soil conditions. Therefore SHH transcript levels were monitored in roots exposed to inhibitory concentrations of aluminium. The SHH transcript levels were found to decline within 2-4 days of treatment. Heat shock, also known to arrest the cell cycle, caused a rapid decrease in SHH transcript within root tips. It was concluded that the decrease in SHH transcript may reflect a decreased requirement for methylation due to decreases in the synthesis of DNA and/or lignin in the growth inhibited roots.

This summarised study does not compare SHH transcript levels in root tips to other wheat tissues, hence not allowing analogies to the comparisons observed in species utilised in this study. However the concluding remarks that SHH transcript abundance declines in cell cycle arrested cells, agrees with conclusions reached from this study. In areas of high metabolic activity such as mechanically separated cells, wounded tissue, meristems and developing anthers, SHH gene activity appears to be greater than in less metabolically active tissue, such as leaf lamina in which rapid cell division and differentiation is not occurring. Due to the varied metabolic pathways outlined in chapter 1 in which SAM-dependent OMT activities are required, and hence SHH activity is utilised, the precise requirements for SHH enzyme activity within each tissue tested cannot be surmised. However, within certain tissues such as stems, the majority of SHH expression can be expected to be needed for the process of lignification. In areas of rapid cell division such as the Arabidopsis stem meristem, SHH may be utilised to remove SAH by-product from DNA methylation.

By studying SHH at the transcript, protein and enzyme levels, it has been concluded that SHH gene expression is controlled mainly at the level of transcription. As SHH was detected in every plant tissue tested, this may suggest SHH is expressed in every tissue at a low housekeeping level, but this is increased upon specialised requirements. Therefore, to further study this transcriptional control, it would be advantageous to clone the promoter regions of an SHH gene and carry out promoter/reporter gene assays.

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Chapter 6

Isolation and sequence analysis of an *Arabidopsis thaliana* SHH genomic clone

6.1 Introduction

The northern analysis presented in the previous chapter provides data on both the transcriptional regulation of SHH within specific organs, and temporal patterns of expression within some of these organs, however it does not comprehensively cover all temporal and spatial SHH expression patterns. Reporter gene technology has provided a convenient method to assay the location of transcript expression.

To isolate potential promoter fragments two main approaches are utilised. Genomic libraries can be constructed and screened for clones hybridising to the sequence of interest. Alternatively inverse PCR has been used to try and isolate gene promoter fragments. This later approach was successfully utilised to isolate an asparagus promoter fragment for a cDNA previously isolated from asparagus separated cell libraries, after the more conventional technique of genomic library screening failed (Warner *et al.*, 1993). One consideration prior to the attempted isolation of genomic sequences, is the possibility of members of small SHIH gene families within species, which may allow specificity of expression to develop within particular family Figure 6.1 Genomic DNA blots hybridised to SHH probes

Figure 6.1A *Arabidopsis thaliana* genomic Southern blot probed with the *Arabidopsis* SHH PCR product amplified with MSPCR1 and MSPCR2 primers.

Lane 1	digested with Eco RI
Lane 2	digested with Sal I
Lane 3	digested with Xho I.

Figure 6.18 Asparagus officinalis genomic Southern blot probed with the AoSHH cDNA.

Lane 1	digested with Eco RI
Lane 2	digested with Hind III
Lane 3	digested with Hind III and Xba I.

Figure 6.1C *Nicotiana tabacum* genomic Southern blot probed with the AoSHH cDNA.

Lane 1	digested with Eco RI
Lane 2	digested with Hind III
Lane 3	digested with Hind III and Xba I.

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B

1 2 3

1 2 3

_ 5.1 _ 4.1

_ 3.1

2.0

_ 1.6

_ 1.0

_ 0.5



members, as found in the phenylalanine gene family of *Phaseolus vulgaris* L. (Shufflebottom *et al.*, 1993). Therefore if a plant species was found to contain more than one SHH gene, the promoter fragment from a member of this putative small gene family may not drive representative reporter gene expression in all tissues where SHH gene expression is observed. Due to this consideration genomic Southern blots were performed on DNA from three model species, prior to the attempted isolation of an SHH promoter.

6.2 Genomic Southern analysis

Southern blot analysis was performed using genomic DNA from *A*. *thaliana*, *A*. *officinalis*, and *N*. *tabacum* as can be seen in figure 6.1. The large number of hybridising bands observed in some *A*. *officinalis* and *N*. *tabacum* DNA restriction digests, suggest that SHH is likely to be a member of a small gene family in these two species. However in *A*. *thaliana* the presence of a simple array of bands suggests that SHH is present as a single copy gene, and therefore this gene should be expressed in all tissues demonstrating SHH enzyme activity. This finding prompted the attempted isolation of the putative single *A*. *thaliana* gene.

6.3 Genomic library screening

An existing *Arabidopsis thaliana* var. Landsberg *erecta* genomic library constructed using λ DASH II (Stratagene) by Dr. M Roberts (Roberts *et al.*, 1993) was available within the laboratory. Prior to screening this library, the presence of SHH within the library was tested by using MSPCR1 and MSPCR2 to PCR a fragment of SHH gene from an aliquot of the library. As this experiment produced a positive result, the library was screened using the cloned *Arabidopsis* MSPCR1/MSPCR2 primed amplification product, as a probe. Approximately 80,000 plaques were plated from the freshly titred library for the primary screen. The resultant plaques were lifted in duplicate and probed with the described radio-labelled SHH DNA fragment. Each of the putative hybridising regions were cored from the plate, eluted in SM and titred for a second round of screening. Following a further two rounds of screening, to

Figure 6.2 Restriction enzyme map of the AtSHH λ clone

Map of the portion of the genomic clone hybridising to probe fragments. The specific probes utilised to map this clone are shown above the restriction enzyme map, and include the four asparagus DB6 cDNA subclones (shown in green), as well as the *Arabidopsis* MSPCR1/MSPCR2 amplification product (shown in orange). The final probe fragment utilised was an 1845bp *Xho* I fragment of the genomic clone itself. This was shown by previous probes to include a small portion of the SHH coding region, but to mainly contain DNA sequences 5' to the translational start site.

The black arrows symbolise the remainder of the lambda clone's insert of approximately 14kb, which was not mapped.



Figure 6.3 Subclones of the AtSHH λ clone

Simplified restriction enzyme map of the mapped region of the genomic clone. Below the restriction map are each of the subclones used to derive the sequence of AoSHH. Those subclones drawn in green were derived from the lambda clone directly, while the clones in blue are subclones of the previously cloned lambda insert fragments. The *Eco* RV/Xho I fragment at the bottom of the figure was utilised as the probe in S1-mapping of the transcriptional start site. Particular note should be made to the presence of several well spaced restriction enzyme sites, present within the potential 1845bp *Xho* I promoter fragment, which would provide a deletion series of promoter fragments.

The black arrows symbolise the remainder of the lambda clone's insert.



achieve plaque purity, one positively hybridising clone was obtained.

6.4 Mapping and subcloning of the SHH hybridising Arabidopsis λ clone

DNA was isolated from the single putative Arabidopsis SHH λ clone as described in section 2.9.7, and restriction enzyme analysis was performed. This showed the λ clone to contain an insert of greater than 20kb, which is within the upper limit of the λ DASH II vector (9-23kb). The restriction enzyme analysis revealed several common restriction enzyme sites to be present within the clone, and therefore generated numerous DNA fragments. Some of these fragments were mapped, using the *Arabidopsis* probe used to isolate the clone, and heterologous asparagus cDNA fragment probes, on Southern blots of these restriction enzyme digests. The resultant map of a portion of the λ clone and the probes used in it's derivation can be seen in figure 6.2. This map suggested most of the coding region of the gene was present within a 4kb Sal I fragment of the genomic clone. Within this fragment a 1845bp Xho I fragment contained a small portion of coding region at its 3' end while the majority of this fragment potentially contained the 5' upstream region controlling the transcription of the gene. These two fragments of the lambda insert were therefore initially subcloned into pBluescript[™]SK- [Stratagene]. Terminal sequencing of these fragments verified the presence of an SHH coding region. To determine the sequence of the 3.5kb span of DNA of interest, it was extensively subcloned, both from the existing subclones and from the lambda insert, as represented in figure 6.3. The map of the lambda insert suggested the presence of a single intron within the SHH gene coding region.

6.5 Sequence analysis of the lambda clone

The final contiguous sequence of all the subclones, encoded a protein of 485 amino acids and confirmed the presence of a single suspected intron within the coding region. The intron is 281bp long, which is slightly larger than the average size of 219bp for a dicot intron (Simpson *et al.*, 1992), and was found between amino acids 237 and 238. Neither of these amino acids' coding regions

Figure 6.4 Sequence of the promoter region of AtSHH

The nucleotide sequence of the 1845bp *Xho* I subclone of the *Arabidopsis thaliana S*-Adenosyl-L-Homocysteine Hydrolase (AtSHH) genomic clone. As mapping studies suggested, this fragment contains the first 30 amino acids of the coding sequence, which are shown below the nucleotide sequence. The untranslated portion of this subclone contains the putative promoter sequence. The sequence is numbered from the initial ATG, the adenosine residue being +1. The probable transcriptional start site at -60 is shown in **boldtype**. The putative TATA box is underlined at -97 to -102. Several nucleotide direct repeat sequences are also underlined.

-1759	CICGAGIGITIGACCITITICIGGTCGATIGAATAGAATCGAATGTCITAATCCAGTACCCT	-1700
-1699	${\tt CCAGCTTTTATTTCGTGTAATTTATTTTCCAAACCTACCACTACCAGTTTCATAACTCTC}$	-1640
-1639	GAATAAATTTATCAAATAGTCTTTTGAGTGCTCAAAGTCTTGGGATAATAAATGGTCAGT	-1580
-1579	GCTATGTATCACCCGGATGTGAAACATTATGGGTGGAGATAGACTATTATAAATTTATTG	-1520
-1519	AAATATACGATTGTTACTCGTTTAATAGCAAAAGTAGTACAATGTATATAGTTTCTATCG	-1460
-1459	agaacaagatct <u>atttaaaatto</u> gaaaagtac <u>atttaaaattc</u> ataaacatataaagata	-1400
-1399	GTAACATGTTAGATCTGCATAGTACCACCAAAACAAGAAAAAGAAACGCACATCGCCAC	-1340
-1339	ATAATTGCTATGATTCTCACTGTCGGCTGCTTTGAAATATTCGATTCTTTTGGTAAATCA	-1280
-1279	$CACAACA \underline{TAATATAATTA} CAATAAATATATATATATATAAAGTATAAAT\underline{\mathsf{TAATATAATTA} AT$	-1220
-1219	ACCACATTGTTTAATTCTGTTTTGATCTTTTTAAGATCAGTCAG	-1160
-1159	CACGCGCAGGTCCAGATCCAAACAGCACACACACACACAC	-1100
-1099	TTGGTGGCTATTGCACTATTGATACTCTTTCTTCAAAAAACAAGTTATTGTTTT	-1040
-1039	TATTTTCAACCCAACTTTAATACGGATTCATACTGGG <u>ATTTAGGT</u> GTTAAATCTGATA <u>AT</u>	-980
-979	TTAGGTITGAATAAGTTGTATATTTGFTTCFTTGATTAAAAAAAGAACCTATATATATAC	-920
-919	$\texttt{AAAA} \underline{\texttt{ATAAATAAAAAGTTCT}} \texttt{AGATTTCAATTTTCCGTATATAGCGGGTTGAATTGTCTAT}$	-860
-859	TTTAATATGAAAATTGXCGGATCIT <u>ATAAACAAAATGTTCTGAAATATG</u> TAAAAGGATIT	-800
-799	AGCCAAAGTTAACCAAAAAAAAAAAAAAAAAAAAAAAAA	-740
-739	$GATCTAAGGCATTAATTTA\underline{GAAATATGT}CGTTACAATAAGCGGAGAACATGGGACGTTTC$	-680
-679	TCGTGGTCCAATCAGACGAACGAGATCTCATAAATTAAATGACTTCAGXCGAGGGAATTC	-620
-619	ATGGCAGAATGATAATGCAACTTAAGTGACTTTAGAGTGAAAATGATACGAGAACAATGC	-560
-559	ATAATCCATATGACCGTTGAGTGAGTGATACCATTAGCGCGATACAAGCGGGACTATAAA	-500
-499	CTGATCTAGATTGTTTTTCTTGGGAAAAAATGTTACAAATTTTAAATATGTAGTTTGAAT	-440
-439	TGTTAAACCAAGATTCAACAGAAATATACCGTAAATAAACAACAGTTGATAATAGTCATC	-380
-379	GAAAAGATATCAACTGATTCTTCACTTGGGCTACTGTGACGGCCCGTTAGGTTCTCAATA	-320
-319	TAAGTCAATAACTACGATCTACGATTCACTGAAACAAATAAAACACAGCCACGTGTCCAC	-260
-259	CCTCCCACATCACCGTCCGATCTAACCCACGACAAGCTTACAACACGGGTCATACCGGCT	-200
-199	CGTGCAGCGTGTTCCGTCATCCACG3GATTACAACTTCTACCAGATCCACCAAACCCTCA	-140
-139	AAACAATCTGAACCGTTCATTTCATTTTGACCTCATC <u>TATATA</u> TTCTCTGTCACTCCCCT	-80
-79	TTCTCTTCTCCCCGCACACACTTCTCTCTCTCTCTCTCTC	-20
-19 1	TCTCAGATCTAGCTCAACCATGGCGTTGCTCGTCGAGAAGACCTCAAGTGGCCGTGAATA M A L L V E K T S S G R E Y	41 14
42 15	CAAGGTCAAAGACATGTCTCAAGCCGATTTCGGTCGTCTCGAACTCGAG K V K D M S Q A D F G R L E L E	90 30

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Figure 6.5 Sequence of the coding region of AtSHH

Nucleotide and deduced amino acid sequence of the *Arabidopsis thaliana S*-Adenosyl-L-Homocysteine Hydrolase (AtSHH) genomic clone coding region. Translated nucleotides are shown in capital letters. The sequence is numbered as in figure 6.4. The predicted amino acid sequence is shown below the nucleotide sequence, with intron acceptor and donor junctions underlined. The transcriptional start site at -60 is shown in **boldtype**. The termination codon is indicated by an asterisk.

-79	ttc	tct	tct	cct	cgc	aca	cac	ttc	tct	ctc	tct	ctc	tct	ctg	cct	cct	ttc	gga	ttc	aaa	-20
-19 1	tct	cag	atc	tage	ctc	aac	CAT M	GGC A	STT L	L	v	CGA E	GAA K	GAO T	CTCI S	AAG S	G G	R	E	ATA Y	41 14
42 15	CAA K	GT V	CAA K	AGA D	CAT M	GTC S	TCA Q	AGO A	CGA D	FTT F	CGG G	TCG R	ICT L	CGA E	ACTV L	CGA E	CT L	CGO A	CGA E	AGT V	101 34
102 35	TGA E	GAT M	эсс Р	rcc G	ACT L	TAD M	GGC A	TTG C	rcc R	TAO T	CGA. B	F	coa G	ACC P	ric s	ICA Q	990C. A	F	CAA K	AGG G	161 54
162 55	CQC" A	rag. R	AAT I	CAO T	CGG G	ATC S	TCT L	TCA H	CAT M	GAO T	CAT I	Q Q	AAO T	cgo A	CGT/ V	ACT L	CAT I	CGA E	AAC T	сст L	221 74
222 75	AAC T	TGC A	TCT L	CGG G	TGC A	TGA B	AGT V	CAG R	ATG W	c c	rto s	CTO C	CAA N	CAT	CTT F	rrc s	CAC T	TCA. Q	AGA D	сса Н	281 94
282 95	COC A	000 A	A A	AGO A	CAT I	CGC A	TCG R	TGA D	сто s	000 A	CGC A	TQC A	TTT F	CGO A	CTG W	GAA K	AGG G	TGA/ E	GAC" T	TCT L	341 114
342 115	TCA Q	GGA B	GTA Y	CTO W	GTG W	crc C	TAC T	CGA B	GCG R	TGC A	TCT L	AGA D	TTG W	GGG G	TCC. P	AQG G	TGG G	TGG G	TCC P	TGA D	401 134
402 135	TCT L	GAT I	TGT V	TGA D	TGA D	TGG G	TGG G	TGA D	coc A	TAC T	TCT L	TTT L	GAT	TCA H	TGA E	606 G	TGT V	TAA K	AGC A	TGA E	461 154
462 155	GGA E	GAT	CTT F	TGA B	GAA K	GAC T	TGG G	TCA Q	AGT V	TCC P	TGA D	TCC P	TAC T	TTC S	TAC T	TGA D	TAA N	CCC P	TGA E	GTT F	521 174
522 175	TCA Q	GAT I	ogr v	GTT L	GTC S	TAT I	TAT I	CAA K	GGA E	AGG G	TCT L	TCA Q	AGT V	TGA D	TCC P	TAA K	GAA K	GTA Y	CCA H	CAA K	581 194
582 1 9 5	GAT M	GAA K	oga E	GAG R	ACT L	TGT V	TOG G	TGT V	cīc s	TGA E	oga B	AAC T	TAC T	CAC T	TOG G	ror V	TAA K	GAG R	GCT L	TTA Y	641 214
642 215	CCA Q	GAT M	QCA Q	GCA Q	AAA N	TGG G	AAC T	TCT L	TTT L	GTT F	CCC P	TGC A	CAT I	taa N	CGT V	TAA N	CGA D	CTC S	TGT V	CAC T	701 234
702 235	CAA K	GAG S	CAA K	Gat	att	gat	ctt	cag	att	gtt	ctt	act	agt	gat	gat	aat	ctg	cta	ggt	ctt	761 237
762	agc	tct	gta	gtt	ttg	atg	cga	tag	gct	taa	caa	caa	cat	aat	cac	taa	gta	gaa	gca	agc	821
822	ttt	tca	ttg	ttg	gtt	gtt	gaa	agt	att	acg	ato	tag	tag	aga	aaa	gtc	ctt	cct	tgg	tta	881
882	cag	aat	ttg	cat	gaa	aag	att	ata	ttt	ctc	atg	ctt	tet	ttg	att	tta	tat	tca	acg	ttt	941
238	. ge	ceu	ouc		.cg t	gee	900	ucc	gut	auu		ucc	.cgc		cyu		ace	F	D	N	240
1002 241	CTT L	GTA Y	G	s s	G	TCA H	S	L	P	TGA D	G	L	M	GAG R	A R	CAC T	TGA D	V	M	GAT I	1061 260
1062 261	COC A	TOG G	K K	GGT V	TCC A	v V	TAT I	стс с	G G	АТА Ү	G G	TGA D	v V	G G	AAA K	.000 G	C C	A N	TQC A	TGC A	1121 280
1122 281	CAT M	GAA K	GAC T	TGC A	G G	TGC A	TAC R	AGI V	CAT I	TGI V	GAC T	TGA E	GAT I	TGA D	лос Р	TAC I	CIC C	TGC A	L	TCA Q	1181 300
1182 301	AQC A	TTT L	gat M	GGA E	AQC G	ACI L	TC Q	UGGT V	TCI L	TAC T	CCI L	TGA E	D D	V V	TGT V	s	AGA B	AGC A	TGA D	TAT I	1241 320
1242 321	CTT F	TGI V	CAC T	CAC T	сас т	G	TAF N	K K	AGA D	I I	CA1 I	CA1 M	V V	D	ICCA H	CA'I M	R	GЛА K	GAT	'GAA K	1301 340
1302 341	GAC T	CAA N	.0000 P	TAT I	v	oro s	T T	CAT I	TOC G	лса н	CTI F	TG D	N N	TGA E	GAT I	TGA D	M	GCC P	TOG G	L	1361 360
1362 361	TGA E	GAC T	Y Y	P	тос G	NGI V	GA/ K	R	TAT I	CAC T	CA'I I	CAA K	AGCC P	Q Q	GAC T	TG4 D	R	GTG W	V V	GTT F	1421 380
1422 381	CCC P	E E	GAC T	САА К	A000	G G	I AA	IAC	TGI V	L	NGGC A	TG E	1000 G	FICC R	JICI L	'GA'I M	'GAA N	L	G	C C	1481 400
1482 401	тсс Р	CAC T	G	лса Н	P P	S S	F	v V	GAT M	STC S	C C	s S	F	T T	CAA N	Q Q	v v	GAT I	TGC A	Q Q	1541 420
1542 421	GCI L	CG4 E	L L	CTC W	KGAZ N	E	GA/ K	AQC A	S S	G	AA/ K	GTA Y	E	AGA/ K	IGAA K	v V	GT/ Y	V V	TCI L	P P	1601 440
1602 441	CAA K	юсл н	L	D D	E	GA/ K	v	TQC A	L	L	GCA H	CTI L	G G	сал К	GCI L	TQC G	ago A	CAG R	E L	TAC T	1661 460
1662 461	AAA K	IGCI L	GTC S	аал К	D	000# Q	ATC S	D	CT7 Y	v V	CAC S	CAT I	TCC P	I AAC	TGA E	1600 G	ACC P	АТА Y	K K	AGCC P	1721 480
1722 481	TCC P	тся н	CT/	CAC R	GT/	ACTO	GAG	GAC	AG	GAC	AG	rcc r	C								1758 485

