MOLECULAR CHARACTERISATION OF THE SERINE ACETYLTRANSFERASE GENE-FAMILY FROM 'ARABIDOPSIS THALIANA'

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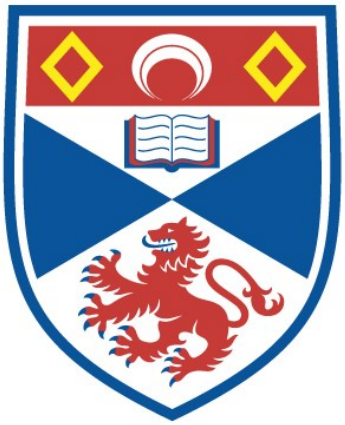
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Molecular Characterisation of the Serine Acetyltransferase Gene-Family from *Arabidopsis thaliana*

By

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Abstract

Formation of *L*-cysteine, from *L*-serine and sulphide, represents the principal route of sulphur incorporation into organic compounds in living organisms. Cysteine biosynthesis in plants is achieved by two enzymes, serine acetyltransferase (SAT) and *O*-acetylserine (thiol) lyase (*O*ASTL), which form a cysteine synthase complex.

Three novel cDNA species, Sat-52, Sat-53 and Sat-106, encoding SAT isoforms from *A. thaliana* were isolated from a collection of cDNAs previously cloned by functional complementation of the *E. coli cysE* mutant strain JM15, which is defective in serine acetyltransferase. Deduced amino acid sequence analysis suggests that Sat-52 encodes a putatively mitochondrial isoform whilst Sat-53 and Sat-106 encode proteins with cytoplasmic locations. Sequence information derived from the Arabidopsis Genome Initiative allows mapping of *Sat-52*, *Sat-53* and *Sat-106* genes to locations on chromosomes V, I and II respectively. A fourth SAT cDNA from *A. thaliana*, Sat-1, was cloned prior to the work detailed here and encodes a putatively plastidic isoform of the enzyme. Southern hybridisation against digested genomic DNA suggests that each SAT gene is represented by a single copy in the *A. thaliana* genome.

DNA probes specific to the SAT gene-family members were designed and used in various studies to examine expression of SAT genes. Northern blotting and hybridisation was used to determine transcript distribution between root, leaf, stem, flower and silique tissues and to study the expression of the gene-family in response to sulphate and nitrate nutrition. Spatial distribution of *Sat-52* and *Sat-53* transcript in root, leaf and stem tissue was also examined by *in situ* hybridisation using specific riboprobes. Both genes are highly expressed in leaf trichomes. The *Sat-52* transcript was also localised to the vascular bundles of root, leaf and stem tissue. The isoforms encoded by *Sat-52* and *Sat-53* are hypothesised to have specific roles in these cell-types. Expression of the *Sat-53* gene in

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Nomenclature

Sat-52.....cDNA encoding serine acetyltransferase

pSAT52.....cloning vector pYES containing the Sat-52 cDNA

JM15/pSAT52.....*E. coli* strain JM15 harbouring the vector pSAT52

Sat-52.....serine acetyltransferase gene and transcribed mRNA

SAT52.....serine acetyltransferase protein

The same nomenclature is used for all serine acetyltransferase isogenes cloned in this laboratory. Clones from other laboratories are assigned their published nomenclature.

Abbreviations List

APS	Adenosine 5'-phosphosulphate
APSK	APS kinase
APSR	APS reductase
APSST	APS sulphotransferase
ATP	Adenosine triphosphate
ATPS	ATP sulphurylase
BAC	Bacterial Artificial Chromosome
Car-SH	Carrier thiol
CIAP	Calf Intestinal Alkaline Phosphatase
CoA	Coenzyme A
CTßL	Cystathionine ß-lyase
CTγS	Cystathionine γ-synthase
DMSP	ß-dimethylsulphoniopropionate
DPNPase	3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase
GSH	Glutathione
LB	Luria-Bertani bacterial growth medium
MSase	Methionine synthase
OAS	*O*-acetyl *L*-serine
OASTL	*O*-acetyl *L*-seine (thiol) lyase
PAPS	3'-phosphoadenosine 5'-phosphosulphate
PAPSR	PAPS reductase
PPi	Inorganic pyrophosphate
PRH	PAPS reductase homologue
SAT	Serine acetyltransferase
SiR	Sulphite reductase
S^{2-}	Sulphide
SO_3^{2-}	Sulphite

SO_4^{2-}	Sulphate
ST	Sulphate transporter
TB	Transformation buffer
Trx_{ox}	Oxidised thioredoxin
Trx_{red}	Reduced thioredoxin
YAC	Yeast Artificial Chromosome

Chapter 1: Introduction

1.1 Sulphur, Plants and the Environment

1.1.1 Environmental sulphur availability

Our understanding of the role of sulphur in plants has never been so important as at present. Although relatively abundant in the environment (up to 0.5 mM in arable soils (Syers et al. 1987)), legislative cuts in SO_2 emissions and fertiliser usage are seeing many crops, previously grown in areas where sulphur deposition exceeded requirement, facing large nutritional deficits. Acquisition of sulphur is now the major nutritional problem facing arable crops throughout Europe (Schnug 1992, 1994), and here in Scotland 100% of oil-seed rape produced is sulphur deficient (Sutton 1994).

Between 1980 and 2003 the British government aims to cut SO_2 emissions by 60%, leading to as much as 50% of our entire crop-lands being at risk of sulphur deficiency (Ceccotti & Messick 1997). Due to the high mobility of sulphate ions, and consequent leaching rates in soil, plants will rapidly experience the effects of such reductions in sulphur availability. Sulphur deficiencies directly lead to reductions in sulphur-containing secondary metabolites, amino acids and total plant proteins (Schnug 1997). As sulphur content affects the quality of a wide range of commercially important plant products, our knowledge of sulphur metabolism in plants must be keenly pursued. Only by understanding the role of the relevant processes involved will we ultimately be able to achieve and control required levels of plant sulphur nutrition in the increasingly austere environment.

1.1.2 Sulphur-containing plant compounds

After nitrogen, sulphur is the second most abundant element taken up and metabolised by plants. The vast majority of this comes from the soil in the form of sulphate, although SO_2 and H_2S can also be metabolised (Ernst 1993). The nitrogen to sulphur ratio of 20:1 found in most plants represents the proportions of the elements in proteins (Dijkshoorn & van Wijk 1967). Only in sulphur-accumulating plants does the N to S ratio decrease due to the storage of such compounds as glucosinolates in members of the *Brassicaceae*, S-alkylcysteine sulphoxides (alliins) in the *Liliaceae* or inorganic sulphates in species such as *Welwitschia*, *Desmarestia*, *Gossypium* and *Lepidium* (Evans 1975; Schnug 1993).

In fact, sulphur is perhaps the most versatile biological element, being incorporated into primary metabolites such as proteins, vitamins and coenzymes, secondary metabolites such as glucosinolates, thiols, thiazoles, cyclic disulphides and alliins (Schnug 1993) and also having a structural role. The secondary metabolites have functions as wide ranging as pathogen defence (Bennett & Wallsgrove 1994), signalling molecules (Matsubayashi & Sakagami 1996) and storage compounds (Srivastava & Hill 1974). Glucosinolates, for example, can be catabolised by plants in the event of sulphur starvation releasing thiocyanates and sulphate for the synthesis of primary products.

Sulphur is also structurally important. Disulphide bonds are found in both intermolecular associations, binding different protein molecules and enzyme subunits, and intramolecular associations maintaining folding and thermostability of enzymes. Specific disulphide bonds between residual proteins complexed with nuclear DNA are responsible for the structural organisation of chromosomes (Struchov et al. 1995).

Unlike nitrogen, sulphur is also required in its oxidised state in a variety of plant compounds. Oxidised sulphur can be bound as a sulphonic acid directly to the carbon skeleton of lipids forming sulpholipids (sulphoquinosovyl diacylglycerol) which are incorporated into the inner chloroplast and chloroplast thylakoid membranes (Heinz 1993). The donor of the sulphur in its oxidised state is likely to be 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (see section 1.2.11), transferred by a sulpholipid kinase, although the oxidation of a previously reduced sulphur source cannot be ruled out (Schmidt & Jäger 1992). PAPS-specific sulphotransferases are involved in the transfer of oxidised sulphur to desulphoglucosinolates forming sulphoglucosinolates (Glendening & Poulton 1990) and sulphation of tyrosine using the sulpho-group from PAPS is involved in phytosulphokine metabolism in *Asparagus officinalis*. Phytosulphokines are sulphated pentapepide growth factors which have recently been shown to induce cell proliferation in plants (Matsubayashi & Sakagami 1996).

1.1.3 Organic incorporation of reduced sulphur

Plant compounds containing sulphur in its reduced state are ultimately derived from the amino acid cysteine. Biologically, the incorporation of sulphur, in the form of sulphide, into cysteine is the sole point of entry of inorganic sulphur into organic combination (Smith & Thompson 1971; Ngo & Shargool 1974; Masada et al. 1975). Cysteine biosynthesis is therefore regarded as a fundamental biological process, particularly due to the fact that the cysteine derivative methionine is one of the nine essential amino acids required by animals. Cysteine itself can only be produced by animals when sufficient methionine is provided in the diet, indicating their dependence on plants for sulphur-containing amino acids (Giovanelli 1990).

Oxidised sulphate is reduced to sulphide in plants by reductive sulphate assimilation which is discussed in section 1.2. The biosynthesis of cysteine from sulphide and *L*-serine is discussed in section 1.3.

1.2 The Reductive Sulphate Assimilation Pathway

1.2.1 Background

As highly oxidised sulphate is the major sulphur source for plants, it must be reduced before incorporation into the amino acid cysteine. This is carried out by a series of reactions known as reductive sulphate assimilation whereby the hexavalent sulphate ion (SO_4^{2-}) receives eight electrons forming divalent sulphide (S^{2-}). These electrons are provided by ferredoxin, thioredoxin or glutaredoxin (discussed later). At present the chloroplast and root plastid are thought to be the only plant cellular compartments capable of sulphate assimilation (Schwenn 1994). However, as sulphate reduction takes place in the mitochondrion of *Aspergillus nidulans* (Bal et al. 1975) and *Euglena gracilis* (Saidha et al. 1988), the plant mitochondrion may also contain some or all of the reductive sulphate machinery.

The mechanisms of sulphate reduction were first characterised in *Salmonella typhimurium* and *Escherichia coli* due to the ease of manipulation and production of cysteine auxotrophic mutants of these organisms (reviewed in Kredich 1993). Work with bacteria and *Saccharomyces cerevisiae* led to the characterisation of sulphate reduction in higher plants. It became clear that higher plants may reduce sulphate by a different mechanism to that of the prokaryotes and lower eukaryotes, and much debate ensued as to the possible pathway(s) involved (reviewed in Schmidt & Jäger 1992; Schwenn 1994; Leustek 1996; Hell 1997; Brunold & Rennenberg 1997; Wray et al. 1998). Only

in recent years with rapid advances in the molecular characterisation of sulphate reduction in higher plants have these contentious questions been suitably addressed. The enzymatic steps of the proposed pathways of reductive sulphate assimilation in plants are shown in Figure 1.1

1.2.2 Uptake and transport of sulphur

Before sulphate can be assimilated it must be taken up from the environment and transported throughout the plant in the xylem. It seems likely that sulphate reaches the vascular system of the root via the symplast, rather than apoplastically, due to the anion-repelling negative charges of the cell wall constituents (Clarkson et al. 1993). Consequently, the initiating step in sulphate metabolism is the direct transport of ions from the soil into the root cells. It has long been known that sulphate is actively transported into plant cells (Legget & Epstein 1956) and that membrane-spanning sulphate transporters allow passage of the ion across the plasma membrane. Plant sulphate transporters may be expected to be of several functional types permitting direct sulphate uptake from the external environment into the root cell, as well as intercellular and intracellular transport within the plant (Cram 1990; Clarkson et al. 1993).