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were split by the presence of the intron (See figure 6.5). The predicted amino acid sequence of the coding region was found to be 89.7% identical and 94.4% similar to the predicted amino acid sequence of the AoSHH cDNA. Therefore the sequenced portion of the genomic clone was called *Arabidopsis thaliana S*-Adenosyl-L-Homocysteine Hydrolase (AtSHH).

Within the intron neither a conserved branch point sequence nor polypyrimidine tract, similar to those found in yeast or vertebrate introns, were found, agreeing with Goodall and Filipowicz (1989) who observed these elements are not essential for plant intron splicing. All previously cloned dicot introns contain a minimum 60% AU content and this intron agrees with these findings at 67.7%. Plant introns on average are 15% more AU rich than exons, with dicot introns on average 71% AU rich, and exons only 55% AU rich. In the case of the Arabidopsis SHH gene intron, these values are 67.7% and 50.7% respectively. Conserved sequences for 5' and 3' splice sites have been proposed (Simpson et al., 1992; Goodall and Filipowicz, 1991) for plants and similar sequences are observed at the AtSHH introns boundaries. As the percentage mismatch at each position of the splice site junction consensus sequences is higher in plants than either yeast or vertebrates, with only 5.2% of plant introns having an exact match to the 5' consensus (Luehrsen et al., 1994), an exact consensus sequence at the splice sites of the AtSHH intron was not expected or observed.

As predicted by the derived map, the 1845bp *Xho* I fragment contained the coding region for the first 30 amino acids of the protein. However this was present at the 3' end of this subclone, which therefore represented a putative promoter. Within this putative promoter sequence several features of previous promoters were observed. Notably the first ATG sequence present after the transcriptional start site is the translational start site. Also a putative TATA box can be observed on figure 6.4, as well as several direct repeats within the promoter sequence. No other long open reading frames were detected within this subclone and the only significant homology (using January 1996 release of EMBL/Gen database) to the nucleotide sequence was to a human enhancer element (Mautner *et al.*, 1995) with 70.6% identity in a 68bp region (numbered -29 to -96 on figure 6.4).

Figure 6.6 S1-transcript mapping

S1-transcript mapping used to identify the probable transcriptional start site of the AtSHH gene. Both lanes 1 and 2 were hybridised to the same RNA population but only lane 2 was treated with S1-nuclease. Post S1-nuclease digestion the products were separated on a denaturing gel with a sequencing ladder run along side as a molecular mass marker (lanes G, A, T, and C). The major protected fragment corresponds to 149bp.



149bp product

6.6 Transcript mapping of AtSHH

Transcript mapping can be utilised to determine the boundaries between transcribed and non-transcribed regions of genes, and also to map intron/exon boundaries. In this experiment it is used for the former purpose. The method relies on the principle that DNA/RNA hybrids are protected from digestion by exonucleases specific for single stranded nucleic acids.

Using a subclone of AtSHH, a probe was prepared from an *Eco* RV/*Xho* I fragment, which can be seen in figure 6.3. This pBluescriptTM subclone was digested with *Xho* I, to linearise the plasmid. Following dephosphorylation of the 5' termini, *Eco* RV cleavage produced a 460bp fragment. This fragment was purified and end labelled using T4 polynucleotide kinase. Therefore a probe with the antisense strand labelled at the 5' end was generated.

Previous data presented in chapter 5 had shown SHH transcript and protein to be present in every plant organ tested. Therefore the RNA used to protect the DNA probe fragment, could be isolated from any tissue, so *Arabidopsis* bud total RNA (donated by Dr. Roberts), isolated from a large size range of buds, was hybridised to the probe at 50°C and subsequently treated with S1-nuclease. The products were run along side sequencing ladder on a 6% polyacrylamide sequencing gel.

The autoradiograph showed one major protected fragment which was 149bp in length (figure 6.6), among a small number of protected fragments.

6.7 Discussion

The genomic Southern analysis of hybridising SHH gene fragments within both tobacco and asparagus, suggested small SHH gene families to exist within these species. The probable occurrence of more than one SHH gene was also reported within parsley (Kawalleck *et al.*, 1992). Further studies published during the course of this work confirm similar results within *Nicotiana sylvestris* and alfalfa (Mitsui *et al.*, 1993; Abrahams *et al.*, 1995). The Southern blot of *N. sylvestris* is not directly comparable to the Southern analysis described in this chapter as *N. tabacum* was utilised in this study as well as different restriction enzymes. *N. sylvestris* is the female progenitor of *N*.

tabacum and therefore some family members within N. tabacum may have arisen from this parent line. The isolation of two separate cDNAs, showing strong identity [\approx 80%] at the nucleotide level, confirms the presence of small SHH gene families within N. sylvestris, and more than one gene family member is transcribed (Mitsui *et al.*, 1993). Within rat, a single genomic clone has been isolated but at least seven pseudogenes have also been identified which confuses Southern analysis (Merta *et al.*, 1995). The possible occurrence of this situation within plant species can not be ignored, with a single gene being transcribed while the other detected gene fragments on Southern analysis may represent non-transcribed pseudogenes. The relatively simple array of hybridising bands observed in *Arabidopsis thaliana* genomic DNA, and this species' small genome size, led to its utilisation in the isolation of an SHH genomic clone.

A single clone was isolated from an *A. thaliana* genomic library, which, upon mapping, subcloning and sequencing, was found to contain an SHH gene. The restriction enzyme map of the genomic clone does not correlate to the genomic Southern due to the use of two different ecotypes for these two experiments. The approximate 1750bp region of the clone encoding the SHH amino acid sequence contains one intron. Regions 5' of the coding region have also been subcloned and putatively contain the promoter regions controlling the transcriptional expression of this gene. These potential promoter fragments were used in further experiments discussed in the next section of work.

The transcript mapping experiment defined the likely position of the transcriptional start site. However, despite the protection of a 149bp DNA fragment by hybridisation to SHH mRNA, the presence of an intron within the 5' untranslated region of the transcript cannot be discounted.

The AtSHH translational start site is the first ATG sequence present after the transcriptional start site. This agrees with Kozak (1987), who proposed the 40s ribosomal subunit binds at the 5' capped end of mRNA and migrates 3' until an AUG is found. Therefore the first AUG is utilised in most cases. The flanking sequences of the first ATG are also important because if they are suboptimum, this first AUG may be bypassed and a later AUG utilised. Kozak's study was based on diverse eukaryotic genes. A study by Joshi (1987) used only plant genes and found that the 'first AUG rule' was consistent in 92% of the 79 plant gene sequences tested. This study postulated a plant consensus sequence region around the AUG of UAAACA<u>AUG</u>GCU, and AtSHH has similar sequence around the utilised AUG (UCAACC<u>AUG</u>GCG). This similarity to the optimum consensus sequence around the first AtSHH AUG probably aids in the use of this site as the translational start site.

An adenine residue is utilised in 85% of plant transcriptional start sites, with pyrimidine bases flanking (Joshi, 1987). This is observed in AtSHH where the S1-mapping studies placed the transcriptional start site at an adenine residue (numbered -60 on figure 6.4), with large (\approx 20 nucleotides) pyrimidine tracts at either side. Leader sequences vary from 9-193 nucleotides with 53% of plant genes having 40-80 nucleotide leader sequences (Joshi, 1987). Most leader sequences (90%) are AU rich with 21% being in the 51-60% AU rich category (Joshi, 1987). Both these observations are also seen in AtSHH.

A TATA site is found in 84% of plant genes tested and a consensus sequence of TCAC<u>TATATATAG</u> has been postulated to occur 37±7 nucleotides upstream of the transcript initiation site (Joshi, 1987). In AtSHH a sequence similar to the consensus is 36bp upstream of the transcriptional start site.

The only significant nucleotide sequence homology found within current databases to the putative AtSHH promoter sequence, is to a human enhancer element region. However this homology is found in the DNA sequence between the TATA box and the translational start site, including the transcriptional start site. This region is comprised of large pyrimidine tracts to which all the identity was observed. The actual portion of human DNA showing homology is not an experimentally proven enhancer but is within a span of DNA in which proven enhancers are found (Mautner *et al.*, 1995).

As previously mentioned one other higher eukaryotic genomic SHH clone has been isolated from rat (Merta *et al.*, 1995). This clone is comprised of 10 exons which are dispersed over 15kb. Two of the introns found within this gene are in exactly the same position as the two introns found in the lower eukaryote nematode *C. elegans* SHH gene. However this conserved intron position is not observed in AtSHH. The intron within AtSHH is found only two codons from the position of an intron within the rat gene.

This isolation of an *Arabidopsis thaliana* SHH genomic clone, allows the utilisation of potential promoter regions within reporter gene constructs, leading to an increased knowledge of SHH transcriptional control, as discussed in the following chapter.

Chapter 7

Construction of AtSHH reporter gene constructs, and their analysis in transgenic *Nicotiana tabacum* and *Arabidopsis thaliana*

7.1 Introduction

Following the isolation of an AtSHH genomic clone, a putative promoter fragment was available to be tested using reporter genes in fusion experiments. This technology assays the location of normal promoter activity by simple histochemical staining of tissue or by quantitative enzyme activity measurements. The *E. coli* &-glucuronidase gene (*gus*; Jefferson *et al.*, 1986) and the firefly luciferase gene (Ow *et al.*, 1986) were chosen as reporter genes as both of these encode enzymes, that are easily assayed (Draper *et al.*, 1988; Ow *et al.*, 1986). The *gus* reporter gene has the added advantage of acting on histochemical substrates which provide a visual marker for promoter driven *gus* gene expression. These AtSHH promoter/reporter gene fusions were designed to provide data regarding the temporal and spatial gene expression of SHH. Tobacco was chosen as the species to be initially transformed as the process of *Agrobacterium* mediated tobacco transformation is simple, repeatable and generates transgenic plants over a relatively short time period Figure 7.1 Assembly of an AtSHH-luciferase construct

The previously constructed vector pKS AoPR1 Full LUC (Warner *et al.*, 1994) was utilised to create pKS AtSHH LUC, an intermediate vector containing an AtSHH promoter next to the luciferase reporter gene. This portion of the intermediate clone was ligated into suitably digested pBI101 AoPR1 LUC (Warner *et al.*, 1994) to create the final binary construct pBI101 AtSHH LUC.



(Draper *et al.*, 1988). However, since an *Arabidopsis thaliana* promoter fragment was being studied, the longer process of *A. thaliana* transformation was also performed (Clarke *et al.*, 1992), to ensure that the observed transcriptional activity within the foreign host species, tobacco, reflected that of the endogenous host. Tobacco is a much larger species than *A. thaliana*, being advantageous for simple dissection of plant organs, such as flower parts, for analysis.

When the reporter gene constructs were designed, the sequence of the entire mapped genomic region (see chapter 6) was unknown. However, mapping and initial sequencing data had demonstrated that a 1845bp Xho I fragment contained the coding region for the first 30 amino acids of the AtSHH protein, at it's 3' terminus. This fragment of the genomic clone was considered an excellent candidate to contain the required promoter elements to direct transcription of the reporter genes. Sequence analysis of this 1845bp Xho I fragment showed that an Nco I site was present at the ATG initiating codon. This allowed fusions of the AtSHH putative promoter to reporter genes with Nco I sites at their ATG translational start site. This forms an exact translational fusion where the initiating methionine is encoded by both halves of the fusion and therefore is referred to as a transcriptional fusion in this work, to differentiate this fusion and another fusion containing the first 30 amino acids of the AtSHH coding sequence. As the transcriptional start site had not been mapped at the onset of the construction of the reporter gene plasmids, the use of this fragment avoided the potential problem of missing the transcription start site from the constructs. Therefore all constructs were either translational fusions, or transcriptional fusions in which the initial methionine codon was the site of fusion. If the first 30 amino acids of the translational fusion encoded a signal peptide sequence, then the fusion protein may be exported, leading to glycosylation of the GUS protein and formation of functionally inactive enzyme (Iturriaga et al., 1989). This was not considered a problem as no predicted regions within the 30 amino acids displayed predicted hydrophobic domains characteristic of signal peptides (von Heijne, 1985) and other SHH proteins are found in the cytosol.

Expression data relating to the cloned parsley SHH cDNA (Kawalleck et

Figure 7.2 Assembly of a transcriptional AtSHH-gus construct

The previously constructed vector pSK PR1a-GUS (donated by S. Firek, at Leicester) was utilised to create pSK AtSHH-GUS, an intermediate vector containing an AtSHH promoter next to the *gus* reporter gene. This portion of the intermediate clone was ligated into suitably digested pBin19 (Bevan, 1984) to create the final binary construct pBin19 AtSHH-GUS.

In this construct the fusion between the AtSHH promoter and the *gus* gene occurs at the translational start site.



al., 1992), and the northern, western and enzyme assay studies presented in chapter 5, suggest SHH to be expressed in every tissue tested, with an increase in this expression occurring upon wounding. Therefore these hypotheses were tested using the transgenic plants obtained in this study.

7.2 Reporter gene constructs

Two separate reporter genes were utilised to ensure that any results obtained were not artifacts due to the use of a specific reporter gene. To allow full comparisons to be made between *gus* and luciferase gene expression, the same promoter fusion was required between the two reporter genes. Therefore, the fortuitous presence of a *Nco* I site at the translational start codon within AtSHH was utilised in fusions to both reporter genes.

7.2.1 Construction of an AtSHH promoter-luciferase gene fusion

Previously constructed plasmids were utilised in the construction of an AtSHH promoter driven luciferase reporter gene construct. An intermediate vector, pKS AoPR1 Full LUC (Warner *et al.*, 1994; see figure 7.1) was digested with *Nco* I and *Xho* I to remove the AoPR1 promoter from the rest of the vector. Using these sites the 1760bp AtSHH promoter fragment was isolated from the appropriate λ subclone and ligated into the plasmid to create pKS AtSHH-LUC, a cloning intermediate.

The binary vector pBI101 AoPR1-LUC (Warner *et al.*, 1994) was digested with *Bam* HI and *Sal* I removing the AoPRI-LUC cassette, and the *Xho* I/*Bam* HI digested AtSHH-LUC cassette from pKS AtSHH-LUC was ligated into the plasmid to create pBI101 AtSHH-LUC.

7.2.2 Construction of AtSHH promoter-ß-glucuronidase gene (*gus*) fusions 7.2.2.1 A transcriptional AtSHH promoter-*gus* fusion

An SHH promoter-reporter cassette was constructed utilising the *gus* reporter gene in place of the luciferase reporter gene using an identical AtSHH promoter fragment, to facilitate direct comparisons between the two reporter genes.

Figure 7.3 Assembly of a translational AtSHH-gus construct

An *Xho* I fragment containing the AtSHH promoter was ligated into the commercially available vector pBI101.1 to create the two possible constructs shown.

In this construct a translational fusion occurs, with the first 30 amino acids encoded by the AtSHH gene being translated before the gus gene.



Initially the plasmid, pSK PR1a-GUS (donated by Dr. S. Firek, at Leicester; see figure 7.2) was digested with *Nco* I/*Xho* I to release the PR1a promoter, leaving a *gus* gene containing an *Nco* I site at its initiating methionine codon. The 1760bp AtSHH promoter was similarly digested and ligated into the vector to create pSK AtSHH-GUS. The *Xho* I/*Bam* HI fragment of this plasmid was then cloned into the *Bam* HI/*Sal* I sites of pBin19 (Bevan, 1984) to create a binary plasmid pBin19 AtSHH-GUS.

7.2.2.2 A translational AtSHH promoter-gus fusion

A simple one step cloning strategy allowed a further gus gene fusion to be made using pBI101 (Jefferson *et al.*, 1987; see figure 7.3). pBI101 is a commercial binary vector (Clontech), which is a derivative of pBin19 containing the gus reporter gene downstream from a cloning site, followed by a NOS terminator region, to direct efficient polyadenylation of the gus message. Upstream from this region is an *nptII* gene cassette that allows selection of transformed plants on media containing kanamycin. These sequences all lie in the Ti plasmid derived left and right borders, that both assist and define the region of DNA transferred into the plant's genome.