Plasma membrane sulphate transporters are driven by a H^+/SO_4^{2-} proton pump and can be inhibited by direct competition from sulphate analogues such as selenate, chromate, arsenate and sulphite (Cupoletti & Segel 1975; Roomans et al. 1979; Lass & Ullrich-Eberius 1984). Depending on the species studied, different ion exchanges have been proposed to balance the electrical charge across these pumps. Cupoletti & Segel (1975) proposed that the sulphate transporter of *Penicillium notatum* co-transported one proton for every sulphate ion, with either Mg^{2+} or Ca^{2+} anions also entering to maintain the electrical gradient, but hypothesised that some of the additional anions are

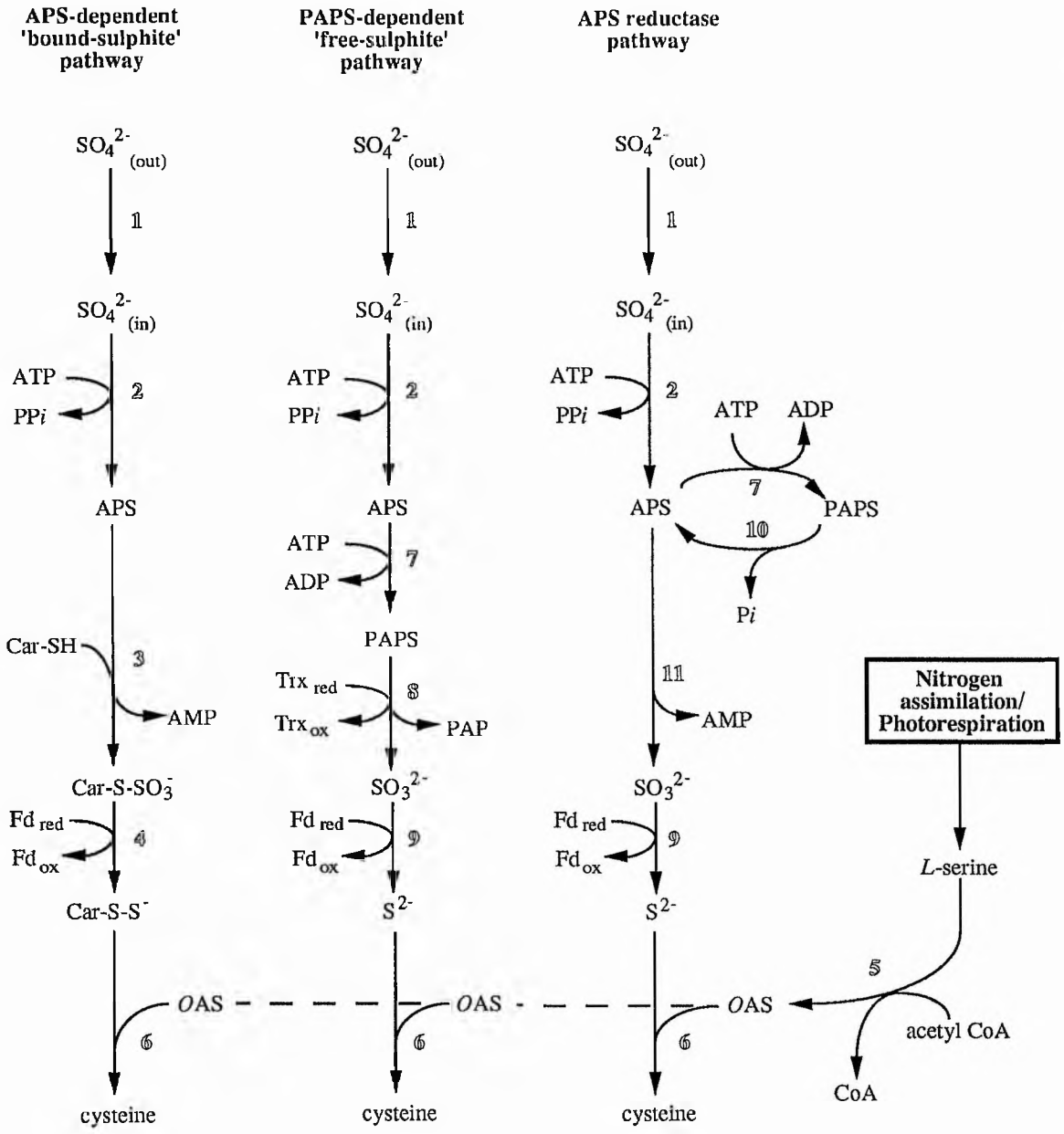


Figure 1.1 The three proposed pathways of reductive sulphate assimilation in higher plants and the subsequent biosynthesis of cysteine. Enzymatic steps are numbered: 1. Sulphate transporter, 2. ATP sulphurylase, 3. APS sulphotransferase, 4. thiosulphonate reductase, 5. serine acetyltransferase, 6. *O*-acetylserine (thiol) lyase, 7. APS kinase, 8. PAPS reductase, 9. sulphite reductase, 10. DPNPase, 11. APS reductase

recycled within the pump. In the main work carried out on a higher plant system (*Lemna minor*), Lass and Ullrich-Eberius (1984) suggested an influx of three protons for every sulphate ion taken up by the transporter, although direct measurements of the stoichiometry of their system were not made. Due to the low availability of protons for free energy change in alkaline sea waters, potassium ions rather than protons drive the sulphate transporters of some marine algae (Raven 1980).

A much greater understanding of sulphate uptake came with the cloning of sulphate transporters. The first sulphate transporter gene was cloned from *Neurospora crassa* (Kettler et al. 1991), followed by those from *Stylosanthes hamata* (Smith et al. 1995a), *Saccharomyces cerevisiae* (Smith et al. 1995b), *A. thaliana* (Takahashi et al. 1996 1997; Yamaguchi et al. 1997), *Sporobolus stapfianus* (Ng et al. 1996) and *Hordeum vulgare* (Smith et al. 1997). The plant transporters were cloned by functional complementation of a *S. cerevisiae* stable deletion mutant YSD1, defective in the plasma membrane sulphate transporter, and were found to form a unique group of membrane-bound proteins. Hydropathy studies predicted sulphate transporters having twelve membrane-spanning domains with cytoplasmically-located, hydrophilic N and C-termini. It is suggested that an extracellular arginine residue between membrane-spanning domains 9 and 10, which is conserved between all known sulphate transporters, may be involved in the binding of sulphate ions (Smith et al. 1995a).

So far, a single cDNA from *H. vulgare* (*hvst1*; Smith et al. 1997), three from *A. thaliana* (*ast56* and *ast68*, Takahashi et al. 1996, 1997; although another five different unpublished sequences are deposited in the GenBank database) and three from *S. hamata* (*shst1*, *shst2* and *shst3*; Smith et al. 1995a) have been characterised in detail. These cDNAs fall into two groups, the first encoding high affinity transporters with a K_m for sulphate in the region of 10μM (SHST1, SHST2, HVST1), and a second group of low affinity transporters with a

K_m for sulphate of approximately 100μM (SHST3, AST56, AST68). The high affinity transporters are expressed solely in roots and are highly inducible under sulphur-stress implicating them in uptake of sulphate from the environment. The low affinity transporters are not so highly inducible by sulphate starvation and are found throughout the plant which may suggest a role for this group of transporters in movement of sulphate from cell to cell . It is unlikely that either of these transporter groups are involved in sulphate transport across intracellular membranes, such as the chloroplast envelope or the tonoplast of the vacuole, as the genes were cloned by functional complementation of a mutant defective in a plasma membrane sulphate transporter (Hawkesford & Smith 1997). Sulphate is believed to enter the chloroplast via the triose-phosphate translocator (Pfanz et al. 1987; Flügge et al. 1989) whereas the mechanism of sulphate transport into the vacuole, the major sulphate storage location of plant cells, remains unclear (Cram 1990; Clarkson et al. 1993).

1.2.3 Regulation of sulphate transport

Unlike the nitrate transporter of higher plants, which is inducible by nitrate (Beevers & Hageman 1980), sulphate transport is increased under sulphate starvation (Hart & Filner 1969). The increase in sulphate transport occurs rapidly and even after long periods of sulphate deficiency, where the plant is showing physical signs of sulphur stress, can rapidly return to normal levels on the addition of sulphate. This indicates that extracellular sulphate concentrations directly affect the influx of sulphate irrespective of the sulphur nutrition status of the plant (Clarkson et al. 1983). The increases in sulphate uptake capacity on sulphate-starvation are controlled by increased transcription of the transporter genes. As with sulphate uptake capacity, transcript abundance

of the barley *hvst1* sulphate transporter increases on sulphate starvation, rapidly decreasing on resupply of sulphate (Smith et al. 1997).

Several internal signals are also known to affect the rate of sulphate transport in higher plants. Glutathione and cysteine have both been shown to repress sulphate uptake in tobacco cells (Rennenberg et al. 1989; Herschbach & Rennenberg 1994) and repress the transport of sulphate into the xylem. The effect on xylem loading is much more sensitive than the effect on uptake of sulphate. Exogenous applications of reduced-sulphur compounds such as glutathione and cysteine have been shown to reduce sulphate entering the xylem by 60-70% (Herschbach & Rennenberg 1991).

The other major factor regulating sulphate uptake is the metabolite *O*-acetylserine (*O*AS) formed by serine acetyltransferase (SAT). *O*AS has been shown to rapidly increase transcription and rate of sulphate uptake of the barley sulphate transporter, even in conditions of adequate nutrition, leading to increased cysteine and glutathione pools. This presumably indicates that *O*AS can over-ride the repressive effects of reduced sulphur compounds and sulphate (Hawkesford et al. 1995; Smith et al. 1997). The regulation of the sulphate transporter is represented diagramatically in Figure 1.2.

1.2.4 Activation of sulphate by ATP sulphurylase

The first step of the reductive sulphate assimilation pathway in plant cells is catalysed by the enzyme ATP sulphurylase (ATPS) (EC 2.7.7.4) which forms adenosine 5'-phosphosulphate (APS) and pyrophosphate (PP*i*) from ATP and sulphate, as shown in Figure 1.1. Whereas sulphate is chemically stable, the high energy anhydride bond formed between the phospho- and sulpho-groups in APS activates the sulphur for subsequent reduction reactions.

Kinetically, the production of APS by ATPS is an unfavourable reaction with an equilibrium constant (K_{eq}) of approximately 10^{-7}. However, APS is produced by ATPS *in vivo* due to the efficient removal of APS and PP*i* products by subsequent pathway enzymes such as APS kinase (APSK), APS reductase (APSR) or APS sulphotransferase (APSST) (reviewed in Schmidt & Jäger 1992; Schwenn 1994; Leustek 1996; Hell 1997; Brunold & Rennenberg 1997; Wray et al. 1998) and inorganic pyrophosphatase (Leyh 1993). Working with purified spinach enzyme, Renosto et al. (1993) found that APS was a potent competitive inhibitor of ATPS. However, it seems unlikely that this would have any regulatory function *in vivo* due to the rapid removal of APS required for assimilatory ATPS activity.

Despite the chloroplast and root plastid being the only sites of sulphate reduction in plants, ATPS activity can also be extracted from the cytoplasmic fraction of higher plant cells (Gerwick et al. 1980; Lunn et al. 1990; Ruegsegger & Brunold 1993). 80% of extractable ATPS activity from spinach was found to be chloroplastic, but the remaining 20% of activity was located in the cytoplasm (Lunn et al. 1990). The chloroplastic and cytoplasmic isoforms of ATPS from spinach were also found to be biochemically distinct, having a K_m for ATP of 0.046 mM and 0.24 mM respectively (Renosto et al. 1993). As ATPS is the only enzyme of reductive sulphate assimilation located in the cytoplasm, the function of a cytoplasmic isoform is unclear. It has been suggested that it may play a role in sulphate assimilation under conditions of high sulphate demand (Leustek 1996). However, the cytoplasmic enzyme's low affinity for ATP, coupled with high cytoplasmic levels of pyrophosphate (Weiner et al. 1987) and lack of subsequent sulphate reducing enzymes suggest that the cytoplasmic ATPS may be redundant or function in the reverse direction to that involved in sulphate assimilation.

Mitochondrial isoforms of ATPS have been detected in *Aspergillus nidulans* (Bal et al. 1975) and *Euglena gracilis* (Li et al. 1991). In *E. gracilis* sulphate reduction takes place on the outer surface of the inner mitochondrial membrane, using ATP from oxidative phosphorylation as an energy source (Saidha et al. 1988). Chloroplastic, cytoplasmic and mitochondrial ATPS activity can all be detected in this organism (Li et al. 1991).

To date plant ATPS cDNAs have been cloned from *Solanum tuberosum* (Klonus et al. 1994), *A. thaliana* (Leustek et al. 1994; Klonus et al. 1995; Murillo & Leustek 1995; Logan et al. 1996), *Brassica napus* (Buchanan-Wollaston & Ainsworth 1997), *Brassica oleracea* (GenBank U69694) and *Zea mays* (GenBank AF016305). Of those which have been characterised, the cDNAs from *Solanum tuberosum* encode chloroplastic and cytoplasmic enzymes which are expressed in leaf, stem and root tissues, but not in the tuber (Klonus et al. 1994). All four cDNAs cloned from *A. thaliana* encode putative plastidic isoforms and are expressed in leaf and root tissue (Leustek et al. 1994; Klonus et al. 1995; Murillo & Leustek 1995). The reason for multiple forms of ATPS being directed to the plastid is not yet clear.

Significant homologies are observed between deduced ATPS protein sequences of plants, fungi and chemoautotrophic bacteria. Homologous regions of the ATPSs from these organisms have been identified as substrate binding sites (Foster et al. 1994). The ATPSs from plants, fungi and chemoautotrophic bacteria are homooligomeric proteins encoded by single genes. ATPS enzymes from *E. coli* and *Rhizobium meliloti*, however, are heterodimers encoded by the *cysD* and *cysN* (*E. coli*) or *nodP* and *nodQ* (*R. meliloti*) genes (Leyh et al. 1988; Mishra & Schmidt 1992; Schwedock & Long 1990, 1992). These two-subunit bacterial enzymes show little homology to plant ATPSs. Despite the structural differences between the homooligomeric and heterodimeric forms of ATPS, functional homology is shown by the ability to clone plant and fungal cDNAs

by functional complementation of *cys*D/*cys*N mutants of *E. coli* (Murillo & Leustek 1995).

Although unable to reduce sulphate to sulphide or to synthesise sulphur-containing amino acids, animal cells do metabolise sulphate. Sulphate is incorporated into PAPS (3'-phosphoadenosine 5'-phosphosulphate), which is involved in sulphate transfer and sulphation reactions (Niehrs et al. 1994). The production of PAPS in animal cells is catalysed by the enzyme PAPS synthetase which carries out a reaction equivalent to those of ATPS and APS kinase (APSK) in plants, converting ATP and sulphate to PAPS. Recent cloning of the genes for PAPS synthetase from the marine worm *Urechis caupo* (Rosenthal & Leustek 1995) and mouse (Li et al. 1995) have revealed single genes, the deduced proteins of which have an N-terminal domain with high homology to plant APSK and a C-terminal domain with high homology to plant ATPS. This suggests intermediate APS channelling within a single bifunctional animal protein. APS channelling may also occur between associated ATPS and APSK proteins in plants, lower eukaryotes and bacteria. If so, the different ATPS isoforms may associate with different secondary enzymes depending on the fate of APS produced. Any association between ATPS and APSK in higher plants may not be involved in reductive sulphate assimilation (see section 1.2.11).

1.2.5 Regulation of ATP sulphurylase activity in plants

The main regulatory factors affecting ATPS activity in higher plants appear to be similar to those involved in the regulation of the sulphate transporters. Activity is highly inducible by sulphate deprivation and repressed by reduced sulphur compounds such as cysteine and glutathione. Activity of ATPS increased 100% and 500% in rose (Haller et al. 1986) and tobacco (Reuveny & Filner 1977) respectively when deprived of sulphate, with similar trends also reported in

Lemna minor (Brunold et al. 1987). It is interesting to note that the derepression of ATPS by sulphur starvation does not function in plants which are also subject to nitrogen starvation. In these circumstances repression of ATPS activity, possibly by reduced supply of *O*AS, maintains balanced levels of sulphur-containing and non-sulphur-containing amino acids available for protein synthesis during periods of low nitrate assimilation (Reuveny et al. 1980). The regulation of ATPS activity is represented diagramatically in Figure 1.2.

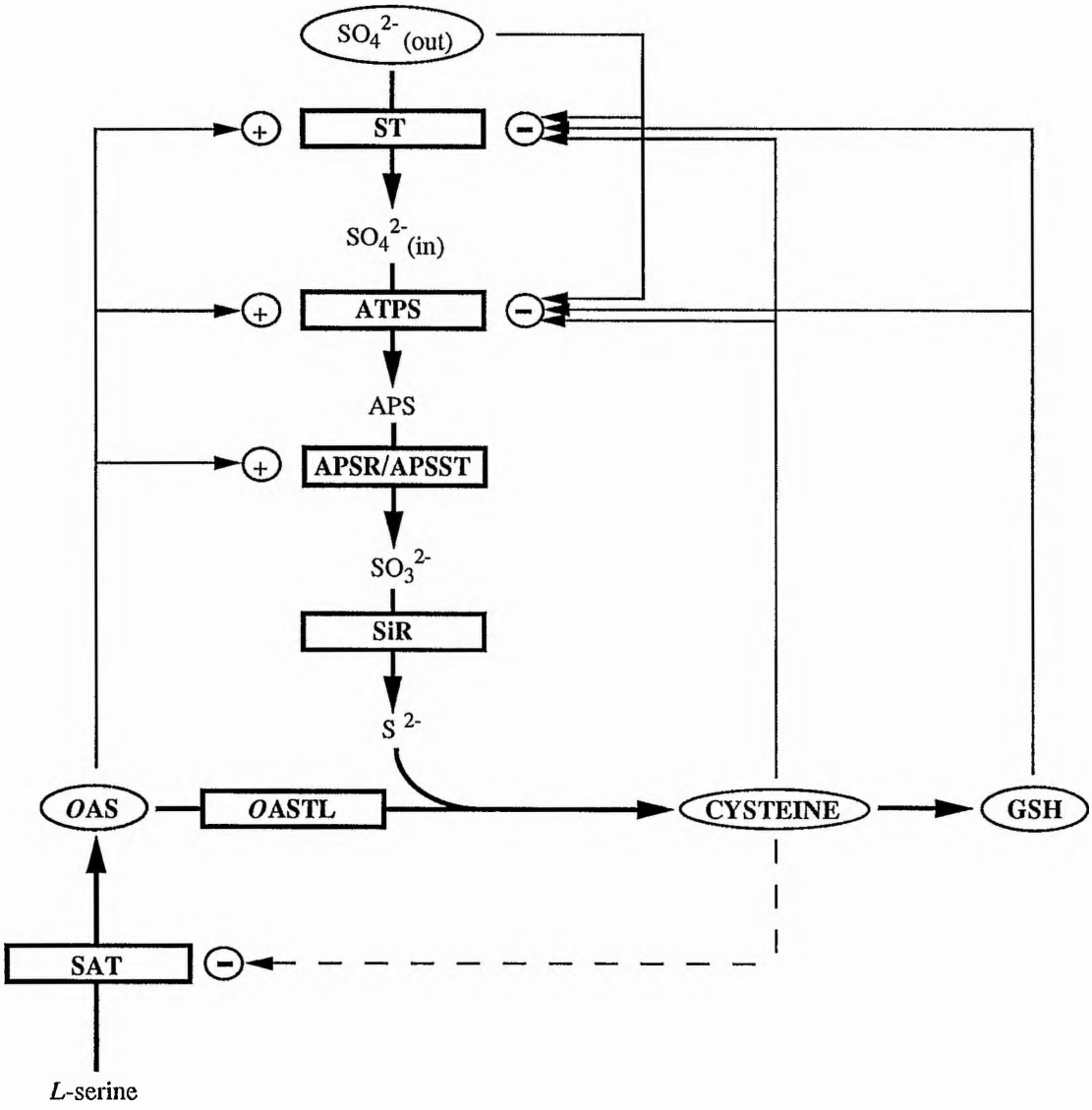
Inhibition of plastidic ATPS from *A. thaliana* by *O*AS has also been reported *in vitro*, but only at concentrations well above those expected *in vivo* (Klonus et al. 1997). This may rule out any direct effect of *O*AS on the ATPS enzyme under physiological conditions. Following the cloning of genes for ATPS from *A. thaliana*, northern analysis has shown the regulation of ATPS activity by sulphate, cysteine and glutathione to be at the level of transcription (Lappartient et al. 1997).

1.2.6 Cleavage of the sulpho-group from APS in reductive sulphate assimilation

The uptake and activation of sulphate have been well characterised in plants as outlined in sections 1.2.2 to 1.2.5. However, the subsequent fate of APS produced by ATPS in plants has been the subject of debate for many years (reviewed in Schmidt & Jäger 1992; Schwenn 1994; Leustek 1996; Hell 1997; Brunold & Rennenberg 1997). The uncertainty as to the fate of APS arose from conflicting biochemical evidence suggesting two different, but not necessarily mutually exclusive, pathways. In bacteria and yeast, APS is further activated to PAPS by the enzyme APS kinase (APSK) and the sulphur is released from this compound in the form of sulphite by the enzyme PAPS reductase (PAPSR). Sulphite is then reduced to sulphide by sulphite reductase (SiR) before

Figure 1.2 Suggested regulatory mechanisms controlling reductive sulphate assimilation and cysteine biosynthesis in higher plants. The pathways of reductive sulphate assimilation and cysteine/glutathione (GSH) metabolism are represented by bold arrows. Constituent enzymes are boxed. Intermediate substrates with regulatory functions are circled. Abbreviations: **ST**, sulphate transporter; **ATPS**, ATP sulphurylase; **APSR**, APS reductase; **APSST**, APS sulphotransferase; **SiR**, sulphite reductase; **SAT**, serine acetyltransferase; ***O*ASTL**, *O*-acetylserine (thiol) lyase; ***O*AS**, *O*-acetylserine; **GSH**, glutathione.

Regulation at the level of gene expression is shown by solid lines. Regulation of enzyme activity is shown by dashed lines.


SO_4^{2-} (out)
ST
SO_4^{2-} (in)
ATPS
APS
APSR/APSST
SO_3^{2-}
SiR
S^{2-}
OAS
OASTL
CYSTEINE
GSH
SAT
L-serine

incorporation into cysteine by *O*-acetylserine (thiol) lyase (*O*ASTL) (Dreyfuss & Monty 1963; Jones-Mortimer 1968; Tsang & Schiff 1976a; Thomas et al. 1990). In plants, a similar PAPS-dependent 'free-sulphite' pathway was suggested (Schwenn 1989; Schiffman & Schwenn 1994; Schwenn 1994). In the second proposed plant sulphate assimilation pathway, the APS-dependent 'bound-sulphite' pathway, sulphur from APS is bound directly to a carrier thiol by APS sulphotransferase (APSST). The bound-sulphite would then be reduced to bound-sulphide by a thiosulphonate reductase (Hodson & Schiff 1971; Schmidt 1972; Goldschmidt et al. 1975; Schmidt 1975, 1976). Both of these proposed pathways of reductive sulphate assimilation in plants are shown in Figure 1.1

1.2.7 The APS-dependent 'bound-sulphite' pathway

Work with the unicellular alga *Chlorella pyrenoidosa* first suggested a different mechanism of sulphate reduction in photoautotrophic eukaryotes to that described in bacteria and yeast (Hodson & Schiff 1971; Schmidt 1972, 1973). It was shown that the sulpho-group of APS was directly transferred to a carrier thiol to produce a bound form of sulphite which could then be further reduced to bound-sulphide by thiosulphonate reductase (TSR) as shown in Figure 1.1. The carrier *in vivo* was not identified but could be replaced by DTT, DTE or glutathione *in vitro*. PAPS could not be utilised as a sulphur donor unless a 3'-phosphonucleotidease was included in the reaction which would convert the PAPS back to APS (Hodson & Schiff 1971; Schmidt 1972; Goldschmidt et al. 1975). The same requirement for a 3'-phosphonucleotidease was later demonstrated in higher plants (Schmidt 1975, 1976).