Sequence data predicted that a fusion with pBI101.1 would generate an active translational fusion between the AtSHH promoter and the *gus* gene with the first 30 amino acids of the translational fusion encoded by the SHH gene. This construct was made by ligating the 1845bp *Xho* I fragment of the SHH promoter into the *Sal* I site of pBI101.1. The resultant clone was named pBI101 AtSHH Correct. The SHH promoter fragment also ligated into pBI101.1 in the opposite orientation, within the same ligation (i.e. in the anti-sense orientation), creating pBI101 AtSHH Wrong. This construct was used as a control in expression studies.

7.3 Initial transformation data

Each of the four described constructs' structures were confirmed by Southern hybridisation experiments, and predictably sized restriction enzyme fragments were found to hybridise to labelled AtSHH 1845bp *Xho* I probe.

The plasmids were introduced into LBA4404 Agrobacterium

Figure 7.4Genomic Southern analysis of Agrobacterium
harbouring the four AtSHH-reporter gene constructs

Southern analysis of transformed *Agrobacterium tumefaciens* LBA4404, harbouring the four named constructs, hybridised with the 1845bp *Xho* I putative AtSHH promoter probe. Lanes 1-4 are digested with *Eco* RI and lanes 5-8 are digested with *Hind* III.

Lanes 1 and 5	pBI101 AtSHH correct
Lanes 2 and 6	pBI101 AtSHH wrong
Lanes 3 and 7	pBin19 AtSHH-GUS
Lanes 4 and 8	pBI101 AtSHH-LUC

The maps of each of these four constructs (seen in figures 7.1-7.3) predict the probe to hybridise to the observed fragments



tumefaciens using electroporation (described in section 2.4). Transformed agrobacteria were selected on NA media containing rifampicin and kanamycin. Pure single colonies were grown in liquid culture and total DNA preparations performed (Draper *et al.*, 1988; described in section 2.4.2). The resultant DNA was digested with appropriate restriction enzymes and Southern blots carried out to confirm the constructs were harboured within the agrobacteria (see figure 7.4).

The transformation of tobacco with all of the above constructs was performed first, due to the speed and ease of production of transgenics.

7.3.1 Tobacco leaf disk transformation

Transformed tobacco plants were generated by standard techniques (Draper et al., 1988), for each of the four reporter gene constructs. Once the putative transformed plants had produced sizable roots in MSO containing kanamycin, they were transferred to compost filled pots and maintained in transgenic growth rooms. At the time of this transfer a small sample of leaf tissue was removed from each line for initial analysis. Data presented within chapter 5 suggested SHH transcript to be present within every tissue tested, therefore reporter gene activity was predicted to be present within these excised portions of leaf tissue. The four kanamycin-resistant plants harbouring the construct, pBI101 AtSHH Wrong, were utilised as controls in a histochemical analysis of leaf tissue of all 36 GUS lines. Unexpectedly, the leaf tissue from pBI101 AtSHH Wrong lines, produced blue colouration within the tissue, as well as all the other GUS lines tested. The experiment was repeated utilising wild-type tobacco plants as negative controls and identical results were obtained for all the previously tested lines, but no blue pigment could be visualised in wild-type controls. This result therefore suggested that the 'negative control' constructs transformed into tobacco, expressed gus activity and would not be suitable for negative controls. Due to time constraints these plants were not analysed further. This histochemical analysis also suggested that GUS activity was greater in vascular tissue within the leaf, than in the leaf lamina. This observation was studied in detail in section 7.5.1.2.

Similar analysis of all 21 individual kanamycin-resistant plants







B

Figure 7.6 Genomic PCR analysis of transgenic tobacco lines

Figure 7.6A PCR amplification of the complete AtSHH promoter, a small fragment of the vector's multi cloning site and a small portion of the 5' end of the luciferase reporter gene, by utilising the vector primer M13-20, and an internal luciferase primer (annealing to the luciferase gene at its 5' end).

Lanes 1 and 10	1KB ladder
Lanes 2-7	Amplification from DNA extracted from
	transgenic lines 5, 10, 11, 14, 16 and 23,
	harbouring the construct pBI101 AtSHH LUC
Lane 8	No template, negative control
Lane 9	Amplification from DNA extracted from
	untransformed tobacco

Figure 7.6B PCR amplification of the complete AtSHH promoter, a small fragment of the vector's multi cloning site and a portion of the 5' end of the *gus* reporter gene, by utilising the vector primer M13-20, and an internal *gus* primer (annealing to the *gus* gene approximately 400bp from its 5' end).

Lane 1	1KB ladder
Lanes 2-7	Amplification from DNA extracted from
	transgenic lines 15, 17, 19, 20, 23 and 24,
	harbouring the construct pBI101 AtSHH Correct
Lane 8	Amplification from DNA extracted from
	untransformed tobacco
Lanes 9-12	Amplification from DNA extracted from
	transgenic lines 4, 9, 20 and 22, harbouring the
	construct pBin19 AtSHH GUS
Lane 13	Positive control amplification from
	Agrobacterium harbouring the construct pBin19
	AtSHH GUS

harbouring the pBI101 AtSHH-LUC construct were performed using leaf protein extractions in a fluorometric assay, for luciferase activity. Due to the positive nature of the results from this and the previous experiment, a sample of lines containing each construct were chosen for further study. Of the 21 individual kanamycin-resistant plants harbouring the pBI101 AtSHH-LUC construct, 6 lines were chosen for further analysis; of the 18 individual kanamycin-resistant plants harbouring the pBin19 AtSHH-GUS construct, 4 lines were chosen for further analysis; and of the 18 individual kanamycinresistant plants harbouring the pBI101 AtSHH-GUS construct, 6 lines were chosen for further analysis. Fluorometric analysis of reporter gene activity was performed on leaf lamina tissue of some of these chosen lines. Similar sized explants were harvested, their protein content extracted in the appropriate extraction buffer and quantified, and a fraction of the extract used for fluorometric assays. The GUS activity was calculated and expressed in terms of picomoles of 4-MU produced/min/mg of total protein. The luciferase activity was calculated and expressed in terms of Luc units/ μg of total protein. The results of this analysis are presented in figure 7.5, which emphasises the varied constitutive levels of reporter gene activity found within leaf lamina tissue of different transformant lines.

Luciferase activity can be seen to vary between the highest expressing plant, line 5 and the lowest expressing plant, line 23, by approximately one order of magnitude. GUS activity can be seen to vary between the highest and lowest expressing lines by a similar degree, within each construct.

DNA extractions were performed on each of the selected lines and PCR analysis performed to confirm the presence of the AtSHH promoter fragment 5' to either the luciferase or β -glucuronidase reporter genes (see figure 7.6).

7.3.2 Arabidopsis root explant transformation

As analysis of the T1 tobacco lines suggested no significant variation in GUS expression between the translational and transcriptional fusions (see later), the relatively time-consuming transformation of *Arabidopsis* was only performed with one construct, pBI101 AtSHH Correct. This GUS reporter gene construct was chosen due to the common use of pBI101 vector within previous



B



AtSHH-reporter gene constructs' expression in tobacco lines' leaf lamina

Figure 7.5A Luciferase activity in the six tobacco lines chosen for study.

Figure 7.5

Figure 7.5B GUS activity in six representative tobacco lines. Lines 15, 17 and 19 harbour pBI101 AtSHH Correct and lines 9, 20 and 22 harbour pBin19 AtSHH GUS.

(Filled columns represent the mean and the vertical bars represent the standard deviation_{n-1} of data obtained from three independent measurements.)

studies, allowing direct comparisons of data.

Arabidopsis thaliana [ecotypes C24 and Columbia] were transformed with pBI101 AtSHH Correct, using the technique described by Clarke *et al.*, (1992). This method requires the T1 plants to set seed *in vitro*, so the first generation available for reporter gene analysis was the T2. The T2 generation is a segregating population of plants in which some offspring of the original transformant will no longer contain a T-DNA, so a large portion of each T1 line seed was selected on kanamycin. Whole three week old kanamycinresistant T2 plants were analysed for GUS activity using fluorometric analysis and no significant variance in GUS expression could be detected between C24 and Columbia transformants (see figure 7.7). Therefore only C24 lines were analysed in the T3 generation.

The segregation ratios for each T2 seed population were subjected to chisquare tests and five C24 lines were found to have a 3:1 ratio suggesting the presence of a single T-DNA insert locus. The resultant seed set from nine individual T2 plants, for each of these five lines, was collected and a portion germinated on kanamycin selection plates. The T3 seedling populations in which all seedlings were kanamycin-resistant were selected as the lines to be studied. These lines are potentially homozygous for a single T-DNA locus insert, however, it is possible that more than one T-DNA copy is present at this locus.

7.4 Wound induced reporter gene expression

Results presented within chapters 3 and 5 have proven SHH transcript and enzyme activity to be induced by wounding within etiolated asparagus seedlings. This induction occurred within one day post-wounding and then the transcript and enzyme activity decreased back towards the basal levels over the following two days. Therefore transgenic plants were analysed to assess if the putative AtSHH promoter fragment utilised would cause wound induction at the level of transcription.

As maturing T1 tobacco plants were to be utilised, this wound induction study could not be performed upon etiolated seedlings as with asparagus.

Figure 7.7 AtSHH-GUS activity in *Arabidopsis* T2 seedlings of two ecotypes

Figure 7.7A GUS activity in four representative *Arabidopsis* ecotype C24 lines.

Figure 7.7B GUS activity in four representative Arabidopsis ecotype Columbia lines.

• (Filled columns represent the mean and the vertical bars represent the standard deviation_{n-1} of data obtained from three independent measurements.)



Therefore a previous method used to study wound inducible reporter gene expression within transgenic tobacco was utilised (Warner *et al.*, 1993). Leaves from transgenic tobacco plants were removed and cut into squares approximately 1cm² in size, avoiding major veins and the midrib. These leaf squares were uniformly stabbed nine times using yellow pipette tips. The wounded leaf pieces were transferred to Petri dishes lined with 3MM Whatman paper dampened with sterile water, and incubated in plant growth rooms for 0-3 days.

7.4.1 Tobacco

Three leaf disks were utilised/sample and the experiment performed in triplicate using different sections of the same leaf for each transformed line assayed. The fluorometric analysis of reporter genes for a representative sample of lines is shown in figure 7.8. This data demonstrates that an increase in reporter gene activity is detectable within one day post-wounding and this is the period of maximal reporter gene activity, with activity reducing over the following two days [2-3 days post-wounding], towards the basal level observed in leaf samples assayed on day zero. This described pattern of wound induced reporter gene expression was observed in all 13 lines tested, with no difference in the temporal induction profile observed between luciferase or ß-glucuronidase reporter genes; or between transcriptional and translational *gus* gene fusions. This fluorometric data confirmed the previously observed variance in expression levels between transformants.

The average increase in reporter gene activity within one day of the wounding of leaf tissue can be expressed in terms of an x fold increase in reporter gene activity in comparison to the levels recorded prior to wounding [day 0 time point]. The average for each construct is as follows: pBI101 AtSHH-LUC constructs had an average 2.34 fold increase in reporter gene activity in response to wounding [standard deviation=0.976]; pBin19 AtSHH-GUS constructs had an average 2.042 fold increase in reporter gene activity in response to wounding [standard deviation=0.508]; pBI101 AtSHH-GUS constructs had an average 1.885 fold increase in reporter gene activity in response to wounding [standard deviation=0.415]. These values give an

Figure 7.8 Wound-induced AtSHH-reporter gene expression in representative tobacco lines

Figure 7.8A Luciferase activity in Line 11 wounded explants, monitored at one day intervals post-wounding.

Figure 7.8B Luciferase activity in Line 23 wounded explants, monitored at one day intervals post-wounding.

Figure 7.8C GUS activity in Line 19 (harbouring pBI101 AtSHH Correct) wounded explants, monitored at one day intervals post-wounding.

(Points represent the mean and the vertical bars represent the standard deviation n-1 of data obtained from three independent measurements.)



average wound induction of reporter gene activity of 2.151 fold [standard deviation=0.744].

Histochemical analysis of a tobacco leaf explant, one day post-wounding, can be observed in figure 7.17. The constitutive *gus* expression in the leaf lamina tissue provides the blue background upon which the more intense blue staining appears at wound sites. The wound sites include the cut edge of the explant as well as the stab holes. This picture also demonstrates the greater staining observed in the vascular tissue of the leaf than in the lamina tissue.

7.4.2 Arabidopsis

Fluorometric analysis of wounded *Arabidopsis* leaves was not performed as the *Arabidopsis* leaves were found to desiccate easily and the tissue started to rot within three days if excess water was used. Therefore, histochemical analysis was performed on fresh leaf tissue, and tissue one day post-wounding (see figure 7.18). As observed in wounded tobacco tissue, more intense staining is observed at wound sites (which include the cut edges of the explant as well as the stab wounds), while constitutive expression in the leaf lamina, with greater staining in the vascular tissue, can also be observed.

7.4.3 Tobacco callus

Once wound induction of reporter gene constructs was observed within transgenic tobacco, analysis of tobacco callus tissue was performed as several wound-induced transcripts are also known to be expressed within callus (for example Firek *et al.*, 1993). As neither the *gus* nor luciferase reporter gene utilised in the reporter gene constructs, contained an intron, expression of the reporter gene may occur within the prokaryotic species *Agrobacterium tumefaciens*. To analyse expression within callus tissue, young, nearly fully expanded leaves were removed from each T1 transgenic plant and identical procedures to a tobacco transformation were performed, without agrobacterial inoculation. Due to these leaves already harbouring a T-DNA, expressing kanamycin resistance, the leaf explants all produced callus upon incubation on MSD4X2 media containing kanamycin and augmentin. These calli were

Figure 7.9AtSHH-reporter gene constructs' activity within
tobacco callus in representative tobacco lines.

Figure 7.9A Luciferase activity in the callus and leaf lamina tissues of the representative lines, 11, 16 and 23.

Figure 7.9B GUS activity in the callus and leaf lamina tissues of the representative lines, 17 (harbouring pBI101 AtSHH Correct), 16 and 23 (harbouring pBin19 AtSHH GUS).

(Filled columns represent the mean and the vertical bars represent the standard deviation_{n-1} of data obtained from five independent measurements for callus tissue and three independent measurements for lamina tissue.)



removed from the leaf disks following a 3 week incubation and analysed for reporter gene activity.

The measured reporter gene activities within callus and leaf lamina tissue of representative lines is shown in figure 7.9, and histochemical staining of a callused leaf explant can be seen in figure 7.17. As predicted from the fluorometric data, callus at the edge of the leaf explant stains to a greater degree than the leaf tissue to which it is attached, but this may also reflect the easier entry of the X-Gluc substrate into the unorganised callus cells, than the leaf tissue. The variable difference between the reporter gene activity measured in the calli and leaf lamina tissue can be partly explained by the methodology of the experiment. Leaf disks of previously transformed tissue were used to produce callus over a 3 week period. During this incubation, some callus could be observed to occur on the surface of the leaf disk and not at the cut edges, often at the site of small puncture wounds arising as a result of forcep handling during the experimental preparation. This leaf tissue has also not experienced normal environmental conditions while in culture, so the leaf lamina incubated on the MSD4X2 kanamycin selection plates was not utilised as the control tissue for leaf expression. The leaf tissue measurements were taken from leaf samples prior to the incubation period. Due to the possible occurrence of contamination during in vitro culture of the callusing leaf disks, a large number of disks and plates were utilised, so several leaves from each transgenic line were utilised to provide sufficient lamina tissue. As the measurement is an average of several leaves this value may not reflect the true activity within the actual leaf sample from which the callus tissue was extracted. To try and overcome this problem, 5 samples of calli were independently assayed for each line, and the average activity plotted in figure 7.9.

Despite the lack of accurate numerical comparative data, a general trend could be observed; the reporter gene activity within callus was greater than that measured in leaf lamina tissue for each reporter gene, each construct and each line assayed.

7.5 Developmental reporter gene expression in untreated transgenic

Figure 7.10 AtSHH-reporter gene constructs' activity within stem tissue of representative tobacco lines

For each graph, stem section A represents the youngest portion of the stem tissue utilised, and section D represents the eldest portion of stem tissue utilised.

Figure 7.10A Luciferase activity in line 11 stem explants.

Figure 7.10B GUS activity in line 19 (harbouring pBI101 AtSHH Correct) stem explants.

Figure 7.10C GUS activity in line 22 (harbouring pBin19 AtSHH GUS) stem explants.

(Filled columns represent the mean and the vertical bars represent the standard deviation n-1 of data obtained from three independent measurements.)


plants

As T1 tobacco material was utilised in all previous studies, it was also used in the analysis of developmental expression in untreated tobacco plants. However, if the analysis of a particular tissue was considered to be detrimental to the normal development of the plant, such as the extraction of root tissue, these structures were not analysed. Analysis of this nature was therefore performed upon the T3 *Arabidopsis* transformants for which numerous, genetically identical plants were available for each line.

Once the first flowers of each tobacco line had set seed, to provide the next generation of transgenic tobacco plants, analysis of the stems of each line was performed. Northern data and SHH enzyme analysis had both suggested a temporal expression profile of SHH transcript and enzyme activity to occur within lignifying tobacco stem tissue (see chapter 5). To test if the *Arabidopsis* SHH promoter causes a similar temporal transcript profile within tobacco, sections of stem tissue were analysed for reporter gene expression (see section 7.5.1.1). As reporter gene activity had not yet been assayed within developing flower buds, because the first flowers were left to set seed, tissue culture of stem explants was performed at the same time, to recover genetic clones of the original T1 transformants (method described in section 2.12.2).

7.5.1 Tobacco

7.5.1.1 Reporter gene activity within tobacco stems

The plants used in this analysis had started to set seed and therefore represented more mature explants than those utilised for northern analysis in chapter 5. Four sections were assayed fluorometrically from each stem of eleven representative transgenic lines, to try and encompass all representative stages of stem lignification. Figure 7.10 presents data for three of these representative lines. Although each graph does not show the greatest reporter gene activity to be present within the same section of stem tissue, this analysis demonstrates the AtSHH promoter does not drive identical reporter gene activity within each stem section. Similar observations were made in chapter 5 following northern and SHH enzyme activity analysis. The inability to utilise Figure 7.11 Comparative AtSHH-reporter gene constructs' activity in leaf lamina, veins and midribs of representative tobacco lines

The three tissues assayed were the leaf midrib, major veins coming from the midrib, and finally leaf lamina tissue in which no large veins were present.

(Filled columns represent the mean and the vertical bars represent the standard deviation $_{n-1}$ of data obtained from three independent measurements.)



leaf lamina
midrib of leaf
vein of leaf

stems from the same age plants, as utilised in northern analysis, may contribute to the differing temporal pattern of expression observed in the reporter gene analysis. Histochemical analysis of a cross-section of stem tissue can be seen in figure 7.17, in which all tissues of the stem appear to stain apart from the secondarily thickened xylem. More intense staining appears to correlate to the phloem tissue of the stems, with both the cortex and pith cells also staining.