Further evidence for the use of APS instead of PAPS in reductive sulphate assimilation in plants came from studies on the reduction of radiolabelled substrates in cell-free spinach extracts. Measuring the acid-volatile

radioactivity released from radioactive substrates, as a quantitation of sulphotransferase activity, both PAPS and APS were found to be possible intermediates in reductive sulphate assimilation. However, non-radioactive PAPS did little to inhibit the use of ^{35}S-APS as a substrate whereas non-radioactive APS drastically reduced the use of ^{35}S-PAPS in the system (Schürmann & Brunold 1988). This strongly implied that APS is the preferred substrate in plants for the transfer of sulphite to the carrier molecule.

APSST activity has been shown to be particularly susceptible to regulation by nitrogen nutrition. Haller et al. (1986) and Brunold and Suter (1984), working with *Rosa* sp. and *Lemna minor* respectively, demonstrated that APSST activity was repressed under nitrogen starvation and induced by nitrogen nutrition. Different nitrogen sources were found to induce APSST activity to different extents with nitrate showing a much less prominent induction than observed with ammonium. In plants nitrate is a slowly assimilated nitrogen source, whereas ammonium is readily assimilated into protein. The relative effects of nitrate and ammonium nutrition on APSST activity, and consequently flux through the reductive sulphate assimilation pathway, ensure balanced production of sulphur-containing amino acids in relation to the rate of non-sulphur-containing amino acid assimilation (Brunold 1993).

Bound sulphite from the proposed APS-dependent pathway must be reduced to bound-sulphide before incorporation into the amino acid cysteine. This would be carried out by thiosulphonate reductase, which is a ferredoxin-dependent enzyme in *Chlorella*, and was found to reduce glutathione-bound sulphite but not free sulphite (Schmidt 1973). One hypothesis drawn from experiments with intact spinach chloroplasts is that bound sulphite is converted to bound sulphide and no free form of sulphite or sulphide is detected as an intermediate (Schmidt & Schwenn 1971; Schmidt 1973). However, some workers argue that if thiosulphonate reductase produced bound sulphide as the end

product of sulphate reduction, a further reductant would be required to liberate sulphide from the carrier prior to incorporation into cysteine. No additional reductant has yet been demonstrated (Schwenn 1994).

1.2.8 The PAPS-dependent 'free-sulphite' pathway

The possibility that a PAPS-dependent 'free-sulphite' pathway may function in plants (Figure 1.1) is partially evidenced by the extraction of APSK (Burnell & Anderson 1973; Schwenn & Schriek 1984), PAPSR (Schwenn 1989) and SiR activities (Aketagawa & Tamura 1980; Krüeger & Siegel 1982) from plant tissues, and the cloning of APSK (Arz et al. 1994; Jain & Leustek 1994) and SiR (Ideguchi et al. 1995; Brühl et al. 1996) genes. It was concluded that all the enzymes and co-factors required for sulphate reduction via the PAPS-dependent 'free-sulphite' pathway were present in higher plant chloroplasts and this pathway would therefore be active (Schwenn 1989).

In the proposed 'free-sulphite' pathway, APSK further activates APS by phosphorylation to 3'-phosphate 5'-adenosinephosphosulphate (PAPS). PAPSR then catalyses the formation of free-sulphite from PAPS using the reducing power of thioredoxin (Schwenn 1989; Thomas et al. 1990). Free-sulphite would then be reduced by a ferredoxin-dependent sulphite reductase (SiR) (EC 1.8.7.1) (Aketegawa & Tamura 1980; Krüger & Siegel 1982; Brühl et al. 1996). Sulphite is presumed to bind to the Fe^{2+} group of the heme of SiR, being reduced to sulphide in a series of cleavages of S-O bonds. Sulphite is known to remain in the active site of sulphite reductase throughout catalysis as no intermediates are detectable, although the exact process is not fully understood (Schwenn 1994). The free form of sulphide is then used by *O*ASTL in cysteine biosynthesis.

1.2.9 Difficulties in distinguishing pathway intermediates obstructs biochemical analysis of the sulphate reduction pathway

The possibility that both the 'bound-sulphite' and 'free-sulphite' pathways of reductive sulphate assimilation function in higher plants, or even that some overlap between the two may occur, cannot be overlooked. For example, an enzyme acting as a PAPS sulphotransferase may transfer the sulpho-group of PAPS to a carrier thiol as has been proposed in *S. cerevisiae* (Wilson & Bierer 1976). Alternatively, as no suitable carrier has been described *in vivo*, free sulphite may be released from APS by a thiol-dependent APS reductase (Schmidt & Jäger 1992; Wray et al. 1998). Until recently no work had unequivocally clarified the exact nature of the sulphate reduction pathway in plants. The reason for this was largely that the cell-free biochemical assays previously employed had been almost impossible to interpret.

The first problem when biochemically studying transferase and reductase reactions involved in reductive sulphate assimilation, is that sulphur can readily change oxidation state non-enzymatically in solution. Sulphite and sulphide can readily interchange (Schiff et al. 1993) and, if a suitable carrier is present, free-sulphite can non-enzymatically form bound-sulphite (Schwenn 1994). This causes obvious problems when trying to assess whether free or bound sulphite is the true sulphate reduction intermediate *in vivo*.

Further problems are encountered when interpreting data on APS metabolism due to the nature of the non-physiological enzyme assays employed. APSST activity is assayed in cell-free extracts by the production of bound sulphite from ^{35}S-APS in the presence of a suitable carrier-thiol, such as DTT or DTE (dithioerythritol). Non-radioactive sulphite is also included to exchange with the bound radioactive label, releasing volatile radioactive sulphur dioxide which can then be quantified (Schiff & Levinthal 1968; Schiff & Hodson 1973).

Similarly, PAPSR (Schwenn et al. 1988) and TSR (Schmidt 1973) assays also involve measuring the release of acid-volatile products from radioactive substrates. Problems arise when interpreting data from such assays as various theoretical reaction products would give the same quantifiable radioactivity on volatilisation. Sulphite and thiosulphate would both produce volatile sulphur dioxide and sulphide would produce volatile hydrogen sulphide. Only by further analytical experiments, such as chromatographic separation of the reaction products, could the exact nature of the radioactive sulphur released from ^{35}S-labelled substrates be determined (Schiff & Levinthal 1968; Li & Schiff 1992). As a consequence the APSST assays employed measured the rate of APS metabolism but did not determine the nature of the radioactive reaction product (Tsang & Schiff 1976b). Measuring enzyme activity by release of acid-volatile radioactive products therefore does not clearly distinguish between sulphotransferase and reductase activity and hence can not be used to determine whether the 'bound-sulphite' or 'free-sulphite' pathway is the true *in vivo* process of sulphate reduction in plants.

1.2.10 Recent molecular advances may indicate the functional pathway of APS metabolism in plants

Molecular cloning of genes involved in APS metabolism, and subsequent analysis of the recombinant enzymes, has given the best indication to date as to the functional pathway of reductive sulphate assimilation in plants. Between 1994 and 1996 plant genes encoding almost all the reductive sulphate assimilation enzymes were cloned. This was usually achieved by the functional complementation of bacterial or yeast mutants, lacking activity of a single pathway enzyme, with plant cDNA expression libraries (reviewed in Leustek 1996; Hell 1997). Due to the lack of an APS-dependent 'bound-sulphite'

pathway in bacteria and yeast, mutants were not available for the cloning of APSST or TSR genes. However, an indication as to the actual process of sulphate reduction in plants came from attempts to clone PAPSR from *A. thaliana* using the *E. coli* PAPSR-deficient mutant JM96 (Guttierrez-Marcos et al. 1996; Setya et al. 1996). Three different cDNAs were found to complement the JM96 mutant to prototrophy. A central domain of the three deduced PRH (**P**APS **R**eductase **H**omologue) proteins showed substantial identity (approximately 55%) to previously cloned microbial PAPSR sequences, indicating a common evolutionary origin. However, the plant PRH proteins differed from the bacterial PAPSRs, having N-terminal and C-terminal extensions. The N-terminal extensions encoded chloroplast targeting peptides and analysis of the C-terminal extensions revealed homology with thioredoxin, the thioredoxin domain of protein disulphide isomerase and with various other enzymes with integral thioredoxin activity known as the thioredoxin superfamily (Gutierrez-Marcos et al. 1996 and references therein). The functional activity of the C-terminal domain was shown by enzyme assays on extracts of JM96 expressing PRH proteins. Exogenous thioredoxin was not required for the conversion of ^{35}S-PAPS to acid-volatile ^{35}S-sulphite by PRH proteins, unlike extracts from wild-type *E. coli* cells possessing PAPSR activity (Guttierrez-Marcos et al. 1996). The role of the PRH proteins in plant sulphate assimilation was further demonstrated by the discovery that they preferred APS to PAPS as a substrate. PRH proteins exhibited two hundred times higher activity with APS as a substrate than with PAPS.

Prh cDNAs were also found to functionally complement the *E. coli* APSK mutant JM81a to prototrophy. This indicated that PRH proteins are able to bypass the requirement of *E. coli* for PAPSR activity by converting APS directly to sulphite. As no conventional microbial-type PAPSRs were found in functional complementation experiments with *E. coli* JM96 mutants it would appear that

rather than plants having a 'free-sulphite' pathway, analogous to that found in bacteria and yeast, they produce sulphite directly from APS by a thioredoxin-independent APS reductase (APSR) (Guttierrez-Marcos et al. 1996; Setya et al. 1996).

The nature of the reductant for the thioredoxin-like domain of APSR *in vivo* is unclear at present. Whilst the C-terminal extensions of the PRH protein share a greater homology with thioredoxin, the redox-active site more closely resembles that of glutaredoxin (Wray et al. 1998). Glutathione may therefore be the source of electrons for APSR *in vivo,* being the reductant for glutaredoxin. Reduced glutathione would readily be supplied by the chloroplast-localised glutathione reductase (Holmgren 1989). Alternatively, the C-terminal domain of APSR proteins may be reduced by the chloroplast ferredoxin:thioredoxin reductase system, which has been shown to regulate the redox states of enzymes involved in photosynthesis (Schürmann 1995).

The recent cloning of ferredoxin-dependent SiRs from *A. thaliana* (Brühl et al. 1996) and *Z. mays* (Ideguchi et al. 1995), together with the discovery of the genes for APSR, suggest that the conversion of APS to sulphide in plants proceeds via the APSR-dependent sulphate assimilation pathway outlined in Figure 1.1, rather than by the 'bound-sulphite' or 'free-sulphite' pathways which have been debated for so long.

The free sulphide product of this APSR/SiR pathway in higher plants would be available for incorporation into cysteine by *O*ASTL as proposed in the 'free-sulphite' assimilation pathway (Schwenn 1994).

Full schematic representation of the proposed 'bound-sulphite', 'free-sulphite' and APSR-dependent sulphate assimilation pathways are shown in Figure 1.1.

1.2.11 The role of PAPS in plants

If the reductive assimilation of sulphate proceeds via the APSR-dependent pathway in plants, as outlined in section 1.2.10, APSK and PAPSR presumably have roles in plant metabolism outside the sulphate reduction pathway.