7.5.1.2 Reporter gene activity within vascular tissue of the leaf

During the initial analysis of leaf tissue from all *gus* gene containing transgenic lines, it was observed that more intense histochemical staining occurred in the vascular tissue of the leaf than in the leaf lamina (see figure 7.17 for an example). This may have arisen as an artifact of the histochemical staining, as the substrate may have preferentially entered the vascular tissue through the wound site at the edges of the leaf explants. Therefore, fluorometric analysis was used to test if greater reporter gene activity could be detected in the vascular tissue of the leaf compared to leaf lamina. To allow standardisation of the tissues compared in this analysis, three clearly defined areas of the leaf were analysed: the leaf midrib; major veins arising from the midrib; and leaf lamina occurring between these veins in which no major veins were present. Three separate extractions were made for each tissue, from a single leaf, for each line and luciferase activity analysed.

This fluorometric analysis of representative transgenic lines, confirmed the observation that greater reporter gene expression occurred in the midrib and large veins of tobacco leaves in comparison to the leaf lamina (see figure 7.11). The use of luciferase expressing lines proves that the histochemical analysis was not an artifact of staining or an artifact of GUS enzyme activity. The results clearly show a decreasing level of reporter gene activity from midribs to veins and finally leaf lamina tissue. An average 1.75 fold [standard deviation_{n-1}=0.85] greater luciferase activity was measured in veins compared to leaf lamina, while an average 2.24 fold [standard deviation_{n-1}=0.772] greater luciferase activity was assayed in the midrib of leaves compared to leaf lamina. Figure 7.12 AtSHH-reporter gene constructs' activity during bud development of representative tobacco lines

For each line assayed the buds were categorised according to size and further divided into tissue type.

Figure 7.12A Luciferase activity during bud development in line 14.

Figure 7.12B GUS activity during bud development in line 24 (harbouring pBI101 AtSHH Correct).

(Filled columns represent the mean and the vertical bars represent the standard deviation_{n-1} of data obtained from three independent measurements.)







This analysis proves that AtSHH driven reporter gene expression does not occur uniformly within leaves, with greater expression in vascular tissue.

7.5.1.3 Reporter gene activity within developing and mature flowers

Initial histochemical analysis of mature open flowers demonstrated *gus* gene expression in all tissues observed. However this analysis demonstrated that pollen stained very quickly compared to other tissues and possibly represented a tissue with high levels of reporter gene expression. Therefore only flowers with non-dehisced anthers were utilised to prevent the dispersal of pollen on flower parts, which would affect the results obtained in fluorometric analysis.

To allow simple staging of bud development, four size ranges were utilised for bud classification. The four categories chosen were based upon those defined by Roberts *et al.* (1993) for the stages of male gametogenesis. Buds <10mm represented those in which sporogenesis has been completed; 10-16mm buds represent those in which microspore development occurs; 16-30mm buds represent those in which microspore mitosis occurs; and finally the open buds, >30mm in length, in which it was observed that the anthers had not dehisced, represented the later stages of pollen maturation. Utilising these stages of bud development allowed any detected reporter gene activity to be compared to the northern analysis of bud development presented in chapter 5. Each category of buds was dissected into three portions for reporter gene analysis. The samples referred to as 'male' were derived from the anthers and filaments. The samples labelled 'petals' were derived from the rest of the bud tissue, namely the developing ovary, stigma and style.

Three separate buds were dissected for each stage and the averaged results of this reporter gene analysis can be seen in figure 7.12, for two representative transgenic lines, one containing the luciferase construct and the other a *gus* gene construct. The temporal and spatial expression profile of each construct can be observed to follow a similar pattern of expression. The 'female' and 'petal' samples of the developing buds display the greatest (up to 2 fold) expression during the 10-30mm stages of development. Meanwhile in the

Figure 7.13 AtSHH-reporter gene constructs' activity in flower organs of two representative tobacco lines

Flower parts were dissected from flowers in which the anthers had not dehisced.

Figure 7.13A Luciferase activity in the tissues of the flower from line 14.

Figure 7.13B GUS activity in the tissues of the flower from line 24 (harbouring pBI101 AtSHH Correct).

(Filled columns represent the mean and the vertical bars represent the standard deviation n-1 of data obtained from three independent measurements.)



'male' tissue, this pattern of a gradual increase in activity is seen in the same two stages, however upon reaching the final stage assayed, the reporter gene activity significantly increases. This represents the stage of pollen maturation following microspore mitosis which culminates in the formation of mature pollen (Roberts *et al.*, 1993). This increase in the reporter gene activity is thought to be due to the high level of reporter gene activity measured in mature released pollen (see next paragraph), also being present in the maturing pollen in the non-dehisced anthers.

To further examine the spatial expression pattern of the reporter genes the >30mm buds with non-dehisced anthers were dissected into individual flower parts and assayed. The results of this analysis can be seen in figure 7.13, for the same two representative lines used in figure 7.12. This analysis demonstrates that the large increase in reporter gene activity observed in 'male' explants during this stage of development (figure 7.12), is due to expression in the anthers rather than the filaments. The expression within the 'petal' portions utilised in figure 7.12, occurs to a greater degree in the petals than in the sepals. The lower comparative expression of the luciferase reporter gene in the stigma compared to the GUS activity, was observed in several lines, and histochemical analysis always demonstrated strong GUS activity to be present in the stigma (see figure 7.17). This analysis shows reporter gene expression in every plant flower part assayed. These results were confirmed by histochemical analysis of transgenic flowers containing a *gus* gene construct, an example of a stained stigma can be seen in figure 7.17.

Analysis of mature pollen from each line containing a luciferase construct resulted in the highest luciferase activity measured in any tissue, with an average of 10,451,816 Luc units/ μ g of total protein for line 14 (standard deviation_{n-1}= 3,134,519). This analysis was not performed on GUS lines as it has been suggested that the *gus* gene may express aberrantly in pollen (Uknes *et al.*, 1993). However the results obtained using luciferase constructs confirm the prediction of high reporter gene activity within maturing and mature pollen. Mature seeds were also analysed giving an average value of 270,732 Luc units/ μ g of total protein for line 14 (standard deviation_{n-1}= 27,670).

The seed set from this tobacco T1 generation was plated on MSO

Figure 7.14 GUS analysis of five T3 homozygous *Arabidopsis* lines, chosen for further study

Figure 7.14AGUS activity, measured at 7 day intervals, during seedling development.

Figure 7.14B Comparative GUS activity in root tissue and aerial tissues of three week old plants grown in kanamycin containing agar media.

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(Filled columns represent the mean and the vertical bars represent the standard deviation n-1 of data obtained from three independent measurements.)



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containing augmentin and kanamycin to study the segregation ratios, but no fluorometric analysis was performed due to this generation being a segregating population unlike the T1 in which all the previous analysis had been performed. Therefore the fluorometric analysis of reporter gene activity during seedling development was performed on transgenic *Arabidopsis* lines (see section 7.5.2.1). Histochemical analysis of *gus* gene expression was performed on four week old kanamycin-resistant T2 seedlings and a typical example can be seen in figure 7.17. This histochemical analysis demonstrates GUS activity throughout the root, including the root hairs, and in the cotyledons and true leaves. Even at this early stage of development greater expression can be observed in the vascular tissue, however the hypocotyl of the seedling does not appear to stain.

7.5.2 Arabidopsis

7.5.2.1 Seedling development

Once the five lines described in section 7.3.2 had been proven to contain a single T-DNA locus, seed collected from the T2 parent lines giving homozygous progeny was sown on soil in petri dishes. Following a three day vernalisation to synchronise seed germination, the petri dishes were incubated in a growth cabinet for a three week period post-planting. At one week intervals (post-planting), 5 seedlings from each line were homogenised in buffer and fluorometrically assayed, this was performed in triplicate for each line. This analysis was performed for three weeks and the results can be seen in figure 7.14A. Overall a gradual increase in GUS activity can be observed over the assay period. Histochemical analysis was also performed on samples from each time point and examples of these can be seen in figure 7.18. The seedlings removed from the soil, one week post-planting, simply consist of the cotyledons, the hypocotyl and a short root, all of which can be seen to stain in histochemical analysis, with the hypocotyl staining least as observed in tobacco. The seedlings removed from the soil two weeks post-planting, had produced their first true leaves as well as extending the root and as observed with tobacco seedlings, the whole root, including root hairs, stained, as did the cotyledons. The first true leaves show greater expression at the leaf tips and once again

Figure 7.15 Analysis of GUS activity in the organs of the five T3 homozygous *Arabidopsis* lines, chosen for further study

Five tissues or stages of development were assayed, for each line.

Figure 7.15A Line 1.

Figure 7.15B Line 4.

Figure 7.15C Line 10.

Figure 7.15D Line 16.

Figure 7.15E Line 20.

(Filled columns represent the mean and the vertical bars represent the standard deviation n-1 of data obtained from three independent measurements.)



lower expression is observed in the hypocotyl. A strong area of staining is visible at the primordia for the next pair of leaves. The final sample, taken three weeks post-planting, contain several true leaves, an example of which can be seen in detail following histochemical staining, in figure 7.18. This histochemical analysis of leaf tissue from a three week old *Arabidopsis* plant suggests greater *gus* gene expression in the vascular tissue of the leaf than in the leaf lamina, as noted in both histochemical and fluorometric analysis in tobacco transformants.

7.5.2.2 Organs

No analysis of reporter gene expression in root tissue was performed on tobacco transformants and therefore *Arabidopsis* homozygous T3 plants were utilised for this purpose. The three week old kanamycin-resistant plants grown on germination media were utilised as a source of root material, as the intact roots could be easily extracted from the agar media. To allow comparative data to be accumulated, the aerial portion of the same plants were also assayed and the results of this analysis can be seen in figure 7.14B. In all lines the GUS activity measured within root tissue is greater than that observed in aerial parts of the three week old plants, with an average 6.9 fold greater expression level in the roots compared to aerial portions of the plant.

Figure 7.15 shows comparative GUS activity in various organs for each of the five T3 homozygous lines studied. Overall this analysis confirms the analysis of tobacco transgenics, with comparatively low levels of reporter gene expression observed in leaf tissue, compared to other tissues, namely siliques, stems and inflorescences. However, once again reporter gene activity is present within every tissue assayed. Histochemical analysis of a whole inflorescence can be observed in figure 7.18. This analysis confirms GUS activity within all structures of the flower as previously noted for tobacco transgenics. Both the stigma and anthers can be observed to display more intense staining.

7.6 Comparative reporter gene and endogenous SHH gene expression

Figure 7.16 Analysis of gus and endogenous SHH transcript levels

For both northern analysis lanes 1-4 correspond to T3 homozygous *Arabidopsis* lines 1, 4, 10 and 16 respectively.

Figure 7.16A Northern analysis of *gus* gene transcripts in whole plant tissues. Total RNAs were hybridised to a *gus* probe.

Figure 7.16B Northern analysis of AtSHH gene transcripts in whole plant tissues. Total RNAs were hybridised to an AtSHH probe.

Figure 7.16C GUS activity in extracts of whole plants used for RNA extractions.

(Filled columns represent the mean and the vertical bars represent the standard deviation_{n-1} of data obtained from three independent measurements.)



To analyse if the putative AtSHH promoter fragment utilised in the reporter gene constructs was driving similar levels of reporter gene transcript expression to endogenous SHH transcript, northern analysis of transgenic *Arabidopsis* was performed. RNA extractions from a number of plants from each of four T3 homozygous lines were quantified and separated by agarose gel electrophoresis prior to blotting. Fluorometric analysis of a portion of the extracts was also performed prior to phenol extractions during RNA extraction. The resultant blot was first probed with a *gus* gene probe and following removal of this probe, an AtSHH probe, labelled to approximately the same specific activity. The results of this analysis can be seen in figure 7.16. The *gus* gene probed blot was exposed for half the time as the AtSHH probed blot to achieve a similar exposure, suggesting GUS transcript to be present at higher levels than the endogenous SHH gene transcript, in all four lines.

7.7 Discussion

A putative AtSHH promoter has been demonstrated to drive reporter gene expression in both *Nicotiana tabacum* and *Arabidopsis thaliana*. The use of two reporter genes, ß-glucuronidase and luciferase, allowed comparative data to be accumulated, which because of its agreement suggests all observed reporter gene expression to be due to the promoter fragment utilised, rather than the reporter gene itself. This is clearly demonstrated in the anther and pollen of flowering tobacco where ectopic expression of GUS activity has been demonstrated (Uknes *et al.*, 1993), however as luciferase activity was also measured in these tissues, this expression is not considered to be an artifact.

The plasmid, pBI101 AtSHH Wrong, contained the 1845bp AtSHH putative promoter fragment in the wrong orientation with respect to the *gus* reporter gene. Histochemical analysis demonstrated GUS activity within leaf tissue of transgenic tobacco plants containing this construct. This result suggests that the 1845bp fragment utilised as a putative AtSHH promoter could drive transcription in either orientation. This situation may have arisen due to two possibilities. Firstly, this fragment could contain a second promoter from a different *Arabidopsis* gene and therefore drives *gus* gene expression when placed next to the reporter gene. Examples of bidirectional promoters present

Figure 7.17 Histochemical localisation of GUS expression in AtSHH-gus T1 tobacco plants

Figure 7.17A wounded leaf explant.

Figure 7.17B four week old seedling.

Figure 7.17C leaf removed from a four week old seedling.

Figure 7.17D callus at the edge of a wounded leaf explant.

Figure 7.17E section of stem tissue.

Figure 7.17F stigma.



on much smaller DNA fragments have been identified. An *et al.*, (1996) identified an actin promoter which also appears to drive expression of an unknown function gene in an antisense orientation. The two TATA boxes of these genes are only 270bp apart and therefore very small promoter sequences are required to drive expression of the genes. As an \approx 1.8kb fragment was utilised in this work, it is very feasible for bidirectional promoters to exist on this fragment. Secondly, strong enhancer elements within the 1845bp fragment may cause transcription of the *gus* gene from cryptic transcriptional start sites near the fusion.

As described in chapter 6, DNA sequence alignments of the 1760bp putative AtSHH promoter to sequences held on the EMBL/Gen databases produced no significant matches. This suggests that if a translational fusion to the *gus* gene has occurred in pBI101 AtSHH Wrong transgenic lines, then the sequence of the fusion gene is not within current databases. However, the translational fusion may be very short or alternatively contain an intron, causing the lack of a significant match. Inspection of the DNA sequence near this end of the 1845bp fragment, identifies possible motif sequences. A putative TATA box can be observed at -1473 to -1470 (numbers refer to those observed on figure 6.4). Upstream from this only two possible methionine codons can be found, -1555 to -1553 and -1649 to -1647. It is possible that neither of these are utilised as the translational start site and a transcriptional fusion to the *gus* gene has occurred.

Both reading frames from the two methionine residues contain one stop codon before the fusion site which may be expected to inhibit gus gene expression in putative translational fusions. However the presence of introns cannot be discounted, leading to the removal of these inhibitory codons for active translational fusions. Several potential 5' and 3' splice sites exist in the sequence.

The second possible explanation for *gus* gene transcription from the inverted AtSHH putative promoter fragment may rely on the removal of this fragment from its endogenous DNA context. Strong enhancer elements may exist within the fragment which can cause transcription from minimal promoters such as a cryptic transcriptional start site (already proven to exist

Figure 7.18Histochemical localisation of GUS expression in
AtSHH-gus T3 Arabidopsis plants

- Figure 7.18A day 0 wounded leaf explant.
- Figure 7.18B one week old seedling.
- Figure 7.18C leaf from three week old seedling.
- Figure 7.18D day 1 wounded leaf explant.
- Figure 7.18E two week old seedling.

Figure 7.18F inflorescence.



 \approx 290bp from the end of the fragment). These elements may not cause transcription when in their endogenous context, but when present in a transgene may drive the observed unexpected reporter gene expression.

The northern analysis of gus gene and endogenous AtSHH gene expression presented in section 7.6, suggests greater accumulation of reporter gene transcript than endogenous AtSHH gene transcript. This may be due to the putative AtSHH promoter fragment causing greater expression when present as a transgene in a different context to the endogenous copy, due to the lack of control of putative enhancer elements. Alternatively this result may represent a difference between mRNA stabilities of the gus gene fusion transcript and the endogenous AtSHH transcript. It should however be noted that both these transcripts contain identical 5' portions up to the fusion.

The large array of expression levels observed within different tobacco transformants (figure 7.5) may be related to the number of T-DNA inserts present within each T1 tobacco transformant. Segregation analysis of the seed set from these lines suggested every line to contain more than one T-DNA, making the segregation analysis very difficult to interpret clearly. The presence of several T-DNA/T1 tobacco transformant has been noted within other work at Leicester (Warner et al., 1993), and may reflect the use of relatively high concentrations of kanamycin [100µg/ml] to select transformants containing pBin19 derived constructs. This plasmid is known to contain a point mutation within the nptII gene which causes a four-fold reduction in the resistance of the transformed plants to kanamycin (Yenofsky et al., 1990). Also, as a small sample of putative transgenics was analysed in this study, the selection of healthy more vigorous plants for further study can not be discounted. As this selection process occurred as the plants were transferred to soil from selection, the choice of vigorous lines may reflect those lines which were less stressed under selection possibly due to the presence and expression of more than one kanamycin-resistance gene.

Alternatively, the sequences surrounding the T-DNA may cause some of this observed variance. Within the five *Arabidopsis* lines proven to contain a single T-DNA locus for which each line is homozygous, the variance observed between lines may be due to T-DNA copy number, however more probably the surrounding context of sequences flanking the inserted constructs influences the level of expression.

Previous data presented within chapters 3 and 5 suggested an increase in AoSHH transcript to occur within one day, as a response to wounding of etiolated seedlings. A similar induction profile was observed upon wound induction studies of transgenic tobacco lines. In all assays, reporter gene activity increased within one day post-wounding and started to return to basal levels by day two. The average increase in reporter gene activity of approximately twofold is a similar magnitude to that observed upon northern analysis presented in figure 3.2B. The numerical increase in SHH enzyme activity within asparagus seedlings upon wounding can not be directly compared to transgenic tobacco data as different tissues and types of wounding were utilised, but the wound induction profile appears to follow the same temporal pattern. It can therefore be concluded that the AtSHH promoter utilised in this study can cause wound-inducible transcript expression. The presence of greater reporter gene activity within callus than measured in leaf tissue from which the callus was derived, is consistent with the wound inducible nature of this promoter. Significantly greater AoSHH transcript was detected in mechanically separated asparagus cells than in wounded seedlings (figure 3.2), and these cells like callus represent a mass of undifferentiated cells, but unlike callus, all intercellular connections have been severed. This may help to account for the level of expression observed in callus, which is similar to or slightly higher than the level in wounded leaves (one day post-wounding) depending on the line assayed.