Only one report has been published on the purification of a protein with PAPSR activity in plants (Schwenn 1989). Also, no PAPSR cDNAs or genes have been cloned, despite concerted attempts to do so by the functional complementation PAPSR deficient *E. coli* mutants (Guttierrez-Marcos et al. 1996; Setya et al. 1996). Although the plant genome may include conventional PAPSR genes it would appear that the enzyme is not of major importance in reductive sulphate assimilation. APSK on the other hand is a well documented plant enzyme (Arz et al. 1994; Schiffman & Schwenn 1994; Jain & Leustek 1994). The most likely role for APSK would appear to be the production of PAPS for use outside sulphate reduction. A PAPS pool could be used as a source of activated sulphate for sulphation reactions and the production of secondary compounds such as flavonol sulphates (Varin & Ibrahim 1989, 1991, 1992), phytosulphokines (Matsubayashi & Sakagami, 1996), glucosinolates (Glendening & Poulton 1990) and sulpholipids (Harwood 1980). The use of PAPS for sulphation reactions would allow the metabolism of sulphated compounds, without the inhibitory effect on sulphate reduction which would result from a build-up of APS. As mentioned in section 1.2.4 APS must be rapidly metabolised for ATPS, the first enzyme of reductive sulphate assimilation, to assimilate sulphate.

The ability of plants to convert PAPS back to APS may also implicate PAPS as a supplementary source of APS in plants. APS can be regenerated from PAPS in plants by the enzyme 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase (DPNPase). Genes encoding DPNPase have been cloned from

rice (Peng & Verma 1995) and *A. thaliana* (Quintero et al. 1996). Together, DPNPase and APSK may cycle APS and PAPS in plants to regulate the size of the available PAPS pool (Murguia et al. 1995). It is even conceivable that sulphate reduction may proceed via an ATPS/APSK/DPNPase/APSR/SiR pathway (Hell 1997), but no study to date has assessed the viability of such a pathway.

The channelling of APS to either PAPS for sulphation reactions or to the APSR-dependent sulphate assimilation pathway has not yet been studied, but may prove interesting and may be coupled to the protein biosynthesis requirements of the plant. Under sulphate starvation, *A. thaliana* APSR transcript abundance increases (Gutierrez-Marcos et al. 1996) and APSK transcript abundance decreases (Takahashi et al. 1997). Whilst this provides no indication of APSR and APSK activities, it is tempting to speculate that when a plant faces sulphur deficiency, and requires increased production of sulphur-containing amino acids for protein production, APS is preferentially channelled into the sulphate reduction pathway via increased APSR activity and decreased APSK activity.

1.2.12 Could previously reported APSST activities be attributed to APSK or APSR?

The existence of the proposed APSR-dependent sulphate reduction pathway also questions previous reports of APSST activity in plants (Schmidt 1975, 1976; Frankhauser & Brunold 1978; Brunold et al. 1987; Suter et al. 1992; Li & Schiff 1991, 1992). As mentioned in section 1.2.9, the accurate interpretation of sulphotransferase assays is a point of contention. It has been suggested that previously reported APSST activities, which were largely detected in partially

purified plant extracts, may be artifacts of APSK (Schiffman & Schwenn 1994) or APSR activities (Gutierrez-Marcos et al. 1996; Bick & Leustek 1998).

In the presence of ATP and reducing thiols, an APSK cloned from *A. thaliana* (Arz et al. 1994) was reported to produce PAPS from APS as expected. However, in the absence of ATP and reducing thiol, Schiffman and Schwenn (1994) reported that APSK formed a 108 kD homotetramer which produced sulphite from APS. The molecular weight of the plant APSK homotetramer was noted to be within the ranges previously described for APSSTs from spinach (Schmidt 1976) and *Euglena* (Li & Schiff 1991). Schiffman and Schwenn (1994) concluded that plant APSK can exhibit sulphotransferase activity *in vitro* and that previously reported APSST activities were artifacts of APSK. However, this hypothesis would appear unlikely as APSST activities from *Euglena* (Li & Schiff 1992) and spruce (Suter et al. 1992) have both been characterised in the presence of up to 5 mM ATP and DTT.

It seems more likely that reported APSST assays actually measure the activity of APS reductases. Similarities have been observed between previous reports of APSST and the activities of the APSR proteins from *A. thaliana* (Gutierrez-Marcos et al. 1996; Setya et al. 1996). Both enzymes prefer APS over PAPS as a substrate and are chloroplast-localised (Gutierrez-Marcos et al. 1996; Wray et al. 1998). Also, the 43 kD molecular weight of PRH proteins corresponds well to the molecular weights of APSSTs from *Porphyra yezoensis* (Kanno et al. 1996) and *Chlorella* (Hodson & Schiff 1971). As optimum assay conditions, kinetic constants and inhibitors of APSR and APSST are also found to be comparable, it would seem likely that they are in fact the same enzyme. If this is the case, glutathione may be required as an electron donor to the C-terminal domain of APSR (section 1.2.10), which then catalyses the reduction of APS to sulphite (Bick & Leustek 1998). Previously, glutathione was thought to be required by APSST as an acceptor of the sulpho-group from APS. In the

absence of genetic evidence for the existence of a plant APSST, it seems that the link between APSST and APSR will be debated further in the future.

1.3 Cysteine Biosynthesis

1.3.1 Sulphide and L-serine are combined to form cysteine

In most organisms cysteine biosynthesis is carried out by a bifunctional enzyme complex known as cysteine synthase (Kredich et al. 1969; Smith & Thompson 1971; Ngo & Shargool 1974; Cook and Wedding 1977). The first enzyme of the complex, serine acetyltransferase (SAT) (EC 2.3.1.30), acetylates the amino acid *L*-serine using acetyl coenzyme A (acetyl CoA) to form *O*-acetylserine (*O*AS) (Brunold & Suter 1982; Denk & Böck 1987; Nakamura et al. 1988; Nakamura & Tamura 1990). The second enzyme, *O*-acetylserine (thiol) lyase (*O*ASTL) (EC 4.2.99.8), then combines sulphide from the reductive sulphate assimilation pathway with *O*AS, forming cysteine (Masada et al. 1975; Ascaño & Nicholas 1977; Nakamura & Tamura 1989). The reactions catalysed by the cysteine synthase complex are summarised below:-

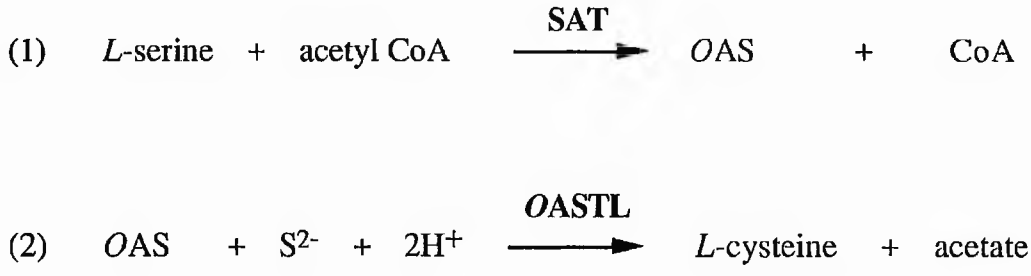


Figure 1.3 The reactions of the cysteine synthase complex. (1) serine acetyltransferase, (2) *O*-acetylserine (thiol) lyase.

By combining sulphide and the amino acid *L*-serine, the cysteine synthase complex effectively represents the point of convergence between the pathways of nitrate and sulphate assimilation in plants. These pathways are closely regulated to maintain the ratio of sulphur and non-sulphur-containing amino acids available for protein synthesis (Brunold 1993). The potential role of the cysteine synthase complex in this regulation makes it of particular research interest.

1.3.2 Serine acetyltransferase

Early work with *E. coli* and *Salmonella typhimurium* highlighted the requirement of acetylated serine, in the form of *O*AS, for cysteine biosynthesis (Kredich & Tomkins 1966). A single SAT protein, encoded by the *cysE* gene, was identified which catalysed this acetylation and was found to associate with the enzyme *O*ASTL. The complex of SAT and *O*ASTL was named cysteine synthase (Kredich & Tomkins 1966) and has also been demonstrated in higher plants (Nakamura et al. 1988; Nakamura & Tamura 1990; Ruffet et al. 1994; Bogdanova & Hell 1997).

SAT activity has been characterised in a wide range of organisms including kidney bean (Smith & Thompson 1971), *Phaseolus vulgaris* (Smith 1972), *S. typhimurium* (Baecker & Wedding 1980), spinach (Brunold & Suter 1982), *Brassica chinensis* (Nakamura et al. 1988), *E. coli* (Denk & Böck 1987; Wigley et al. 1990), *Allium tuberosum* (Nakamura & Tamura 1990) and pea (Ruffet et al. 1994). When purified from plant extracts, most SAT protein is co-eluted with *O*ASTL in a complex of 300 to 350 kD (Nakamura et al. 1988; Ruffet et al. 1994). The SAT can, however, be dissociated from the cysteine synthase complex in the presence of > 0.1 mM *O*AS (Kredich et al. 1969). Isolation of SAT

under these conditions allowed the size of the enzyme from various species to be calculated. SAT subunits, free from *O*ASTL, were found to range between 30 and 34 kD (Denk & Böck 1987; Baeker & Wedding 1980; Ruffet et al. 1994). In recent work, using the yeast two-hybrid system, Bogdanova and Hell (1997) confirmed the occurrence of a cysteine synthase complex *in vivo* with a 4 SAT + 4 *O*ASTL heterooctamer stoichiometry, and found that the area of interaction between the *O*ASTL/SAT and SAT/SAT proteins in the complex coincided with the catalytic domains of the two enzymes. This had previously been suggested by sequence analysis of the chloroplast SAT isoform from *A. thaliana* (Roberts & Wray 1996) and strongly suggests the metabolic channelling of OAS within the complex.

The activity of free SAT does not appear to differ significantly from that when complexed with *O*ASTL. The apparent K_m of isolated pea enzyme for the substrates *L*-serine and acetyl CoA was found to be 2.29 mM and 0.35 mM respectively (Ruffet et al. 1994). The equivalent K_m values for SAT from *Allium tuberosum*, complexed with *O*ASTL, were measured at 5.1 mM and 0.26 mM respectively (Nakamura & Tamura 1990). *O*ASTL activity, on the other hand, is almost twice as high when complexed with SAT than when in its free state (Kredich et al. 1969).

Cellular localisation studies have detected SAT activity in the chloroplast, cytoplasm and mitochondrion of plants (Smith & Thompson 1969; Smith 1972; Brunold & Suter 1982; Droux et al. 1992; Ruffet et al. 1994, 1995), indicating a much wider distribution than the enzymes of reductive sulphate assimilation, which are almost exclusively localised in the chloroplast. In their work with pea, Ruffet and co-workers (1995) found 76% of SAT activity was located in the mitochondrion, 14% in the cytoplasm and 10% in the chloroplast. The mitochondrial SAT activity is extremely high when compared to the 10% of total *O*ASTL activity in this compartment (Ruffet et al. 1995). The 300:1 ratio of

*O*ASTL to SAT activity in the pea chloroplast compared to the 3:1 ratio in the mitochondrion, suggests a different interaction between the enzymes in these cellular compartments. Whilst purifying SAT from pea organelles by gel filtration, Ruffet et al. (1994) found that all the SAT activity from the chloroplast was complexed with *O*ASTL. However, co-elution of SAT and *O*ASTL activities from the mitochondrion was not observed (Ruffet et al. 1995). It is possible therefore that free SAT may have a specific metabolic function in the mitochondrion. It is known that *L*-serine is released in the mitochondrion of C_3 plants, by serine hydroxymethyl transferase, as part of the photorespiration cycle (Kisaki et al. 1971). Mitochondrial SAT may be involved in the metabolism of some of this *L*-serine source.