The data derived for stem tissue cannot be directly compared to the northern or SHH enzyme analysis presented within chapter 5, as the age of the plants utilised varied. Due to the use of mature transgenic tobacco, as a seed set was required, the four stem sections utilised were not identical to the four sections taken from any of the plants from which RNA was extracted. The histochemical analysis of stem sections suggests the greatest GUS activity to occur in the phloem tissue which is in agreement with the GUS data obtained for SAM synthetase promoters isolated from *Arabidopsis* and used to express GUS in transgenic tobacco (Peleman *et al.*, 1989a and 1989b). However unlike

SAM synthetase where this was the only tissue in which GUS activity was observed, the AtSHH driven reporter gene expression occurs elsewhere. Expression within leaf tissue was not uniform within all tissues, with greater expression in the vascular tissue compared to leaf lamina. Northern analysis of SHH transcript abundance within tobacco organs, presented in chapter 5, suggested greater SHH transcript abundance in root tissue compared to leaves, as noted by Mitsui *et al.* (1993). This was confirmed within transgenic *Arabidopsis* plants (figure 7.14B) in which \approx 7 fold greater GUS activity was observed in the roots compared to the aerial portions of the plants. The reporter gene analysis also confirmed northern analysis presented in chapter 5, from which it was concluded that greater SHH transcript is present within the anthers of flowers in which pollen maturation is nearly complete.

Overall the use of the reporter genes has allowed simple assays of AtSHH promoter driven expression, which upon histochemical and fluorometric analysis appears to occur in every organ assayed and perhaps every tissue within each organ assayed. These findings led to the comparison of numerical expression data, to data available for the widely used promoter CaMV35s. As none of the tobacco lines utilised in this study were thought to contain a single T-DNA insert, comparisons were not made to these results. The five independent transgenic Arabidopsis lines, from which homozygous T3 plants were analysed, were known to contain T-DNA at a single loci. Despite the possibility that each of these lines may contain tandem T-DNAs at this locus, these lines were used for comparative purposes. In a control transformation experiment by Clarke et al. (1992), 71 independent Arabidopsis ecotype C24, T1 transformants were generated using the plasmid pBI121 (Jefferson et al., 1987). This plasmid is simply pBI101 in which a CaMV35s promoter has been cloned 5' to the gus gene to drive its expression. Therefore this construct is identical to pBI101 AtSHH Correct, except for the promoter fragment and the presence of AtSHH coding region on the 5' end of the resultant transcript, leading to a GUS fusion protein. Of the 71 transformed lines, the highest GUS activity measured in the leaf tissue was 12,040 pmoles 4MU/min/mg of total protein, which is less than the average GUS activity of 13,725 pmoles 4MU/min/mg of total protein, measured in leaf tissue of the

five *Arabidopsis* lines studied in this work. The CaMV35s promoter has been shown to be active in most tissues, but not all, within several plant species (for example see Battraw and Hall, 1990; Benfey *et al.*, 1990). As observed in this study using *Arabidopsis* seedlings, CaMV35s driven GUS activity is also low within the hypocotyl causing very weak histochemical staining (Holtorf *et al.*, 1995).

The highest AtSHH driven reporter gene activities observed in the transgenic plants correlate to tissues in which high metabolic turnover is generally regarded to occur, such as wounded leaf, callus and mature pollen.

Northern analysis, (figure 7.16) suggests that the GUS transcript is present in the same tissue as the endogenous SHH transcript, but at greater abundance. It is unknown whether this is due to the GUS transcript being more stable than the SHH transcript or alternatively, the removal of the AtSHH promoter from its endogenous DNA context causing it to drive greater expression.

The AtSHH promoter fragment utilised in this work appears to cause high level transcription of transgenes in most tissues of more than one plant species, with a wound inducible increase in this expression, which may provide several important uses for this promoter, as discussed in chapter 9.

Chapter 8

The attempted perturbation of SHH enzyme activity by the use of partial sense or antisense technology

8.1 Introduction

When a transgene, containing sequences homologous to an endogenous gene[s], is introduced into plants, expression of both the introduced transgene and the homologous host gene[s] can be suppressed (van der Krol *et al.*, 1990). This phenomenon is often referred to as cosuppression (reviewed by Flavell, 1994; Matzke and Matzke, 1995). The cellular mechanisms responsible for the various forms of reported transgenic silencing remain unknown, although several models have been proposed (reviewed by Flavell, 1994; Matzke and Matzke, 1995).

The isolation of AtSHH (described in chapter 6) and PCR-derived SHH gene fragments from other species (described in chapter 4) led to the development of the molecular tools to manipulate endogenous SHH gene expression, and hence perturb the biochemical pathways utilising SHH. Several examples exist in which the complete coding region of a transgene have been expressed in transgenic plants leading to two possible phenotypes; the first phenotype arises due to the transgene being overexpressed, and the second phenotype arises due to cosuppression of both the trans- and endogenous gene (for example van der Krol *et al.*, 1990; Hart *et al.*, 1992; Boerjan *et al.*, 1994). The switch between these two states can be developmentally controlled, leading to more complicated interpretation of results (see Boerjan *et al.*, 1994 for examples). To alleviate these problems, this study used an attenuated [partial] sense construct which would not result in overexpression, as the complete coding sequence had not been introduced as a transgene, and therefore would hopefully only lead to cosuppression. This approach was successfully utilised by Smith *et al.* (1990) to cosuppress polygalacturonase gene expression in ripening tomato fruit.

Cosuppression of SAM synthetase was observed to lead to the following phenotype; stunted plants with thick, leatherlike, asymmetric and torn leaves, which emitted a 'cabbage-like' smell (Boerjan *et al.*, 1994). However as discussed in chapter 1, SAM, whose synthesis would be inhibited by lack of SAM synthetase, not only donates the methyl group in many transmethylation reactions but can also donate its four-carbon moiety for ethylene or polyamine synthesis. Therefore if similar levels of cosuppression of SHH were achieved in this study, the phenotypes previously noted in cosuppressed SAM synthetase plants may not be observed. It was predicted that transmethylation reactions would be directly inhibited by the accumulation of SAH, due to cosuppressed SHH. However, the potential alteration in SAM/SAH ratio may lead to effects on ethylene and polyamine synthesis resulting from the potential decreased usage of SAM for transmethylation.

The promoter-GUS fusion data for SAM synthetase and that observed for AtSHH-GUS fusion studies in this work differ considerably, despite both genes encoding important housekeeping enzymes. For example, an *Arabidopsis* SAM synthetase promoter was found to preferentially drive reporter gene expression in vascular tissue (Peleman *et al.*, 1989a), whereas, the reporter gene analysis described in chapter 7, for AtSHH promoter constructs, proved reporter genes to be expressed in every tissue and developmental stage tested. The SAM synthetase cosuppression phenotype described earlier, arose from a construct in which the CaMV35s promoter of the cauliflower mosaic virus was used to express the complete *Arabidopsis* SAM synthetase coding region [*sam-1*] in tobacco (Boerjan *et al.*, 1994). A similar construct in which the CaMV35s promoter was utilised to drive partial sense SHH gene expression was designed so comparisons between SHH and SAM synthetase cosuppression could be made. However as the reporter gene studies of these two genes' promoters had suggested a distinct difference in their respective gene expression profiles, the use of a 'constitutive' promoter to drive the SHH transgene may lead to more deleterious effects in the greater number of tissues in which SHH gene expression normally occurs. This may only result in the production of viable transgenic lines in which low levels of cosuppression occurs. The lines in which high level cosuppression of SHH occurs may die during the transformation process.

As discussed in chapter 7, the AtSHH promoter caused reporter gene expression levels similar to or greater than the levels observed using CaMV35s driven reporter gene constructs, in all the tissues tested. This led to the design of another construct which utilised the AtSHH promoter to drive the SHH partial sense gene fragment. Therefore these two constructs represented 'constitutive' expression cassettes.

Previous attempts to transform SAM decarboxylase sense transgene constructs under the control of the CaMV35s promoter into plants have resulted in very low transformation frequencies [analysed by callus counts], and each of the transformed calli died early in development. Inducible promoters were then utilised to control expression of these deleterious sense transgenes (Kumar *et al.*, 1996).

As a similar phenomenon was one of the anticipated results of cosuppressing SHH, two further partial sense constructs were utilised. A tobacco PR1a promoter (kindly donated by Simon Firek, at Leicester) has been demonstrated to drive reporter gene expression upon induction by treatment of transgenic *Arabidopsis* plants with 1mM salicylic acid. By utilising this promoter to drive SHH partial sense gene transcription, the expression of the engineered gene can be induced by salicylic acid treatment. One further construct was also designed as the PR1a promoter is known to also cause reporter gene expression in several plant tissues upon the onset of flowering (Uknes *et al.*, 1993). An anther-specific promoter (kindly donated by Michael Roberts, at Leicester) was utilised to control expression of the final SHH partial

Figure 8.1 Assembly of SHH partial sense constructs

Assembly of one example of an *Arabidopsis* SHH partial sense construct, namely an AtSHH driven SHH partial sense construct. The construct pAtSHH cassette was designed to provide a cassette containing the partial sense SHH gene fragment and a polyadenylation signal sequence. The second construct shown, pAtSHH-AtSHH, is an intermediate construct in which, in this example, the AtSHH promoter has been cloned 5' to the cassette. The final construct, pBinAtSHH-AtSHH is the binary plasmid which was transferred into *Agrobacterium* for transformation of *Arabidopsis*.

Identical schemes of construct design were utilised for the other three promoters used in this study. The promoter fragments used to make each of the four partial sense constructs were derived from the following sources:

Double CaMV35s	removed from pJIT163 (Guerineau et al.,
	1992)
APG	removed from a plasmid kindly donated
	by Dr. M. Roberts at Leicester
AtSHH	removed from pKS AtSHH-LUC
	(described in chapter 7)
PR1a	removed from a plasmid kindly donated
	by Dr. S. Firek at Leicester



sense construct. The APG gene was isolated from *Arabidopsis* and reporter gene studies suggested the APG promoter directed anther-specific transcription (Roberts *et al.*, 1993). This result was further confirmed by the use of an APG driven ribonuclease [*barnase*] construct (Roberts *et al.*, 1995). The use of the cytotoxic *barnase* gene confirms APG to not cause a constitutive low level of expression previously undetected by GUS assays, but does not rule out expression in minor cell types or within small numbers of cells in particular organs (Roberts *et al.*, 1995). This work does suggest that the APG promoter drives high level, tissue specific expression of a transgene, which is what is required for the final SHH partial sense construct. The northern analysis presented in chapter 5 suggested SHH gene expression to occur in tobacco anthers, during the window of expression of APG. Therefore it was anticipated that the expression of the APG driven SHH transgene in the anthers, may affect pollen viability.

Each of the above described transcripts were utilised in an *in planta*, *Agrobacterium* mediated transformation of *Arabidopsis*, as described in section 8.4.1. When it was discovered that this technique had failed to produce any transgenic seed, even in control transformations, the longer procedure of *Arabidopsis* root transformation was attempted. It was unknown if this would produce transgenic plants for analysis within the remaining time period of this project and therefore further experiments were initiated. Tobacco transformation produces transgenic lines much quicker than the transformation of *Arabidopsis* and therefore was utilised in the further studies. A simple one step cloning procedure produced an antisense SHH construct (see section 8.3) which was driven by CaMV35s.

8.2 Construction of SHH partial sense constructs

The overall scheme of the construction of one example of an *Arabidopsis* SHH partial sense constructs can be seen in figure 8.1. To allow each fusion of the AtSHH coding region to the described promoters, to be exactly the same in all cases, the first \approx 370bp of the AtSHH coding region was utilised. Therefore each fusion occurred using the *Nco* I site found at the AtSHH translational start site. Initially a cassette containing the AtSHH coding

Figure 8.2 Assembly of the tobacco SHH antisense construct

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A simple one-step ligation of a *Bam* HI/*Sac* I tobacco SHH gene fragment into identically digested pROK II (described in the text), resulted in the final construct, pTOBSHH-anti.


region and a termination signal, to produce efficient polyadenylation, was constructed. This was performed using pSL301 (Invitrogen), a vector with an *Nco* I site within its polylinker. Therefore, *Nco* I/*Eco* RI digested pSL301 was utilised in a tripartite ligation with the \approx 370bp *Nco* I/*Xba* I fragment of the AtSHH genomic clone and an \approx 220bp *Xba* I/*Eco* RI fragment from pKS AtSHH-LUC (described in chapter 7) containing the cauliflower mosaic virus 35s terminator sequence. The resultant plasmid was named pAtSHH cassette. The 'cassette' was then cloned after each of the four previously described promoters, namely APG, AtSHH, CaMV35s and PR1a, within a pBluescriptTMSK- derived plasmid. To achieve this, *Kpn* I/*Nco* I promoter fragments, *Nco* I/*Pst* I cassette fragments and *Kpn* I/*Pst* I digested pBluescriptTMSK- were ligated together to form four intermediate constructs, an example of which can be seen in figure 8.1.

The Kpn I/Sma I fragments of each intermediate construct, were cloned into similarly cut pBin19 (Bevan, 1984), to form the final binary constructs. Due to an extra Sma I site carried from a previous polylinker into the intermediate plasmid containing the APG promoter, a Sma I fragment was ligated into pBin19 to form the binary plasmid from this plasmid. The content of each binary plasmid was confirmed by several diagnostic restriction enzyme endonuclease reactions. All four binary plasmids were transformed into LBA4404 Agrobacterium tumefaciens. Total agrobacteria DNA extractions were prepared and Southern blots performed to confirm that the constructs were harboured within the Agrobacterium (data not shown).

8.3 Construction of an SHH antisense construct

The overall scheme of the construction of a tobacco SHH antisense construct can be seen in figure 8.2. pROK II is essentially the binary plasmid pBin19 with two sequences previously cloned into the multiple cloning site (MCS). Firstly, a CaMV35s promoter has been cloned into the *Hind* III/Xba I sites of the MCS, and secondly a nopaline synthase termination site (NOS) has been cloned into the *Sac* I/*Eco* RI sites of the MCS. This therefore resulted in the construction of a plant binary vector to allow CaMV35s driven expression

of a desired sequence which will be polyadenylated, when this plasmid is transformed into plants. A simple one step cloning process allows the insertion of the previously cloned tobacco SHH MSPCR1-MSPCR3 PCR product (as described in chapter 4), in the antisense orientation with relation to the CaMV35s promoter, so that antisense SHH RNA will be transcribed. This tobacco PCR product was cloned into pBluescript™SK- via Xba I sites found at the end of both primers. Fortunately the orientation of the clone was such that on removal of the insert from pBluescript with the enzymes Bam HI/Sac I (no recognition sites for either of these enzymes are found in the PCR product itself), the clone is in the antisense orientation with respect to the position of the CaMV35s promoter in pROK II. Therefore following ligation to identically digested pROK II, the resultant plasmid contains the tobacco PCR product cloned into pROK II in an antisense orientation. This was confirmed by several diagnostic restriction enzyme endonuclease reactions, and the resultant plasmid named pTOBSHH-anti. This plasmid was transformed into LBA4404 Agrobacterium tumefaciens. PCR analysis of the bacterial transformants confirmed that the constructs were harboured within the Agrobacterium (data not shown).

8.4 Transformation of A. thaliana with SHH partial sense constructs

8.4.1 In planta A. thaliana transformation

To ascertain whether expression of the SHH partial sense transgene was deleterious in some of the constructs, numerical analysis of transformation frequency was considered to be advantageous. Therefore the *in planta*, *Agrobacterial* mediated transformation of *Arabidopsis* was performed (Bechtold *et al.*, 1993). This transformation procedure utilises whole *Arabidopsis* plants which are subjected to vacuum infiltration of a suspension of *Agrobacterium* harbouring the binary construct of interest. If deleterious effects occur in the transformed lines this may be observed, as the T1 generation is available for analysis within this transformation procedure. The actual transformation event is thought to occur during seed formation in the flowers (Bechtold *et al.*, 1993), however it should be noted that sectors of

transformed tissue have been noted in the adult T0 plants, following vacuum infiltration.

As the final optical density of the inoculating culture is critical to the experimental technique, this standardises the number of *Agrobacterium* used to infiltrate the plants. By utilising one tray of fifty plants, all sown at the same time and subsequently grown in identical conditions, the quantity and maturity of the *Arabidopsis* plants utilised was also standardised. Transformation was performed using this technique, with all four partial sense constructs and one *gus* reporter gene construct (pBI101 AtSHH correct, described in chapter 7) as a control for transformation frequency. Selection for kanamycin-resistant seedlings, from the entire seed set from all fifty plants utilised in each of the five transformations, yielded no transgenic seedlings, suggesting this *Arabidopsis* transformation procedure had failed.

8.4.2 A. thaliana root transformation

Following the failure of in planta transformation of Arabidopsis, the alternative method of root explant transformation was performed, according to Clarke et al. (1992). This method was not considered to be as ideal as in planta transformation, as many variables are involved in the frequency of transformation. Although bacterial density, explant age and cocultivation period are identical in every transformation, other factors affecting transformation frequency can vary. The removal of excess Agrobacterium following cocultivation is considered important to prevent overgrowth of root explants with Agrobacterium, which affects plant viability. The plating density of root explants has been shown to be critical for efficient transformation (Clarke et al., 1992). Although roots from the same number of seedlings were used for each transformation, the distribution of these roots within the agar could not be standardised. Despite these problems in assessing comparative data, transformations for all four partial sense constructs and one control gus reporter gene construct (same as before) were performed. Further inconsistencies of this transformation system were also observed. Unlike in planta transformation, this procedure proceeds via a callus stage. In tobacco the AtSHH promoter was shown to drive reporter gene expression in callus tissue,

Figure 8.3 Arabidopsis transformation frequencies

,

Table of the observed *Arabidopsis* transformation frequencies for each partial sense construct and the control construct, pBI101 AtSHH correct.

ABC1ConstructNumber of calliNumber of lines in whichNumber of2observedno shoot arose fromno seed s3APG2344APG2345APG2346AtSHH2057CaMV1748PR1a1829control201					,	
1ConstructNumber of callNumber of lines in whichNumber of2observedno shoot arcse fromno seed s3observedsexcised callusexcised4234sexcised callus5APG2346AISHH2057CaMV1749PR1a18210control201		A	œ	C	D	
2 observed no shoot arcse from no seed s 3	-	Construct	Number of calli	Number of lines in which	Number of lines in which	
3 excised callus excised 4 APG 23 4 5 APG 23 4 6 AISHH 20 5 7 CaMV 17 4 8 PR1a 18 2 9 control 20 1	N		observed	no shoot arose from	no seed set arose from	
4 APG 23 4 5 APG 23 4 6 AISHH 20 5 7 CaMV 17 4 9 PR1a 18 2 9 Control 20 1	ω			excised callus	excised shoots	
5 APG 23 4 6 AISHH 20 5 7 CaMV 17 4 8 PR1a 18 2 9 Control 20 1	4					
6 AtSHH 20 5 7 CaMV 17 4 8 PR1a 18 2 9	თ	APG	23	4	4	
7 CaMV 17 4 8 PR1a 18 2 9	თ	AtSHH	20	5	2	
8 PR1a 18 2 9 control 20 1	7	CaMV	17	4	з	
9 control 20 1	œ	PR1a	18	2	o	
10 control 20 1	9					
	0	control	20	-	-	

therefore any transformants containing an AtSHH or CaMV35s promoter construct may not develop beyond the callus stage if expression of the partial sense construct is deleterious to plant growth or development.