Recent molecular cloning has confirmed that a SAT gene-family encodes specific organelle-targeted proteins. Putative chloroplastic (Bogdanova et al. 1995; Roberts & Wray 1996), cytoplasmic (Ruffet et al. 1995) and mitochondrial (Howarth et al. 1997) isoforms of SAT have been cloned from *A. thaliana*. A cytoplasmic isoform from watermelon (Saito et al. 1995) and a putative mitochondrial isoform from spinach (GenBank D88530 and Saito et al. 1997) have also been cloned. The availability of cDNAs has allowed the study of SAT expression in plants. Northern analysis has shown that all the SAT genes cloned are expressed in leaf and root tissue (Bogdanova et al. 1995; Saito et al. 1995) and that the chloroplastic isoform from *A. thaliana* is induced in both root and leaf tissue by sulphate starvation (Takahashi et al. 1997). Expression of the cytoplasmic SAT2 protein from watermelon may also be induced by sulphate starvation. Promoter analysis of the *sat2* gene has revealed a SEF 4-like transcription-factor recognition site. SEF 4 induces transcription of the β-conglycinin gene in soybean in response to sulphur-deficiency (Lessard et al. 1991).

1.3.3 O-acetylserine (thiol) lyase

*O*ASTL activity has also been characterised in a wide range of organisms including kidney bean (Smith & Thompson 1971), rape (Masada et al. 1975; Nakamura & Tamura 1989), wheat (Ascaño & Nicholas 1977), *Phaseolus* sp. (Bertagnolli & Wedding 1977), spinach (Lunn et al. 1990; Droux et al. 1992; Saito et al. 1992; Yamaguchi & Masada 1995), *Capsicum* (Römer et al. 1992), cauliflower (Rolland et al. 1992) and *Datura* (Kuske et al. 1996). *O*ASTL proteins were found to have molecular weights between 26 and 40 kD (Droux et al. 1992; Römer et al. 1992; Saito et al. 1992).

Although SAT and *O*ASTL function as a complex in the biosynthesis of cysteine, in both bacteria and plants there is a large excess of free *O*ASTL. Kredich and Tomkins (1966) found a 20:1 ratio of *O*ASTL to SAT activity in *E. coli*. SAT from rape leaf extract co-purified with 1.2% of the total *O*ASTL activity (Nakamura et al. 1988) and Ruffet and co-workers found a 345:1 *O*ASTL to SAT activity ratio in spinach chloroplasts (Ruffet et al. 1994) and a 300:1 ratio in pea chloroplasts (Ruffet et al. 1995). As a ratio of 380:1 was observed to be optimum for cysteine production in a cell-free system from spinach (Ruffet et al. 1994) it would appear that the levels of *O*ASTL and SAT activity in the chloroplast are around optimal levels for cysteine biosynthesis. Free *O*ASTL in bacteria has a K_m for *O*AS four times higher than observed when complexed with SAT, and has twice the activity (Kredich et al. 1969).

As has been demonstrated with SAT, *O*ASTL activity is located in the chloroplast, cytoplasm and mitochondrion of plant cells (Lunn et al. 1990; Rolland et al. 1992; Ruffet et al. 1994, 1995; Kuske et al. 1996). Studies on the distribution of *O*ASTL activity in spinach showed a 14%, 44% and 42% distribution of activity between the mitochondria, cytoplasm and chloroplast respectively (Lunn et al. 1990).

cDNA clones representing *O*ASTL have been cloned from *Capsicum* (Römer et al. 1992), spinach (Saito et al. 1992, 1993, 1994b; Rolland et al. 1993; Hell et al. 1993), *A. thaliana* (Hell et al. 1994; Hesse & Altman 1995; Barroso et al. 1995), wheat (Youssefian et al. 1993) and watermelon (Noji et al. 1994). These *O*ASTL clones include putative chloroplastic, cytoplasmic and mitochondrial forms.

Northern analysis of *O*ASTL gene-expression in plants indicates expression in leaf, root, stem and flower tissues (Youssefian et al. 1993; Saito et al. 1993; Hell et al. 1994; Barroso et al. 1995; Bogdanova et al. 1995; Saito et al. 1995; Gotor et al. 1997). *In situ* hybridisation studies further localised cytoplasmic *O*ASTL transcript to specific cell types in *A. thaliana* (Gotor et al. 1997). In roots, the xylem parenchyma and cortex both showed *O*ASTL expression and in flowers transcript was exclusively localised in the anthers and sepals. In the stem cortex and leaf blade a general background distribution of *O*ASTL transcript was observed, but extremely high levels of transcript were detected in epidermal trichomes. This indicates a possible specialist requirement for cysteine biosynthesis in these structures. It was hypothesised by the authors that the trichomes may synthesise proteins and peptides with a high cysteine-content such as phytochelatins, metallothioneins and glutathione (Gotor et al. 1997). These compounds are known to be involved in pathogen defence and heavy metal detoxification in the trichomes of some plants (Salt et al. 1995; Foley & Singh 1994; Mehdy 1994). *In situ* hybridisation experiments will be very useful in future to our understanding of the regulation and roles of sulphate assimilation and cysteine biosynthetic enzymes in specific cell types.

1.3.4 Regulatory functions of SAT and OAS

SAT and its product *O*AS are believed to have a central role in the regulation of cysteine biosynthesis and the reductive sulphate assimilation in bacteria and plants.

In *Phaseolus vulgaris* SAT activity is reduced 65% by 1 mM cysteine (Smith & Thompson 1971) and SAT from spinach chloroplasts is almost completely inhibited in this concentration (Brunold & Suter 1982). Watermelon SAT activity was shown to be inhibited by cysteine concentrations as low as 2.9 μM, well within the range of physiological levels, indicating a negative feedback on SAT activity by excess cysteine *in vivo* (Saito et al. 1995).

*O*AS has also been shown to exert regulatory control over sulphate reduction in plants. Neuenschwander and co-workers (1991) found that the rate of sulphate reduction in *Lemna minor* cultures was greatly reduced when transferred to dark conditions. This was indicated by a reduction in APSST activity to 10% of that found in light cultivated plants. Previously this response had been attributed to the dependence of sulphate assimilation on the light-activated thioredoxin system of the chloroplasts. However, the addition of *O*AS could increase APSST activity to 50% of levels observed in the light. This showed that the supply of *O*AS was a limiting factor for sulphate reduction in the dark and that *O*AS can induce the activity of the reductive sulphate assimilation pathway. Light dependence of nitrate assimilation is already known (Gupta and Beevers 1984). Neuenschwander's results show that *O*AS acts as a positive signal, coupling reductive sulphate assimilation to the plants potential for nitrogen assimilation. The *O*AS mediated increase in reductive sulphate assimilation in the light co-ordinates assimilation of sulphate and nitrate into the amino acids essential for balanced protein biosynthesis (Neuenschwander et al. 1991).

The positive effect *O*AS has on the reductive sulphate assimilation pathway suggests that SAT activity and *O*AS supply may be a major limiting factor for cysteine biosynthesis. This was implied by experiments with transgenic tobacco chloroplasts overexpressing a spinach *O*ASTL cDNA fused to a ribulose-1,5-bisphosphate chloroplast targeting-peptide. Despite *O*ASTL activities being 2 to 3 times higher than in control chloroplasts, there was no detectable increase in chloroplast cysteine levels. Cysteine concentrations in the transgenic chloroplasts were only found to increase when exogenous *O*AS was supplied. This resulted in 10 to 34-fold increases in chloroplastic cysteine concentration, depending on the concentrations of *O*AS and reduced sulphur supplied (Saito et al. 1994a).

The inhibition of SAT activity by cysteine feedback and the positive control of sulphate reduction by *O*AS suggests a regulatory control of cysteine biosynthesis in plants similar to that reported in bacteria (Kredich 1987; Ostrowski & Kredich 1989, 1990). It has been shown in *E. coli* and *S. typhimurium* that feedback inhibition of SAT by cysteine closely links *O*AS production to the size of the cysteine pool. Additionally, genes for the sulphate transporter, reductive sulphate assimilation enzymes and *O*ASTL are transcriptionally controlled by an activating protein encoded by the *cysB* locus. The CYSB protein is only active when associated with *O*AS and is inhibited by sulphide which acts as a competitive anti-inducer (Monroe et al. 1990; Ostrowski & Kredich 1990). This system sets up a sophisticated control of cysteine biosynthesis. When cysteine availability is limiting for protein production, SAT is free of the inhibitory effects of cysteine and produces *O*AS. *O*AS then interacts with the CYSB activating protein inducing the transcription of the genes involved in sulphate reduction which in turn increases sulphide availability to *O*ASTL. As the cysteine pool builds up, *O*AS production by SAT is allosterically inhibited and the CYSB protein becomes inactive, down-regulating

the transcription of sulphate reduction pathway genes, and the rate of cysteine biosynthesis is decreased. These regulatory interactions are shown in diagramatic form in Figure 1.2.

From the various published studies which have covered the co-ordination of cysteine biosynthesis in higher plants, it is apparent that several regulatory mechanisms exist to ensure optimal levels of sulphur-containing amino acids are provided for protein synthesis.

1.4 Methionine biosynthesis

The cysteine derivative methionine is the only other sulphur-containing protein-amino acid and is one of the nine essential amino acids required in animal diets (Giovanelli 1990). The methionine biosynthetic pathway will be briefly described here.

To ensure a balanced supply of sulphur-containing amino acids for protein synthesis, a proportion of the cysteine produced in plant cells must be further metabolised to methionine. This is carried out by a series of chloroplast localised enzymes. The first step in methionine biosynthesis involves the attachment of one molecule of *O*-phosphorylhomoserine to the sulphur atom of cysteine. This is catalysed by cystathionine-γ-synthase (CTγS) and results in the formation of one molecule of cystathionine and the release of inorganic phosphate (Ravanel et al. 1995). Cystathionine-β-lyase (or cystathionase) (CTβL) then hydrolyses the cystathionine to homocysteine and pyruvate (Droux et al. 1995; Eichel et al. 1995) before the homocysteine is converted to methionine by methionine synthase (MS). Methionine synthase attaches a methyl-group from N^5-methyltetrahydrofolic acid to homocysteine in this reaction (Eichel et al. 1995).

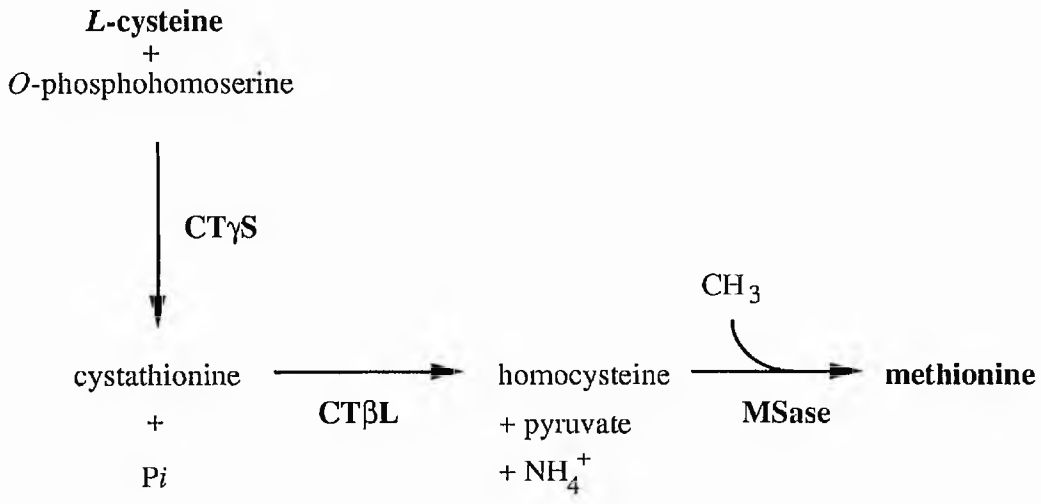


Figure 1.4 The production of methionine from cysteine in higher plants. Abbreviations: CTγS, cystathionine γ-synthase; CTβL, cystathionine β-lyase; MSase, methionine synthase; P*i*, inorganic phosphate

The regulation of methionine biosynthesis has not yet been studied in great detail but the availability of cDNA clones which represent the intermediate enzymes (Kreft et al. 1994; Droux et al. 1995; Eichel et al. 1995; Kim & Leustek 1996) will facilitate such studies in the future. Combining our knowledge of cysteine and methionine biosynthesis will allow a greater overall understanding of the control of protein production in plants. Figure 1.4 shows a schematic representation of methionine biosynthesis.