As previously described in chapter 7, the first generation available for analysis using this transformation procedure is the T2. This is a further problem as any deleterious effects which occur in the T1 generation, such as inviable pollen, may result in the loss of the T2 generation.

8.4.3 Transformation frequencies

The transformation frequencies were recorded for each construct and figure 8.3 tabulates the results obtained. The variance observed in the number of individual calli, putatively representing the number of individual transformation events, is small and may be due to the density of root explant plating. It was concluded that each partial sense construct generated transformants at approximately the same frequency as the control construct. However, it was noted that a larger proportion of the putative individual transformation events [calli] did not produce viable seed in each of the partial sense transformations, in comparison to the control transformation. The significance of this result is hard to interpret with such small sample size populations. Also, the number of lines in which the resultant seed set was kanamycin sensitive, representing escapes or silenced T-DNA inserts, was greater in the control experiment than in the four partial sense experiments. Therefore the overall number of individual transformations.

The results of this analysis identified 6 APG-SHH lines, 6 AtSHH-SHH, 9 CaMV35s-SHH and 9 PR1a-SHH lines for further analysis.

8.4.4 Initial analysis

The T2 was the first generation available for analysis, so a portion of the seed set from each T1 transformed plant was sown on germination media, containing 35μ g/ml kanamycin and 100μ g/ml augmentin (Clarke *et al.*, 1992). Following germination and 3 weeks growth, the resistant plants were analysed.

To confirm the presence of intact T-DNA in the kanamycin-resistant

putative transformants, total protein, total RNA and genomic DNA miniextractions were performed on grouped samples of tissue from a large number of plants from each line. The analysis of these extractions is described later.

8.5 Transformation of N. tabacum with an SHH antisense construct

The transformation of tobacco leaf disks was performed by standard techniques (Draper *et al.*, 1988). Once again, the same *gus* reporter gene construct was utilised as a control for the frequency of transformation. As previously described for the transformation of *A. thaliana*, the presence of a callus stage in the transformation protocol may be problematical, due to the expression of the antisense gene fragment by the CaMV35s promoter in this tissue.

Two separate transformation experiments were performed with both pTOBSHH-anti and a control reporter gene construct, pBI101 AtSHH correct. The number of calli arising on each leaf disk in the first transformation were counted upon removal of shoots for rooting. However analysis this late in the transformation procedure may bias the results, as any calli arising from transformation events in which deleterious expression of the antisense construct occurs may not be observed. Therefore, a second transformation experiment was performed in which the whole transformation experiment was sacrificed within two weeks of Agrobacterium inoculation, to try and accurately count the number of calli arising. The number of calli observed on 42 individual leaf disks for both pTOBSHH-anti and control transformations, were noted. The average number of calli observed in antisense construct transformations was 4.64 (standard deviation_{n-1} 1.71) while an average of 4.68 (standard deviation_{n-1} 1.18) calli were observed on disks transformed with the reporter gene construct. Therefore the transformation frequency observed for the SHH antisense construct in tobacco appears to be identical to that observed in control studies.

To ensure that each shoot chosen in the original transformation arose from a separate transformation event, only one shoot was excised from each leaf disk. This procedure was also performed on control transformation shoots to continue studies through the rooting process. Although a number of shoots containing each construct would not root, the observed variance between each construct was not significant, and was similar to previously observed levels. When roots had grown, each of the small plantlets was transferred to soil and grown to maturity in transgenic growth rooms. During this period frequent observations were made to assess any phenotypic difference between pTOBSHH-anti containing lines and *gus* reporter gene transformants. No phenotypic variance was observed in this T1 generation and therefore molecular analysis was performed. To standardise the material used in this analysis, a portion of the seed set of the T1 lines was sown on germination media containing $100\mu g/ml$ kanamycin and $100\mu g/ml$ augmentin to select for putative transformants.

Once again, despite the proven presence of kanamycin resistance in each seedling, the existence of a complete T-DNA is not assured and therefore similar extractions were performed, as those previously described for A. *thaliana* transformants.

8.6 Western analysis of transgenic plants

To assay if the presence of the T-DNA and putative expression of the partial or antisense SHH coding regions, had any effect on endogenous SHH protein levels within transgenic plants, western analysis was performed utilising the anti-AtSHH antibody raised in this project.

8.6.1 T2 Arabidopsis plants

Utilising the total protein extracted from grouped samples of kanamycin resistant plants from each line, western analysis was performed. To allow comparative data to be achieved the accurate loading of equal quantities of total protein was imperative. To achieve this, Bradford assays (Bradford, 1976) were performed on diluted samples of each extract. Once the protein concentration of each extract was known, all extracts were diluted to the same concentration, prior to the addition of SDS gel loading buffer.

To ensure that the above procedure resulted in equal loading of each sample, each SDS-PAGE gel was run in duplicate. One gel was stained with

Figure 8.4 Western analysis of *Arabidopsis* SHH partial sense transgenic lines

Figure 8.4A Western blot, of the protein sample extracted from control plants, probed with anti-SHH antibody. The samples loaded were always at the same concentration of $0.832\mu g/\mu l$, and only the volume of sample loaded was adjusted, as outlined below.

Lane 1	15µl	Lane 4	7.5µl
Lane 2	13.5µl	Lane 5	4.5µl
Lane 3	9.5µl	Lane 6	1.5µl

Figure 8.4B Western blot, probed with anti-SHH antibody, of equal quantity protein samples from T2 plants of each kanamycin-resistant CaMV35s driven partial sense transgenic *Arabidopsis* line. The lane numbers correspond to the following lines;

Lanes 1 and 11	Kanamycin resistant control line
Lanes 2-10	CaMV35s partial sense lines 1a, 2b, 3b, 5a, 6a, 7a,
	8a, 9b
	and 15b, respectively

Figure 8.4C Same as figure 8.4A using protein samples from T2 plants of each kanamycin-resistant PR1a driven partial sense transgenic *Arabidopsis* line. The lane numbers correspond to the following lines;

Lane 1 and 11	Kanamycin resistant control line
Lanes 2-10	PR1a partial sense lines 2b, 3a, 4b, 5a, 7a, 10b, 11a,
	12a and 18a, respectively

Figure 8.4D Same as figure 8.4A using protein samples from T2 plants of each kanamycin-resistant AtSHH driven partial sense transgenic *Arabidopsis* line. The lane numbers correspond to the following lines;

Lane 1	Kanamycin resistant control line
Lanes 2-7	SHH partial sense lines 1a, 2b, 3a, 7a, 10a and 18,
	respectively

Figure 8.4ESame as figure 8.4A using protein samples from T2plants of each kanamycin-resistant APG driven partial sense transgenicArabidopsis line. The lane numbers correspond to the following lines;

Lane 1	Kanamycin resistant control line
Lanes 2-7	APG partial sense lines 4b, 6a, 7b, 10b, 11a and
	14b
	respectively



Coomassie to visualise the proteins and confirm equal sample loading, while the other gel was western blotted. Following blotting, the proteins transferred to the membrane were visualised by Ponceau staining to ensure identical loading patterns to the Coomassie stained gel.

Each gel contained a control protein sample, treated as above, which was extracted from transgenic kanamycin-resistant *Arabidopsis thaliana* var. C24, 3 week old plants which had been sown on germination media containing 35μ g/ml kanamycin and 100μ g/ml augmentin, at the same time as the partial sense seed. This seed was donated by David Martin at Leicester, and was known to contain a T-DNA with no deleterious effects on the resultant plant.

To assess the amounts of SHH protein which could be distinguished within this assay, a further western blot was also performed. This blot only contained protein from the control plants and can be seen in figure 8.4. As described in the legend to figure 8.4A, decreasing volumes of protein extract were loaded in each lane. This result demonstrates that in the first three lanes, representing 15µl, 13.5µl and 9.5µl of protein extract respectively, the degree of SHH immuno-detected dye development is very similar. Only on comparison of the lane containing 9.5µl of extract to those lanes containing less extract can a clear visual difference be observed. This result suggests that between 12.48µg and 7.904µg of total protein this assay is saturated, however at quantities below 7.904µg a visual difference is observed.

Taking the results of this experiment into consideration, the blots for each of the partial sense constructs were developed. These blots can be seen in figure 8.4B-E. Within each blot no visual difference between each line or between the partial sense lines and the control sample can be observed. As the AtSHH promoter has been shown in the last chapter to drive reporter gene expression in every tissue tested, including all portions of 3 week old *Arabidopsis* plants, it was anticipated that this promoter, as well as the CaMV35s promoter, would drive high levels of expression of the SHH partial sense transgene in these tissues. Therefore, any marked variance in SHH protein levels were anticipated on blots from these lines. However both PR1a and APG promoter driven SHH partial sense lines were anticipated to have no variance in this experiment, unless DNA-DNA interactions occurred (see Figure 8.5 Western analysis of *Arabidopsis* SHH partial sense transgenic lines and tobacco SHH antisense transgenic lines.

Figure 8.5A Western blot, probed with anti-PR1a antibody, of equal quantity protein samples from T2 plants of some kanamycin-resistant PR1a driven partial sense transgenic *Arabidopsis* lines. The lane numbers correspond to the following lines;

Lanes 1-8PR1a partial sense lines 2b, 3a, 4b, 5a respectivelyLane 9Empty

Lanes 10 and 11 Kanamycin resistant control line

Two samples have been ran for each line, the odd numbered lanes were treated with salicylic acid before protein extraction and the even numbered lanes with water, as a control.

Figure 8.5B Western blot, probed with anti-SHH antibody, of equal quantity protein samples from T2 plants of some kanamycin-resistant PR1a driven partial sense transgenic *Arabidopsis* lines. The lane numbers and treatments correspond to the same lines as in figure 8.5A.

Figure 8.5C Western blot, probed with anti-PR1a antibody, of equal quantity protein samples from T2 plants of some kanamycin-resistant PR1a driven partial sense transgenic *Arabidopsis* lines. The lane numbers correspond to the following lines;

Lanes 1-6PR1a partial sense lines 7a, 10b, 11a respectivelyLanes 7 and 8Kanamycin resistant control line

Two samples have been ran for each line, the odd numbered lanes were treated with salicylic acid before protein extraction and the even numbered lanes with water, as a control. The converse is the case for the control samples.

Figure 8.5D Western blot, probed with anti-SHH antibody, of equal quantity protein samples from T2 plants of each kanamycin-resistant PR1a driven partial sense transgenic *Arabidopsis* lines. The lane numbers and treatments correspond to the same lines as in figure 8.5C.

Figure 8.5E Western blot, probed with anti-SHH antibody, of equal quantity protein samples from T2 plants of each kanamycin-resistant antisense transgenic tobacco line. The lane numbers correspond to the following lines;

Lanes 1	Kanamycin resistant control line
Lanes 2-7	Antisense lines 1, 2, 3, 4, 5, and 7 respectively



discussion), as the promoters would not drive expression of the SHH partial sense transgene in this tissue. A further experiment was performed using PR1a partial sense transgenic lines at this developmental stage. Twelve, three week old plants from sample lines were removed from the germination media and six plants floated on 1mM salicylic acid while the other six were floated on water, overnight. Protein was extracted, treated as before, and each set of equal concentration protein samples ran in triplicate on SDS-PAGE gels. As before one gel was stained with Coomassie to ensure equal loading while the other two were blotted and subsequently stained with Ponceau. Therefore, for each set of protein extracts, one blot was probed with anti-AtSHH antibody, while the other was probed with a PR1a antibody (kindly donated by Luis Mur at Leicester). The results of these western blots can be seen in figure 8.5. The anti-PR1a probed blots demonstrate the salicylic acid mediated induction of endogenous PR1a expression and no induction of PR1a by water. However, once again the anti-AtSHH probed blot shows no significant variance in SHH protein concentration between salicylic acid and water treated samples.

The lack of SHH protein variance between lines of each construct in all of the above experiments suggested two possibilities; firstly, the partial sense transgenes were not being expressed and hence would have no effect on SHH protein levels, or secondly, the degree of transgene expression is not causing a dramatic effect on SHH protein level leading to no obvious variance of SHH protein in this assay.

8.6.2 T2 tobacco plants

An identical experiment was performed using four week old kanamycin-resistant tobacco plants. The control plants for this experiment consisted of a previously analysed transgenic line containing pBI101 AtSHH correct, as described in chapter 7. The protein was extracted, and quantified in the same manner as previously described before being analysed in duplicate on SDS-PAGE gels. One gel was stained with Coomassie and the other blotted and stained with Ponceau. The resultant blot, following probing with SHH antibody, can be seen in figure 8.5E. As observed with the *Arabidopsis* samples no marked variance in SHH protein levels could be observed between lines or

Figure 8.6 RT-PCR primers and mechanism

A schematic representation of the described (see text) RT-PCR reaction utilised to analyse SHH transgene expression at the RNA level. The OG2 primer allows first strand cDNA synthesis due to its stretch of 17 T residues annealing to mRNA poly (A) tails. The resultant first strand cDNA product is represented by the green line to distinguish it from the mRNA, represented by the red line. By utilising an SHH specific primer (represented by the blue arrow) in the PCR utilising the first strand cDNA as a template, an SHH specific sense strand is first synthesised (represented by the purple line extending from the SHH specific primer). Using this sense strand as a template, the primer OG9 can anneal to allow production of the antisense strand due to the complementary nature of the synthesised 3' end of the sense strand. Therefore an SHH specific product is produced.



the control sample. Therefore the conclusions regarding the partial sense constructs could also be drawn for the antisense tobacco lines.

8.7 RT-PCR analysis of transgenic plants

Due to time constraints, northern analysis of each line was not performed, but to ensure that each line contained a transgene which was being expressed, and hence check one of the possible conclusions reached in the previous section, RT-PCR [reverse transcriptase polymerase chain reaction] was performed. First strand cDNA was produced from the total RNA extracts taken from the kanamycin-resistant T2 seedlings, as outlined in section 2.13.4. To prime this synthesis, primer OG2 was utilised, the sequence of which can be seen in figure 8.6. This primer contains a stretch of 17 T residues to allow annealing to all mRNA poly (A) tails. Therefore, all first strand cDNA contains this primer at it's 5' end. As well as containing a stretch of T residues this primer also encodes a further 18 base sequence which contains a Bam HI and Xho I site and is complementary to a further primer, OG9 (see figure 8.6). Therefore in the PCR, in which the first strand cDNA population was used as a template, OG9 annealed to the complementary region of OG2 to prime amplification. To produce a specific product the other primer utilised in the PCR reaction was specific for SHH. The SHH primer utilised varied with respect to the construct being analysed. In PCR from transgenic lines containing partial sense constructs, the SHH primer MSPCR1 was utilised (described in chapter 4), whereas in transgenic lines containing the antisense construct the SHH primer MSPCR3 was utilised (described in chapter 4). In utilising an MSPCR1 and OG2/9 primer combination, the endogenous SHH transcript could be amplified as well as the transgene.

8.7.1 Arabidopsis partial sense lines

The RT-PCR products from CaMV35s driven partial sense lines and AtSHH driven partial sense lines can be seen in figure 8.7. Within all lines, including control lines, the endogenous SHH product was amplified. The smaller partial sense transcript was also amplified from all partial sense transgenic lines. This result suggests all transgenic lines assayed were Figure 8.7 RT-PCR from *Arabidopsis* SHH partial sense transgenic lines

Figure 8.7A MSPCR1- and OG9-primed PCR products, amplified from RT synthesised first strand cDNA, ran on a 1% agarose gel. This proves all transgenic *Arabidopsis* containing a CaMV35s driven SHH partial sense transgene were expressing this transgene as mRNA. The lane numbers correspond to the following lines;

Lanes 1 and 12	1KB ladder
Lanes 2-9	CaMV35s partial sense lines 1a, 2b, 3b, 5a, 6a, 8a,
	9b and 15b, respectively
Lane 10	negative control for PCR (no template)
Lane 11	control for RT reaction (kanamycin resistant
	control line)

Figure 8.7B MSPCR1- and OG9-primed PCR products, amplified from RT synthesised first strand cDNA, ran on a 1% agarose gel. This proves all transgenic *Arabidopsis* containing an AtSHH driven SHH partial sense transgene were expressing this transgene as mRNA. The lane numbers correspond to the following lines;

Lane 1	1KB ladder
Lanes 2-7	SHH partial sense lines 1a, 2b, 3a, 7a, 10a and 18,
	respectively
Lane 8	negative control for PCR (no template)





_ endogenous product _ transgene product

B



_ endogenous product _ transgene product expressing the partial sense transgene.

8.7.2 Tobacco antisense lines

The RT-PCR products from seven transgenic antisense containing lines can be seen in figure 8.8A. This RT-PCR was not intended to be quantitative but to simply prove the transcription of the transgene to be occurring in the plant tissue. This is demonstrated within six of the seven lines tested. No product could ever be produced from line 5 in repeat experiments. Therefore genomic PCR was performed on the lines using the DNA extraction obtained from the 4 week old kanamycin-resistant plants. The result of this PCR, utilising a primer which anneals in the CaMV35s promoter and another which anneals 3' to the multiple cloning site, proved six of the seven lines contained this portion of the T-DNA (see figure 8.8B). This PCR product contains a portion of the CaMV35s promoter, 3' to the primer annealing site, the antisense SHH gene fragment, and the polyadenylation signal. To ensure that the DNA extraction from line 5 would allow PCR amplification, two primers annealing within the nptII gene were utilised to prove that all seven lines contained this portion of the T-DNA (see figure 8.8C). Therefore it was proposed that line 5 possibly contained an attenuated T-DNA in which no expression of the antisense SHH gene portion could occur. This line was therefore only utilised further as a control.

These experiments therefore demonstrated that in the six lines in which the whole T-DNA had been transferred, antisense SHH transcript was being expressed, despite the lack of effect on SHH protein levels observed upon western analysis.

8.8 Further analysis of the transgenic lines

As the western analysis revealed no significant reduction in SHH protein levels, despite proven transgene transcription, each line was left to mature with regular observation for any phenotypic effects.