1.5 Concluding Introductory Remarks

The advent of modern molecular biological techniques has opened up interesting new approaches to studying the function and regulation of biochemical pathways in higher plants. In the few years since the first plant genes involved in reductive sulphate assimilation and cysteine biosynthesis were cloned, there has been a renewed surge in our understanding of these important processes. Detailed analysis of recombinant protein activity has partially resolved the on-going debate as to the mechanism of APS metabolism (Guttierrez-Marcos et al. 1996; Setya et al. 1996), transgenic studies have given us molecular insights into the regulation of cysteine metabolism (Saito et al. 1994a) and the *in vivo* molecular interactions involved in the cysteine synthase complex have been demonstrated for the first time (Bogdanova & Hell 1997). The basic molecular knowledge is now in place to study cellular localisation, transcription and protein expression of all the reactions involved in sulphate reduction and cysteine biosynthesis. Genomic cloning and genome sequencing projects are making gene and promoter sequences readily available (almost a third of the *A. thaliana* genome has been sequenced at the time of this thesis being completed). Genomic sequences, the availability of mutant populations of several research plant species and efficient reporter-gene systems (such as β-glucuronidase, GFP

and Luciferase (Guivarch et al. 1996; Leffel et al. 1997; Wood 1998)) are allowing us to analyse the function of promoter elements and to isolate and study genes which have regulatory control over biochemical pathways. As our understanding of sulphur metabolism at a molecular level increases, it will be possible to begin regulating the production of sulphur-containing amino acids transgenically, improving the quality of commercially important plant proteins in increasingly sulphur-deficient soils.

Due to the key regulatory roles of SAT and its product *O*AS (discussed in section 1.3.4), it seems that this enzyme should be one of the first involved in sulphate reduction and cysteine biosynthesis to benefit from such molecular research. The aim of this PhD is to isolate and characterise cDNAs representing the members of the SAT gene-family from *A. thaliana*. The role SAT plays in the various compartments of the plant cell remains unclear. The availability of cDNA clones which encode the various SAT isoforms will allow the regulation and expression of the gene-family in *A. thaliana* to be studied in detail at the levels of gene transcription and translation. Transgenic plants exhibiting enhanced or reduced SAT activity will also be produced, allowing the regulation of reductive sulphate assimilation and interactions between sulphate and nitrate assimilation to be further analysed. The work in this PhD should provide a detailed molecular characterisation of the SAT gene-family from *A. thaliana*.

Chapter 2: Materials and Methods

2.1 Chemicals and Reagents

2.1.1 Suppliers

All general laboratory chemicals and reagents were purchased from Sigma-Aldrich Ltd, UK or BDH Laboratory Supplies, UK unless otherwise stated.

Radioactive [α–^{35}S]dATP, [α–^{35}S]dUTP, [α–^{32}P]dCTP and [γ–^{32}P]dATP were purchased from ICN Biomedicals Ltd, UK

All restriction and modification enzymes and their respective reaction buffers were purchased from Promega UK Ltd unless otherwise stated.

2.1.2 Abbreviations for chemical names

BSA	Bovine serum albumin
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
IAA	Iso-amyl alcohol
IPTG	Isopropyl β-D-thiogalactopyranoside
KAc	Potassium acetate
MES	2-[N-Morpholino]-ethane sulphonic acid
MOPS	3-[N-Morpholino]-propane sulphonic acid
NaAc	Sodium acetate
NBT	Nitro Blue Tetrazolium
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid]
PVP	Polyvinyl pyrrolidone
SDS	Sodium dodecyl sulphate

TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) methylamine
Tween 20	Polyoxyethylenesorbitan monolaurate

2.1.3 Composition of common laboratory buffers and reagents

The pH of buffers was adjusted using NaOH or HCl solutions where required

10x MOPS:

200 mM MOPS
500 mM NaAc
10 mM Na_2EDTA
pH 7.0

10x PBS:

137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4
pH 7.4

20x SSPE:

3 M NaCl
200 mM NaH_2PO_4
20 mM Na_2EDTA
pH 7.4

20x SSC:

3 M NaCl
300 mM Na_3 citrate
pH 7.0

10x TBE:

890 mM Tris-HCl, pH 8.0
890 mM Orthoboric acid
20 mM Na_2EDTA

50x TAE:

40 mM Tris-acetate
2 mM Na_2EDTA
pH 8.5

50x Denhardt (1966) reagent:

1% (w/v) Ficoll
1% (w/v) BSA
1% (w/v) PVP

10x *Taq* DNA polymerase buffer:

500 mM KCl
100 mM Tris-HCl, pH 9.0
1% (v/v) Triton®X-100

10x *Pfu* DNA polymerase buffer:

200 mM Tris-HCl, pH 8.2
100 mM KCl
60 mM $(NH_4)_2SO_4$
20 mM $MgCl_2$
1% (v/v) Triton®X-100
10% (w/v) BSA

10x DNA polymerase Klenow fragment:

500 mM Tris-HCl, pH 7.2
100 mM $MgSO_4$
1 mM DTT

5x AMV reverse transcriptase buffer:

250 mM Tris-HCl, pH 8.3
250 mM KCl
50 mM $MgCl_2$
50 mM DTT
2.5 mM spermidine

10x T4 DNA ligase buffer:

300 mM Tris-HCl, pH 7.8
100 mM $MgCl_2$
100 mM DTT
10 mM ATP

5x RNA polymerase buffer (T3, T7, SP6):

200 mM Tris-HCl, pH 7.9
50 mM NaCl
30 mM $MgCl_2$
10 mM spermidine

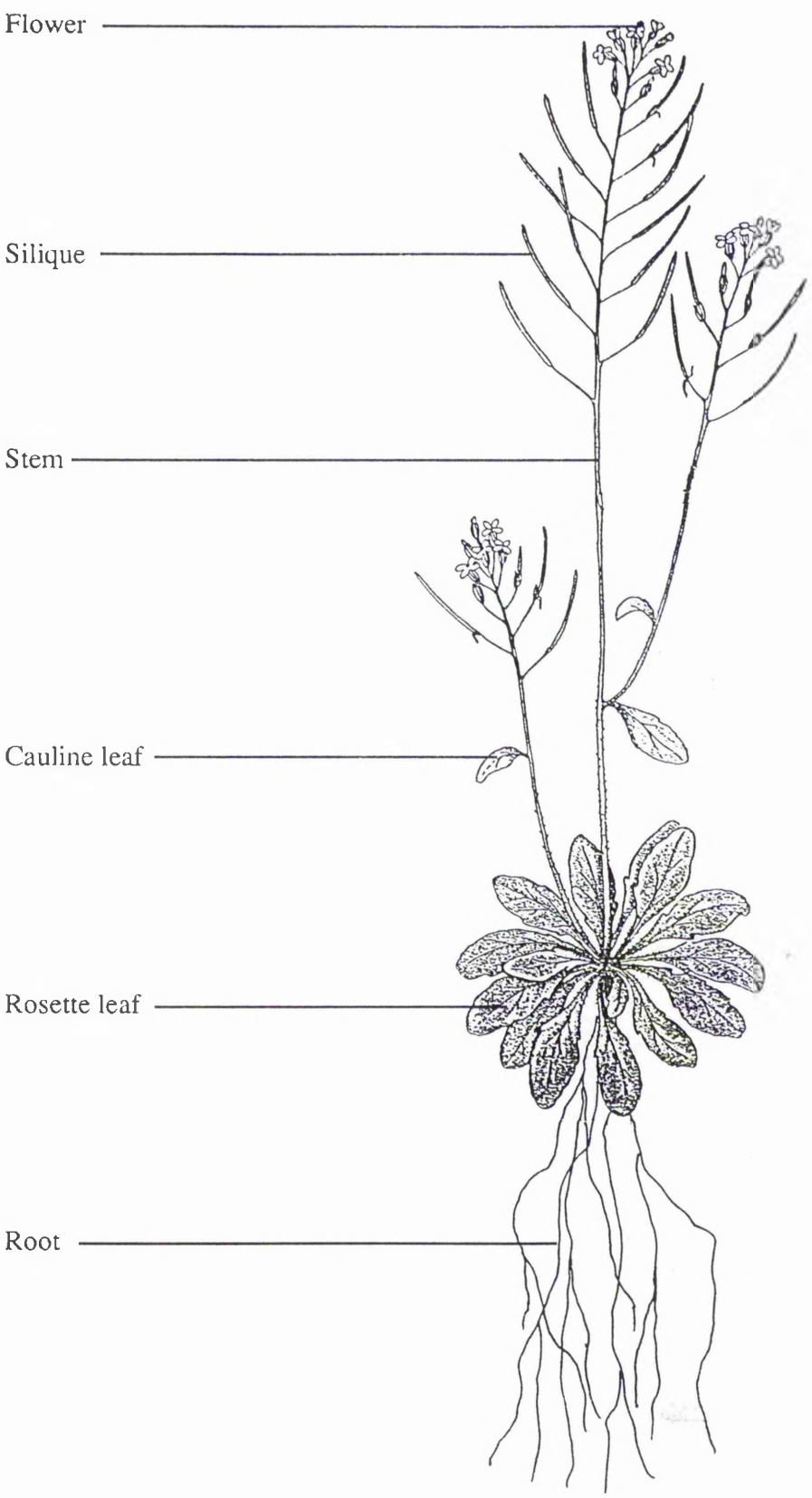
10x CIAP reaction buffer:

500 mM Tris-HCl, pH 9.3
10 mM $MgCl_2$
1 mM $ZnCl_2$
10 mM spermidine

2.2 Plant Material and Growth Conditions

Arabidopsis thaliana cv Columbia seed was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). The morphology of an *A. thaliana* plant is shown in Figure 2.1.

Figure 2.1 The morphology of fully grown *Arabidopsis thaliana* L. (Heynh.). Approximately to scale.


Flower
Silique
Stem
Cauline leaf
Rosette leaf
Root

2.2.1 Growth of plants on compost

Seed was sown on to a mixture of two parts Levington M3 compost (Fisons plc, UK) to one part horticultural grade vermiculite (Silvaperl, UK) in plastic trays and grown in growth cabinets with a 16 hour light (Photosynthetic photon flux density (PPFD) 250-280 $\mu mol/m^2/s$), 8 hour dark cycle at 21°C. Unless otherwise stated plants were watered from above with a fine spray whilst at the seedling stage and with a watering-can when this could be carried out without damaging the plants.

2.2.2 Growth of plants on vermiculite

For growth of *A. thaliana* in conditions of controlled nutrition, seed was sown directly onto vermiculite dampened with half strength Hoagland's medium (Hoagland & Arnon 1938; section 2.4.1) modified to suit the required nutrient regime. Plants were then watered from below every second day by dipping the trays in the required solution. Lighting conditions were as outlined in section 2.2.1.

2.2.3 Growth of plants in vitro

In vitro growth of *A. thaliana* tissue was carried out by sowing sterilised seed into MS or modified MS medium (Murashige & Skoog 1962; section 2.4.1) in sterile Ehrlenmeyer flasks or onto solid medium containing 1% (w/v) agar in sterile Petri dishes. Lighting conditions were as outlined in section 2.2.1.

Seed was sterilised by agitating for 2 mins in 1 ml 70% (v/v) ethanol in a microfuge tube, 50 mins in 1 ml 50% (v/v) household bleach containing 0.05% (v/v) Tween 20 and rinsed in four changes of sterile distilled water.