8.8.1 Arabidopsis lines

Although APG driven partial sense SHH transgenic lines were analysed

Figure 8.8 RT-PCR and genomic PCR from tobacco SHH antisense transgenic lines

Figure 8.8A MSPCR3- and OG9-primed PCR products, amplified from RT synthesised first strand cDNA, ran on a 1% agarose gel. This proves five transgenic tobacco lines containing an SHH antisense transgene were expressing this transgene as mRNA. The lane numbers correspond to the following lines;

Lane 1	1KB ladder
Lanes 2-7	Antisense lines 1, 2, 3, 4, 5, and 7 respectively
Lane 8	negative control for RT reaction (kanamycin
	resistant control line)
Lane 9	negative control for PCR (no template)

Figure 8.88 CaMV and M13-27 primed PCR products amplified from genomic DNA, ran on a 1% agarose gel. This proves five transgenic tobacco lines contain the portion of the T-DNA encoding the transgene. This PCR product could not be amplified from line 5 DNA template. The lane numbers correspond to the following lines;

Lanes 1 and 10	1kb ladder
Lanes 2-7	Antisense lines 1, 2, 3, 4, 5, and 7 respectively
Lane 8	positive control for PCR (plasmid template)
Lane 9	negative control for PCR (untransformed plant)

Figure 8.8C KanI and KanII primed PCR products amplified from genomic DNA, ran on a 1% agarose gel. This proves all transgenic tobacco lines contain the portion of the T-DNA encoding the kanamycin selection gene. The lane numbers correspond to the same lines as in figure 8.8B.



by western analysis as three week old plants, no effect was expected due to the APG promoter only driving expression in the anthers. As discussed in the introduction to the chapter, these lines were produced in case all the other constructs proved to be too deleterious to the plants to generate transformants. The analysis of SHIH protein within the anthers of transgenic plants was not attempted due to its technical difficulty. Therefore to assess if the presence and expression of this construct had any effect on these plants, the quantity of seed set was analysed. Every remaining plant from each line was grown to maturity and left to set seed. This analysis therefore prevented the removal of anthers from flowers to extract RNA for RT-PCR analysis. The resultant seed set from each plant, of each line was collected and weighed. Although large variations in seed set weight were observed between each plant, as observed in the control set of plants, no significant variance could be observed between the seed set of one line or another, between APG driven partial sense lines and other partial sense lines or even the control lines.

As mentioned all other partial sense *Arabidopsis* plants were also left to mature and no phenotypic effects could be observed in any of these plants.

8.8.2 Tobacco lines

The T1 generation of each antisense line had previously been grown to maturity *in vivo* to achieve the T2 seed set, with no observed phenotypic effects. Despite this, the T2 plants were also left to mature in the greenhouse, to confirm the lack of phenotype.

8.9 Discussion

To perturb the endogenous expression of SHH in plants, constructs driving the overexpression of partial sense SHH constructs were tested in transgenic *Arabidopsis* and tobacco. It was anticipated that the cosuppression of SHH, leading to a decrease or removal of SHH enzyme activity, may cause dramatic phenotypic effects as all transmethylation reactions would be affected by SAH accumulation. These inhibited reactions would possibly include DNA methylation, an important mechanism in transcriptional regulation, as well as causing effects in several biochemical pathways (reviewed in chapter 1). Two mutations of the SHH gene itself have been studied and demonstrated to deleteriously affect transmethylation reactions. The previously described SHH null mutant in Rhodobacter capsulatus (Sganga et al., 1992; see chapter 1) is not lethal if growth supplements are provided. However, in a higher organism, mouse, an embryo lethal mutation [lethal nonagouti] has been attributed to a deletion of the SHH gene (Miller et al., 1994). Although the agouti gene controls coat colour in mice, the mutation in the above allele is proposed to be a deletion in which some 3' controlling elements of the agouti gene are deleted. However, it has been proposed that the embryo lethal nature of this mutant is due to this deletion also containing the mouse SHH gene. Experiments have demonstrated SHH mRNA to be present in heterozygous embryos at the time of homozygous embryo death, and the chemical inhibition of SHH activity in cell cultures causes a specific inhibition in inner cell mass development. The timing of embryo death correlates to a switch from long cell cycle times with low levels of DNA methylation, to a period associated with dramatic increases in both DNA methylation and cell proliferation. This data suggested that the lack of SHH activity in homozygotes caused embryo lethality (Miller et al., 1994). Therefore, the only two examples of SHH null mutations both caused phenotypic effects, but within the higher organism this was observed in a more dramatic manner.

It was predicted that a similar lethal phenotype may occur, if cosuppression led to inhibition of all SHH gene expression. However, as noted previously the cosuppression of SAM synthetase led to a distinct phenotype which was viable (Boerjan *et al.*, 1994). Although a significant reduction in measured SAM synthetase activity was observed in cosuppressed tissue in which the transgene could not be detected, SAM synthetase activity could still be detected. Therefore none of the cosuppressed plants contained a null phenotype. As previously highlighted, the inhibition of SAM synthetase activity and hence the reduction in the production of SAM was predicted to affect more pathways than the removal of SHH activity, due to the use of SAM in ethylene and polyamine biosynthesis. Therefore a 'constitutive' CaMV35s construct was utilised to copy this experiment. The use of the AtSHH promoter served a dual purpose. Firstly, if phenotypes were observed using the CaMV35s promoter it was anticipated that similar phenotypes may be observed using AtSHH, therefore proving its potential use as a 'constitutive' promoter. The second reason AtSHH was used, relates to the possible mechanisms controlling gene inactivation (reviewed by Flavell, 1994; Matzke and Matzke, 1995). One main distinction in the several models available for the mechanism of gene silencing is whether the silencing results from transcriptional inactivation, or a post-transcriptional process such as RNA turnover. It has been proposed that most cases of cosuppression involve a post-transcriptional process, presumably RNA turnover (Matzke and Matzke, 1995). A biochemical switch model suggests that RNA accumulates until a threshold is reached, at which point RNA degradation is initiated. This threshold may only be reached due to the expression of the homologous transgene as well as the endogenous gene, hence causing cosuppression. Transcriptional inactivation meanwhile can cause gene silencing in cases where only the promoter fragment of the transgene shows homology to the suppressed endogenous gene (see Park et al., 1996 for an example). It is thought that the pairing of homologous unlinked loci is responsible for the silencing effects, and this DNA-DNA interaction can generate inactive genetic states through either *de novo* methylation or heterochromatin formation (reviewed by Matzke and Matzke, 1995). Therefore if the levels of SHH partial sense transgene mRNA production were not sufficient to cause post-transcriptional silencing, the use of the Arabidopsis SHH promoter fragment may lead to transcriptional inactivation due to homology with the endogenous SHH gene's promoter. This phenomena may also possibly occur between the SHH partial-sense gene fragment and the endogenous gene. One proposed mechanism of this silencing, too complex to explain in detail here, is thought to operate via DNA methylation, one of the predicted affected pathways of SHH perturbation (Matzke and Matzke, 1995).

Following the failure of *in planta Arabidopsis* transformation (Bechtold *et al.*, 1993), *Arabidopsis* root transformation (Clarke *et al.*, 1992) was performed, which was not considered as ideal a system, due to the aforementioned points. The transformation frequency analysis performed during the transformation event suggested no significant difference between partial sense and control transformation experiments (later confirmed upon

completion of this experiment). The conserved nature of the SHH coding region between divergent plant species was demonstrated in chapter 4, and therefore the partial sense constructs based on the AtSHH sequence would probably interact with the endogenous tobacco SHH genes. Examples of heterologous transgene expression causing cosuppression have been observed (for example Dwivedi et al., 1994). Therefore each construct and the control construct were utilised in a tobacco transformation (results not shown). The resultant transformation frequencies showed no significant variation from the control transformation, and the resultant plants were phenotypically normal. Therefore no further experiments were performed on these transgenics. The result of this and the ongoing Arabidopsis transformation suggested that the partial sense constructs were not having the desired effects. Therefore the previously described tobacco antisense construct was made. Antisense transgene expression is also thought to act as a post-transcriptional process but with a slightly different mechanism to partial sense. An RNA degradation pathway is activated due to the presence of the antisense RNA species which may form RNA-RNA duplexes with the endogenous transcript. Therefore by utilising the tobacco antisense construct, it was hoped that gene silencing may occur via a separate mechanism.

As noted in the results both species transformation with each respective construct led to no significant variance in transformation frequency in comparison to control experiments. Although the sample size for the *Arabidopsis* transformation was small, the tobacco transformation was performed on a larger scale and still no variance was observed. These results suggest that the anticipated possible lethal effects of SHH cosuppression in callus and young differentiating tissue did not occur.

The western analysis of endogenous SHH protein within the transgenic plants suggested no single line contained a significantly lower quantity of SHH protein than any other transgenic or control line. The western blot containing different volumes of the same protein extraction, clearly demonstrated this assay to be able to distinguish between the specific quantities of SHH protein. However if the reduction in SHH protein levels were simply very low, for example a 5% reduction, then this assay was not sensitive enough to be able to distinguish these lines. If this did occur, this small reduction in SHH protein level had no effect on the plants' phenotype.

Since these experiments were designed and initiated, one example of SHH antisense expression in tobacco has been published (Masuta *et al.*, 1995). This study utilised the complete coding sequence of a cloned tobacco SHH cDNA in an antisense orientation behind the CaMV35s promoter. This resulted in some transgenic lines, with reduced endogenous mRNA levels, in which no endogenous protein could be detected by western analysis. These plants also contained an altered SAM/SAH ratio, although only half of the transgenics showed any phenotypic effect, simply described as growth alterations (Masuta *et al.*, 1995).

Due to the lack of similar results in this study, such as distinct differences in endogenous SHH protein levels upon western analysis, RT-PCR was performed to confirm that expression of the transgenes was occurring. If no expression of each transgene occurred then the mechanisms of posttranscriptional gene silencing described above could not occur. The RT-PCR analysis proved transgene expression in the CaMV35s and AtSHH driven partial sense constructs as well as the antisense construct. The use of the primer OG2 ensured that only polyadenylated transcript could be used as a template for first strand cDNA synthesis. This confirms that the transgene transcripts are polyadenylated but this does not necessarily mean that each transcript is stable. It is possible that the transgene transcripts are rapidly turned over due to being recognised as 'foreign' transcripts. This may reduce the possibility of cosuppression occurring. The RT-PCR result therefore simply proves transcription to occur and not whether this transcript is stable. One transgenic tobacco line was identified to contain an attenuated T-DNA, in which an intact *nptII* gene had been transferred and integrated into the genome unlike the antisense SHH portion of the T-DNA. Results of this kind may be eliminated in the future by the use of more recently developed binary vectors, for example the pPZP series (Hajdukiewicz et al., 1994), in which the selectable marker gene is positioned next to the left border T-DNA sequence. As the right border T-DNA sequence enters the plant DNA first in any Agrobacterial-mediated transformation, the sequences next to this region are more likely to be

transferred than those next to the left border. Therefore this causes most transgenics, selected by use of the selectable marker gene product, to have had the rest of the T-DNA transferred into the genome during the transformation event.

The lack of any phenotypic effects as well as no measurable decrease in SHH protein concentration, despite demonstrated transgene transcription, leads to two possible conclusions. Firstly, the perturbation of SHH gene activity was not achieved in this study, or secondly, any perturbation which did occur simply reduced the level of the SHH activity to such a small degree it was immeasurable within the confines of the assay used in this study, and caused no phenotypic effects.

If more time had been available, further work would have been performed upon these transgenic plants. This would have included SHH enzyme assays to measure activity within transgenic tissues compared to control tissues. However as the original analysis provided no indications of which plants were possibly cosuppressed, analysis of every plant generated was not performed in this study due to lack of time. Further transformation experiments would also be performed to generate a larger population of transgenic plants. In the study by Masuta et al. (1995), 230 transgenic tobacco lines were generated from which only four lines were studied in detail. However, this study utilised a simple assay to choose which lines to study prior to molecular analysis. Each line was inoculated with TMV, which it was hypothesised would not be able to replicate because of the lack of 5' cap mRNA methylation due to the altered SAM/SAH ratio. Those lines with reduced symptoms of viral infection were studied further (Masuta et al., 1995). However this study does not address how the endogenous transcripts are methylated at the 5' cap if the TMV transcripts are not. Also, no control experiment exists for inoculation of an 'unhealthy' tobacco plant (stunted phenotype) with TMV. However this study did result in the isolation of some transgenic plants from a large population, which have a reduced endogenous SHH protein level. Similar perturbation experiments also produce larger numbers of plants than derived in this work, and involve analysis of only the cosuppressed lines (for example, Boerjan et al., 1994).

Therefore, in conclusion, it is unknown whether this section of work did not achieve the desired aim of cosuppressing the endogenous SHH gene, or whether any cosuppression simply resulted in a very small reduction in SHH protein levels with no phenotypic effects.

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Chapter 9

Discussion

9.1 Summary of work in this thesis

The work described in this thesis originated with a clone isolated from a library constructed using poly (A) RNA isolated from mechanically separated cell suspension cultures. Preliminary studies of this clone, DB6, were performed prior to the onset of this thesis. These studies had putatively assigned DB6 as an asparagus SHH cDNA which appeared to be upregulated by the mechanical separation of asparagus cells.

Upon completion of the sequence analysis of DB6, the putative identity of the asparagus cDNA was confirmed by strong sequence conservation (90.1% identity at the amino acid level) to the recently published PcSHH sequence (Kawalleck *et al.*, 1992). This sequence analysis proved this clone was derived from a single transcript, but that it was truncated at both ends. The isolation of AoSHH (formerly DBF) provided a full length cDNA. RNA hybridisations demonstrated that AoSHH expression was not only upregulated by the mechanical separation of asparagus cells but also by the wounding of etiolated asparagus seedlings. AoSHH is therefore a wound inducible gene.

Sequence data base searches performed with the predicted protein sequence of the AoSHH clone highlighted the strong sequence conservation of the SHH gene throughout evolution. A single anomaly to this observation was analysed using a PCR based approach. This demonstrated that SHH proteins from all five diverse plant species tested contained an extra amino acid motif which had not been found in non-photosynthetic bacterial or animal genes. Similar analysis also showed that three of the five plant species analysed contain an intron in exactly the same position within the SHH gene, suggesting conservation of SHH gene structure as well as sequence.

Analysis of SHH protein levels and enzyme activity confirmed the induction of SHH in mechanically separated asparagus cells, and this activation was observed to be mainly regulated at the level of transcription. SHH transcript, protein and enzyme activity was detected in all tobacco or *A*. *thaliana* tissues assayed, confirming the housekeeping nature of this gene. The greatest levels of transcript abundance correlate to areas of high metabolic activity.

Genomic Southern analysis of three plant species demonstrated that both asparagus and tobacco probably contain small SHH gene families. This led to the isolation of an SHH genomic clone from *A. thaliana*, providing a putative promoter for analysis using reporter gene constructs in transgenic plants.

Reporter gene activities within transgenic tobacco confirm the wound inducible nature of SHH and its relatively high activity within the metabolically active cells in callus tissue. This analysis also confirmed the promoter driven expression of SHH in every tissue tested.

The attempted perturbation of SHH gene expression within transgenic plants by using partial and antisense technology did not produce the anticipated results. To definitively decide on the outcome of these experiments, further analysis would need to be performed (see section 9.2).

9.2 Proposals for future work

The following sections set out the proposed ideas to further analyse SHH gene expression, activity and its role.

9.2.1 Further analysis of the AtSHH promoter

As described in chapter 7, a presumed negative control construct for

reporter gene expression, gave positive results. This construct contained the AtSHH promoter utilised in the described experiments, in the incorrect orientation with respect to the gus gene in pBI101.1. Therefore it was concluded that the 1845bp fragment utilised as a putative AtSHH promoter could drive transcription in either orientation. This situation was thought to be due to one of two possibilities. Either the fragment contained a second promoter from a different A. thaliana gene, or strong enhancer elements within the fragment cause transcription from cryptic transcriptional start sites near the fusion. It was noted that the first explanation was very feasible as such a large fragment of DNA was tested for its putative promoter activity. If this promoter is to be used to drive expression of transgenes in future experiments (see section 9.3), it would be advantageous to be able to use a smaller promoter fragment with the same ability to drive gene transcription. Therefore a series of promoter deletion experiments should be performed to define the limits of the SHH promoter within the original DNA fragment utilised. These studies may also help explain the ability of the original promoter fragment to drive gene expression in both orientations. Current approaches to this type of study include analysis of 5' deleted promoter-reporter gene fusions, either transiently by direct introduction into protoplasts, or by stable transformation of plants. Several convenient restriction endonuclease recognition sites appear throughout the 1845bp Xho I fragment initially used for promoter constructs, allowing simple 5' deletions to be created. These 5' deletions may corroborate the presence of another promoter, as a viable explanation to the bidirectional activity of the original promoter fragment. In these studies each deletion would need to be placed in the vector in either orientation.

The 5' deletion analysis may also be complemented by 3' deletion analysis. This technique relies on fusing upstream regions of the promoter to minimal sequences that can drive transcription (for example, the -40bp CaMV35s promoter). This analysis would complement the 5' deletion analysis since rather than looking for 'loss of activity', with the 3' deletion fusions the 'gain of activity' is sought.

9.2.2 Further experiments on perturbation studies

Due to the lack of time and expected phenotypic results, the transgenic lines containing constructs designed to perturb SHH gene expression were not fully analysed. If, as predicted, the lines undergoing the most severe perturbation had developed a phenotype, then these lines could have easily been chosen for further study. As no such phenotypic indications were observed in the described study (chapter 8), unlike those of Masuta *et al.* (1995), too many lines existed to perform further analysis. Had more time been available or lines with altered phenotypes been produced, then northern analysis of endogenous and transgene SHH transcript would have been performed and compared to wild-type lines to assess if decreased expression levels had occurred. SHH enzyme analysis would also have been performed to assess if the actual activity of SHH enzyme within these transgenic lines was decreased. If more time had been available more transgenic lines would have been produced, leading to an increased probability of positive results being obtained for each of the constructs.

9.2.3 Plant SHH enzymes: copper-binding proteins?

As described in chapter 1, SHH enzyme from mice has been proposed to be a bifunctional protein, not only catalysing SAH hydrolysis but also acting as a copper-binding protein (Bethin *et al.*, 1995a). This aspect of possible SHH enzyme function has not been assayed using SHH enzyme isolated from a plant source. Results presented within this thesis suggest SHH enzyme levels are induced upon the mechanical separation of asparagus cells. This may constitute an ideal starting tissue for the isolation of SHH enzyme [utilising one of the previously published methods for SHH purification from plant tissue (Poulton and Butt, 1976; Guranowski and Pawelkiewicz, 1977; Sebestova *et al.*, 1984)] to analyse its putative copper-binding activities. Once active enzyme has been isolated, similar studies to Bethin *et al.* (1995b) could be performed to assess if the isolated enzyme had copper attached, as observed with mouse SHH, or if any copper associated with the purified protein would exchange with radioactive copper. These studies would provide evidence for the copper-binding role of SHH proposed in mice, also occurring in plants.

9.3 Concluding remarks on SHH and possible applications

Upon the isolation and subsequent sequencing of AoSHH, which at the time, was only the second plant SHH cDNA derived and the first from a monocot species, analysis of sequence conservation was performed. As previously discussed in chapters 3 and 4 the percentage sequence identity observed between AoSHH and the dicot parsley, the bacterium R. capsulatus and the mammalian human predicted amino acid sequences were 90.1%, 58.7% and 58.1% respectively. This concurred with previous observations of a high degree of primary amino acid sequence conservation in SHH cDNAs from diverse species (Kasir et al., 1988; Sganga et al., 1992). This conservation was also observed to occur at the nucleotide level (chapters 3 and 4), allowing the use of degenerate PCR primers to amplify portions of SHH genes from diverse plant species and also from some bacterial species. Sequence analysis of SHH PCR products followed by alignments of the predicted amino acid sequence allowed dendrogram alignments of SHH sequence conservation between diverse plant species to be drawn (chapter 4). This form of analysis is utilised as a major tool in molecular evolution studies. These analyses compare slowly evolving RNA and protein sequences to try and identify when two species last shared a common ancestor (see Mooers and Redfield, 1996). As SHH is a gene with a proven conserved amino acid sequence throughout diverse species, it may be utilised for this type of work in the future. For analysis of plant species evolution, the primer sets already constructed and utilised in this study could be used to amplify portions of SHH sequence from many more plant species. Not only can amino acid sequence conservation be studied but also the presence or absence of the conserved position intron found in this analysis within certain plant SHH genes (chapter 4). Intron position and presence has previously been used in studies of molecular evolution of plant genes (Niesbach-Klösgen et al., 1987).

It has been suggested that by only assaying one protein sequence, results of such studies may be biased. Therefore a number of different protein sequences should be compared. The largest example of this type of study was used to try and evaluate the time of divergence of the major biological groupings (Doolittle *et al.*, 1996). This study utilised 57 different proteins, each of which had to comply to several criteria, including its isolation from at least three species including an animal, a plant or fungus and a bacterium. SHH fulfilled all of the criteria but for an unstated reason was not utilised in this study. This may be due to the presence of the extra amino acid motif in SHH sequence from photosynthetic species. However, as demonstrated by Aksamit *et al.* (1995), the SHH gene can be split into regions for the basis of sequence identity. Therefore, the values obtained from comparisons of sequence from the initial methionine to the amino acid preceding the extra amino acid motif, or from the first amino acid following the extra amino acid motif to the last amino acid, could be used. Therefore on this basis SHH could be another enzyme utilised in the study by Doolittle *et al.* (1996).

In conclusion, it has been demonstrated that SHH is a good candidate gene for molecular evolution studies between both plant species and other more diverse organisms.

An SHH cDNA was initially derived from a library containing transcripts from mechanically separated asparagus cells, a model system for the study of wounding. Northern analysis confirmed the wound induction of AoSHH (chapter 3), while western and enzyme activity analysis confirmed this data in tobacco as well as asparagus (chapter 5). Upon generation of transgenic plants containing AtSHH promoter-driven reporter gene constructs, the wound-inducible nature of SHH was verified at the transcriptional level from a third species, A. thaliana (chapter 7). It can therefore be concluded from these several lines of evidence that SHH is a housekeeping gene, expressed in all tissues assayed, whose expression can be upregulated by a wound stimulus. A promoter providing this type of transcriptional activity may be commercially useful, as a possible alternative to the use of the CaMV35s promoter. Comparative data presented in chapter 7 suggested equal if not higher reporter gene activity in transgenic A. thaliana seedlings containing similar gus gene constructs driven by either the AtSHH promoter or CaMV35s promoter. Therefore the AtSHH promoter may be utilised in the future as an alternative to CaMV35s for the general expression of a transgene. One of the most common uses of CaMV35s is to drive the expression of the selectable marker gene within T-DNA constructs. As the AtSHH promoter drives upregulated
expression in both wounded and callus tissue it would be ideal for this purpose in transformation experiments which occur via this tissue, such as tobacco transformation. As well as being an alternative promoter for transgene expression, AtSHH may be used in conjunction with CaMV35s within the same experiment. For example, when a transgenic plant is transformed with a second construct or crossed to another transgenic line, a second selectable marker gene is utilised to be able to select the double transformant. As described in chapter 8 the presence of the same promoter on both T-DNAs may lead to a cosuppression event (for example, Park *et al.*, 1996), hence the use of AtSHH as an alternative to CaMV35s.

One further advantage and possible application for the AtSHH promoter related to its upregulation by wounding, would be to drive expression of a defence gene. This gene would be expressed in all tissues so far noted to contain SHH gene expression (see chapter 7), and this expression would increase upon a wound stimulus leading to greater accumulation of the defence protein. Studies similar to these are currently being performed by ZENECA.

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Appendix I Solutions

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<u>Agrobacterium</u> Culture Dilution Media Gamborg's B5 Salts Solution 20g/l glucose 0.5g/l MES Adjust to pH5.7 with 1M KOH.

Anode Buffer 1 300mM Tris-HCl pH10.4 10% Methanol

Anode Buffer 2 25mM Tris-HCl pH10.4 10% Methanol

Anti-Proteinase Extraction Buffer 50mM Sodium Phosphate Buffer pH7.0 1mM EDTA pH8.0 0.2mM PMSF 1µM Pepstatin A 2µg/ml Leupeptin 1µM E64

Asparagus Medium: Modified from Nagata and Takebe (1971).ComponentConcentration (mg/l)NH4NO3825.000KNO3925.000CaCl2.2H2O220.000

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MgSO4.H2O	1233.000
KH2PO4	680.000
Na2EDTA	37.300
FeSO4.7H2O	27.800
H3BO3	6.200
MnSO4.4H2O	22.300
ZnSO4.7H2O	10.580
KI	0.830
NaMoO4.2H2O	0.250
CuSO4.5H2O	0.030
Mannitol	30,000.000
Sucrose	10,000.000
Myo Inositol	100.000
Thiamine HCl	1.000
NAA	1.000
6-BAP	0.300

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Adjust pH to 5.8 with KOH and just prior to use add 3.4ml of filter sterile glutamine (2.35g/100ml) per 80ml of medium.

ATP Buffer 50mM HEPES pH7.6 20mM MgCl2 10mM ATP

Automatic Sequencing Reaction Premix 4µl 5X TACS Buffer 1µl dNTP Mix 1µl DyeDeoxy™ A Terminator 1µl DyeDeoxy™ T Terminator 1µl DyeDeoxy™ G Terminator 1µl DyeDeoxy™ C Terminator 0.5µl AmpliTaq[®] DNA Polymerase (all of the above are purchased from Applied Biosystems as a kit)

<u>BCIP Buffer</u> 100mM Tris-HCl pH9.5 5mM MgCl₂ 100mM NaCl

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<u>Blot Strip Solution</u> 0.1X SSC 0.1% SDS 0.2M Tris-HCl pH7.5

<u>10X Blunt End Ligation Buffer</u> 0.66M Tris-HCl pH7.6 50mM MgCl₂

50mM DTT 0.1M ATP 0.1M Spermidine 10mM Hexamine Cobalt Chloride

Bradford Solution 600mg/l Serva Blue G250 stain 2% Perchloric acid

<u>Callus Inducing Media</u> Gamborg's B5 medium (Gamborg *et al.*, 1968) 0.5g/1 MES 20g/1 glucose Adjust pH to 5.8 with 1M KOH 8g/1 Difco Agar Following autoclaving add:-5mg/1 sterile silver thiosulphate 0.5mg/l sterile 2,4-dichlorophenoxyacetic acid 0.05mg/l sterile kinetin

Cathode Buffer 25mM Tris-HCl pH9.4 40mM 6-Aminohexanoic Acid (also called Amino-*n*-caproic acid) 20% Methanol

<u>CIP Buffer</u> 50mM Tris-HCl pH8.0 0.1M EDTA

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<u>Coomassie Stain</u> 0.25g Coomassie Brilliant Blue R/100ml of 10% acetic acid and 10% methanol

2X CTAB Extraction Buffer 100mM Tris-HCl pH8.0 1.4M NaCl 20mM EDTA 2% CTAB

<u>10% CTAB</u> 10% CTAB 0.7M NaCl

CTAB Precipitation Buffer 50mM Tris-HCl pH8.0 10mM EDTA pH8.0 1% CTAB

6X DNA Loading Buffer 0.25% Xylene Cyanol FF 0.25% Bromophenol Blue 15% Ficoll (Type 400)

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Denaturing Solution 1.5M NaCl 0.5M NaOH

50X Denhardt's 1% B.S.A.(Bovine Serum Albumin) 1% P.V.P (Poly Vinyl Pyrrolidone) 1% Ficoll (Type 400)

Depurinating Solution 0.25M Hydrochloric Acid

Germination Medium Half-strength Murashige and Skoog Medium (Murashige and Skoog, 1962) 10g/l sucrose Adjust pH to 5.8 with 1M KOH 8g/l Difco Agar Following autoclaving add sterile silver thiosulphate to a final concentration of 5mg/l

<u>Glycine Running Buffer</u> 25mM Tris 250mM Glycine pH8.3 0.1% SDS

GUS Extraction Buffer 50mM Sodium Phosphate Buffer pH7.0 10mM EDTA 0.1% Triton X-100 0.1% Sarkosyl 10mM &-mercaptoethanol <u>GUS Fluorometric Assay Buffer</u> 1mM methyl umbelliferyl glucuronide in GUS extraction buffer

GUS Histochemical Buffer 50mM Sodium Phosphate Buffer pH7.0 1mM EDTA 0.5mM potassium ferricyanide 0.5mM potassium ferrocyanide 1mg/ml X-Gluc (5-bromo-4-chloro-3-indoyl glucuronide)

<u>High Salt Buffer</u> 3M Sodium Acetate 0.01% Bromophenol Blue pH7.9 with glacial acetic acid

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Hybridisation Solution (DNA) 5X SSPE 5X Denhardt's 0.5% SDS 100µg/ml boiled Herring Sperm

Hybridisation Solution (RNA) 5X SSPE 5X Denhardt's 0.5% SDS 100µg/ml boiled Herring Sperm 50% Formamide

Infiltration Medium Murashige and Skoog macro and micronutrients (used as per manufacturers instructions) 10µg/l 6-benzyl amino purine 5% sucrose 5X Ligation Buffer 0.25M Tris-HCl pH7.6 50mM MgCl₂ 5mM ATP 5mM DTT 25% PEG8000

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Luciferase Extraction Buffer 100mM phosphate buffer pH7.5 1mM DTT

10X MOPS 0.2M MOPS pH7.0 80mM Sodium Acetate 10mM EDTA pH8.0

MSD4X2

As MSO with the addition of:-0.1mg/l naphthalene acetic acid (NAA) 1mg/l 6-benzyl amino purine (BAP)

<u>MSO</u>

MS salts (Flow Laboratories) 10g/l sucrose Adjust to pH5.8 with 0.1M KOH. To solidify the media technical agar was added to 0.8%.

<u>Neutralising Solution</u> 1.5M NaCl 0.5M Tris-HCl, pH8.0

Nuclease-S1 Hybridisation Buffer 40mM PIPES pH6.4 1mM EDTA pH8.0 0.4M NaCl 80% Formamide

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Nuclease-S1 Mapping Buffer 0.28M NaCl 0.05M Sodium Acetate pH4.5 4.5mM ZnSO4 20µg/ml single-stranded DNA (carrier DNA) 100-1000 units/ml nuclease-S1

<u>Nuclease-S1 Stop Buffer</u> 4M Ammonium Acetate 50mM EDTA pH8.0 50µg/ml tRNA (carrier RNA)

<u>N.E.W. Wash</u> 50% ethanol 100mM NaCl 10mM Tris-HCl pH7.5 1mM EDTA

Oligolabelling Buffer The final buffer is made by mixing Buffers A,B and C in the ratio 2:5:3. OLB A 625µl of 2M Tris-HCl pH8.0 25µl of 5M MgCl2 350µl of H2O 18µl of &-mercaptoethanol 5µl each of 3µM dATP, dGTP, dTTP OLB B 2M HEPES pH6.6 OLB C Random hexadeoxyribonucleotides (Pharmacia) suspended in 3mM Tris-HCl pH7.0, 0.2mM EDTA at 90 OD₂₆₀ units/ml.

11X PCR Buffer

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167μl 2M Tris-HCl pH8.8
83μl 1M Ammonium Sulphate
33.5μl 1M MgCl₂
3.6μl β-mercaptoethanol
3.4μl 10mM EDTA pH8.0
75μl of each 100mM dNTP stock
85μl 10mg/ml BSA

PCR Extraction Buffer

100mM Tris-HCl pH8.0 50mM EDTA pH8.0 500mM NaCl 1.4% SDS 10mM ß-mercaptoethanol

PEG/Salt Solution 2.5M NaCl 20% PEG 6000

Phage Precipitation Buffer 20% PEG8000 2M NaCl

Phenol/Chloroform

The following reagents were mixed in the ratio 25:24:1 respectively. Tris-HCl pH8.0 equilibrated Phenol Chloroform Isoamyl Alcohol <u>Phosphate Buffer</u> Mix K₂HPO₄ and KH₂PO₄ in a 82ml:18ml ratio to achieve a pH of 7.5.

Ponceau Stain 0.5% Ponceau 1% acetic acid

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Reverse Transcription Mix4µl 25mM MgCl22µl 10X Reverse Transcription Buffer2µl 10mM dNTP Mix0.5µl Recombinant RNasin Ribonuclease Inhibitor0.8µl AMV Reverse Transcriptase2µl OG2 Primerxµl Template RNAyµl of dH2O to yield a final volume of 20µl

10X Reverse Transcription Buffer 100mM Tris-HCl pH8.8 500mM KCl 1% Triton X-100

RNA Denaturing Solution 65% Formamide 8% Formaldehyde 1.3X MOPS

RNA Extraction Buffer 50mM Tris-HCl pH9.0 150mM LiCl 5mM EDTA pH8.0 5% SDS RNA Formaldehyde Gel 1X MOPS 2.2M Formaldehyde 0.8g Agarose Make up to 100ml with H₂O.

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<u>RNA Grinding Buffer</u>
6% 4-Amino Salicylate
1% Tri-Isopropyl Napthalene (TNS)
6% Phenol
50mM Tris-HCl pH8.4

RNA Loading Buffer 50% glycerol 1mM EDTA pH8.0 0.25% Bromophenol Blue 0.25% Xylene Cyanol FF

2X SDS Gel Loading Buffer
100mM Tris-HCl pH6.8
4% SDS
0.2% Bromophenol Blue
20% Glycerol
200mM DTT (added from a 1M stock just prior to use)

Sequencing Annealing Buffer (5X) 200mM Tris-HCl pH7.5 100mM MgCl₂ 250mM NaCl

Sequencing Enzyme Dilution Buffer 10mM Tris-HCl pH7.5 5mM DTT
0.5mg/ml BSA

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Sequencing Gel Mix 7M urea 5.7% acrylamide 0.3% bis-acrylamide 1X TBE

Sequencing Labelling Mix 1.5μM dGTP 1.5μM dCTP 1.5μM dTTP

Sequencing Stop Solution 95% formamide 20mM EDTA 0.05% Bromophenol Blue 0.05% Xylene Cyanol FF

Sequencing Termination Mixtures Each mixture contains 50mM NaCl and, 80μM dGTP 80μM dATP 80μM dTTP 80μM dCTP In addition, the 'G' mix contains 8μM dideoxy-dGTP; the 'A' mix contains 8μM dideoxy-dATP; the 'T' mix contains 8μM dideoxy-dTTP; and the 'C' mix contains 8μM dideoxy-dCTP.

<u>Shoot Elongation Media</u> As Germination Media without the addition of Silver Thiosulphate.

SHH Enzyme Assay Buffer

0.1M Tris-HCl pH8.0 5mM DTT

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SHH Enzyme Extraction Buffer SHH Enzyme Assay Buffer plus the addition of:-10mM Sodium Metabisulphite 10mM Ascorbic Acid

Shoot Inducing Media Gamborg's B5 medium 0.5g/1 MES 20g/1 glucose Adjust pH to 5.8 with 1M KOH 8g/1 Difco Agar Following autoclaving add:-850mg/1 sterile vancomycin 35mg/1 sterile kanamycin 5mg/1 sterile 2-isopentenyladenine 0.15mg/1 sterile indole-3-acetic acid

<u>SM</u> 100mM NaCl 8mM MgSO4 50mM Tris-HCl pH7.5 0.01% gelatin

Shoot Overlay Media As Shoot Inducing Media except the 8g/l Difco Agar is replaced by 8g/l of low melting-point agarose.

20X SSC 3M NaCl 0.3M Sodium Citrate

<u>20X SSPE</u>

3.6M NaCl 0.2M NaH2PO4 0.02M EDTA pH8.0

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0.3M NaCl 10mM Tris-HCl pH8.0 1mM EDTA

10X T4 Polynucleotide Kinase Buffer 0.5M Tris-HCl pH7.6 0.1M MgCl₂ 50mM dithiothreitol 1mM Spermidine HCl

1mM EDTA pH8.0

<u>10X TAE</u>

0.4M Tris-acetate pH8.0 10mM EDTA

<u>10X TBE</u>

108g Tris Base 55g Boric acid 5.8g EDTA Dissolve in a 900ml of H₂O and pH to 8.3 before making up to 1 litre.

<u>TBS Tween</u> 200mM NaCl 50mM Tris-HCl pH7.4 0.1% Tween 20

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Wash Solution A 3X SSC 0.5% SDS

Wash Solution B 0.5X SSC 0.5% SDS

Appendix II Bacteriological Media

Liquid media

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 \mathbb{NB} (nutrient broth) was purchased as a pre-made powder from Difco Laboratories and dissolved in water.

LB: per litre -	10g NaCl
	10g bacto-tryptone
	5g Yeast Extract
2X YT: per litre -	10g NaCl

16g bacto-tryptone 10g Yeast Extract

NZY: per litre - 5g NaCl

2g MgSO4 · 7H2O 5g Yeast Extract 10g NZ Amine (casein hydrolysate)

 $\ensuremath{\mathbb{NZCYM}}$: per litre - As for $\ensuremath{\mathbb{NZY}}$ with the addition of 1g of Casamino Acids

Solid media

Media was solidified by the addition of bacto-agar to 1.5%, except for Top Agar which is NZY with the addition of 0.7% bacto-agar.

NA (nutrient agar) was purchased as a pre-made powder from Difco Laboratories and dissolved in water in individual bottles.

To sterilise media it was autoclaved at 120°C for 20 min.