2.3 Bacterial Strains

2.3.1 Escherichia coli strains

JM15	*cys*E50 *tfr*-8(Jones-Mortimer 1968)
DH5α	*sup*E44 Δ*lac*U169 (ø80 *lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 (Hanahan 1983; Bethesda Research Laboratories).
BL21	*hsd*S *gal* (λc*Its*857 *ind*1 S*am*7 *nin*5 *lac*UV5-T7 gene1) (Studier & Moffatt 1986).
HB101	*sup*E44 *hsd*S20(r_B^- m_B^-) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*- 5 *mtl*-1(Boyer & Roulland-Dussoix 1969)

2.3.2 Agrobacterium tumefaciens strain

GV3101	(Koncz & Schell 1986)

2.4 Plant and Bacteriological Growth Media

2.4.1 Composition of plant growth media

MS (Murashige & Skoog 1962):
18.8 mM KNO_3, 3 mM $CaCl_2$, 2.5 mM MES, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 0.5 mM myo-inositol, 0.1 mM H_3BO_4, 0.1 mM $MnSO_4$, 0.1 mM $FeSO_4$, 0.1 mM Na_2EDTA, 30 µM $ZnSO_4$, 5 µM nicotinic acid, 2.4 µM pyridoxine-HCl, 1 µM H_3BO_4, 1 µM Na_2MoO_4, 0.3 µM thiamine-HCl, 0.1 µM $CuSO_4$, 0.1 µM, $CoCl_2$; pH 5.7

Half strength Hoagland's medium (Hoagland & Arnon 1939):
3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM $NH_4H_2PO_4$, 50 µM EDTAFeNa, 25 µM H_3BO_4, 4.5 µM $MnCl_2$, 0.5 µM Na_2MoO_4, 0.35 µM $ZnSO_4$, 0.15 µM $CuSO_4$; pH 5.7

2.4.2 Removing sulphates from agar

When plants were to be grown on solid growth medium in the absence of sulphates, contaminating salts were washed from agar by the method outlined in Davies et al. (1994). Agar was washed twice by stirring on a magnetic stirrer in 3 volumes of 96% (v/v) ethanol. The agar was then washed with 10 volumes of sterile distilled water which was drawn through the agar by vacuum using a Buchner funnel. Agar was then dried in an oven at 50°C for 24 hours.

2.4.3 Composition of bacterial growth media

Luria-Bertani (LB):		
	Tryptone	10 g/l
	Yeast extract	5 g/l
	NaCl	10 g/l
	pH 7.5	

SOB:		
	Tryptone	20 g/l
	Yeast extract	5 g/l
	NaCl	0.5 g/l
	$MgCl_2$	10 mM
	KCl	2.5 mM
	pH 7.0	

M9:		
	Na_2HPO_4	50 mM
	KH_2PO_4	22 mM
	NH_4Cl	20 mM
	NaCl	9 mM
	$MgSO_4$	2 mM
	$CaCl_2$	0.1 mM
	Mannitol	0.2% (w/v)
	pH 7.0	

Antibiotic	working conc.	stock conc.	storage
Ampicillin	50 µg/ml	50 mg/ml	H_2O, -20°C
Kanamycin	40 µg/ml	40 mg/ml	H_2O, -20°C
Tetracycline	15 µg/ml	5 mg/ml	96% ethanol, -20°C
Gentamycin	25 µg/ml	50 mg/ml	H_2O, -20°C
Rifampicin	150 µg/ml	25 mg/ml	methanol, -20°C

Table 2.1 Antibiotic concentrations used in bacterial selection media

2.5 Vectors

pBI121	(Clontech Laboratories Inc., USA)
pBluescript KS	(Stratagene Ltd, UK)
pCYB-4	(New England Biolabs Ltd, UK)
pGEM-T	(Promega UK Ltd)
pGEX-4T-1	(Pharmacia Biotech, UK)
pRK2013	(Ditta et al. 1980)
pSLJ4K1	(Jones et al. 1992)
pSLJ438A2	(Jones et al. 1992)
λYES	(Elledge et al. 1991)

2.6 Maintenance and Manipulation of Bacterial Strains

2.6.1 Preparation of competent bacterial cells

Competent *E.coli* cells were made using a variation of the method outlined in Sambrook et al. (1989). A single colony was inoculated into 5 ml of LB medium and incubated at 37°C over night at 250 rpm in an orbital incubator. 1 ml of this culture was then used to inoculate 100 ml of LB medium which was grown at 18°C for 40 hours at 250 rpm to an OD_{550} of approximately 0.5. The bacterial culture was chilled on ice for 10 mins and centrifuged at 4°C for 15 mins at 3000 xg in a Sorvall RC5C centrifuge with a GS-3 rotor (Du Pont Ltd, UK). The pellet was then suspended in 40 ml of transformation buffer (TB) (10 mM PIPES pH 6.7, 15 mM $CaCl_2$, 250 mM KCl, 50 mM $MnCl_2$) and chilled on ice for 10 mins before centrifuging as before. The pellet was again resuspended in 4 ml of TB before mixing with 0.3 ml of DMSO. 100 µl aliquots were immediately frozen in liquid nitrogen ready for use.

2.6.2 Transformation of bacteria

Transformation of plasmid DNA into competent bacterial cells was carried out as described in Sambrook et al. (1989). 10 to 50 ng of plasmid was added to 50 µl of competent cells and incubated on ice for 30 mins. The cells were then given a heat shock at 42°C for 45 seconds before quenching on ice for 2 mins. 200 µl of SOB medium, prewarmed to 37°C, was then added to the cells which were incubated for a further 60 mins at 37°C. Transformed cells were then selected by plating on LB medium solidified with 1% agar containing appropriate antibiotics.

2.6.3 Isolation of bacterial plasmid DNA

Bacterial plasmid DNA was isolated on a mini or midi scale, from 5 ml or 100 ml LB cultures respectively, using Qiagen plasmid preparation columns (Qiagen UK Ltd) as directed in the manufacturers instructions.

2.7 PCR Amplification of DNA

2.7.1 PCR amplification using Taq DNA polymerase

PCR (Polymerase Chain Reaction) amplification of DNA was performed using an MJResearch PTC-100™ Programmable Thermal Controller using methods described in McPherson et al. (1991). The reaction mixture contained:-

$MgCl_2$	1.5 mM
dNTPs	0.4 mM
Primer 1	0.5 μM
Primer 2	0.5 μM
Taq polymerase	0.5 Units
Target DNA	
in 1x Taq polymerase buffer.	

The PCR reaction involved 30 cycles of a primer annealing step at 55°C for 2 mins, chain extension at 72°C for 1 min per kilobase of DNA to be amplified and denaturation at 94°C for 1 min, unless otherwise stated.

2.7.2 PCR amplification using Pfu DNA polymerase

PCR reactions using high efficiency *Pfu* DNA polymerase (Stratagene Ltd, UK) were performed essentially as outlined in section 2.7.1. An extension step of 2 mins per kilobase DNA to be amplified, a maximum of 24 cycles and 2.5 Units of enzyme per reaction were used as recommended by the manufacturer. *Pfu* DNA polymerase incorporates errors 15- to 30-fold less frequently than *Taq* polymerase (Lundberg et al. 1991).

2.8 Subcloning DNA

2.8.1 DNA digestion

Restriction endonuclease digestion of DNA for subcloning purposes was carried out using enzymes and buffers purchased from Promega UK Ltd. DNA fragments for subcloning were extracted from 1% agarose, 1x TAE gels using QIAEX DNA Gel Extraction columns (Qiagen UK Ltd) according to the manufacturers instructions.

Partial digestion of DNA for subcloning was carried out as follows. 100 µl of DNA solution, in 1x restriction buffer, was separated into aliquots of 30, 20, 20, 20 and 10 µl which were numbered 1,2,3,4 and 5 respectively and kept on ice. The appropriate amount of restriction endonuclease was then added to tube 1 and mixed. 10 µl from tube 1 was then added to tube 2 which was mixed and 10 µl added to tube 3. This serial dilution of the enzyme was continued until all five tubes contained 20 µl. Digestion mixtures were then incubated at the optimum digestion temperature for 10 minutes before inactivating the enzyme by heating to 65°C for 10 minutes. Partial digestion products were then sized by electrophoresis as outlined in section 2.9.1 and the fragment of the desired size extracted from the agarose gel using QIAEX DNA Gel Extraction columns (Qiagen UK Ltd)

2.8.2 Precipitation of nucleic acids

Precipitation of nucleic acids from solution was carried out by the addition of 0.1 volumes of 3 M NaAc, pH 5.2 and 3 volumes of ice-cold 96% ethanol followed by incubation at -70°C for 30 mins or -20°C for 2 hours. After centrifugation at 10000g for 15mins at 4°C in a bench-top microfuge, the pellet was washed in 70% ethanol, centrifuged as before and allowed to dry for 5

mins at room temperature in a vacuum desiccator. The pellet was then resuspended in a suitable volume of 10 mM Tris-HCl, pH 7.5 or H_2O.

2.8.3 Alkaline phosphatase treatment of digested plasmid DNA

To prevent self-ligation of compatible ends of plasmid DNA during subcloning, digested plasmid was treated with calf intestine alkaline phosphatase, CIAP. Restriction endonucleases in the digestion reactions were inactivated by heating to 65°C for 10 mins. 0.4 units of CIAP and 0.08 volumes of phosphatase buffer were then added, as outlined in manufacturers instructions, and the reaction incubated at 37°C for 30 mins. The CIAP was then inactivated by the addition of SDS and EDTA, pH 8.0 to final concentrations of 17 mM and 5 mM respectively and heating to 75°C for 10 mins. CIAP-treated plasmid was purified by phenol/chloroform extraction followed by ethanol precipitation. One volume of phenol/chloroform/IAA (25:24:1) was added and the mixture vortexed for 1 min before centrifuging at 10000 xg for 5 mins in a bench-top microfuge. The upper aqueous layer was removed and added to one volume of chloroform/IAA (24:1) before vortexing and centrifuging as before. DNA was then precipitated from the upper aqueous phase as outlined in section 2.8.2.

2.8.4 Ligation of insert and plasmid DNA

Ligation of DNA fragments into plasmid vectors was carried out according to the method outlined in Sambrook et al. (1989). 50 ng of vector and an appropriate amount of insert DNA to give a 1:1 molar ratio of compatible ends were mixed in a minimum volume of 1x ligation buffer with 3 units of T4 DNA ligase. Appropriate amounts of vector and insert DNA for ligation reactions were calculated as follows:-

$$\frac{\text{ng vector x kb insert}}{\text{kb vector}} = \text{ng of insert required for 1:1 molar ratio of vector to insert.}$$

Reactions were incubated over night at 15°C. Control reactions containing plasmid but no insert DNA were also set up to indicate the level of plasmid self-ligation expected.

2.8.5 Selection of positive subcloned constructs

Identification of plasmid constructs containing correctly ligated DNA inserts was carried out by bacterial colony PCR. The ligation reaction mixture was added to appropriate bacterial strains, transformed as detailed in section 2.6.2 and incubated over night at 37°C on LB selection plates containing appropriate antibiotics. Resulting colonies were numbered and picked from the plate using sterile toothpicks, touched on a fresh selection plate (divided into numbered sections for future reference) and the toothpick dipped into 20 µl of PCR reaction mix (section 2.7.1). Colonies were boiled in the reaction mix for 2 mins before amplification. Amplification products of the expected size when reactions were separated on agarose gels (section 2.9.1) indicated which bacterial colonies contain constructs with the desired, correctly orientated insert.

2.9 DNA Analysis

2.9.1 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis of DNA samples was carried out as described in Sambrook et al. (1989). 1x TAE, 1% agarose gels containing 0.5 µg/ml ethidium bromide were run in 1x TAE reservoir buffer. DNA samples were mixed with 0.1

volume of gel loading buffer (80% (v/v) glycerol, 0.02% (v/v) bromophenol blue in 1x TBE) and separated by electrophoresis at 60 V. DNA was then visualised using a short wavelength UV transilluminator. Gels were photographed under UV light using a Polaroid MP-4 camera and Polaroid Type 667 black and white instant film (Polaroid Corp., USA). 1 μg of λHindIII markers (λ DNA digested to completion with HindIII) were run alongside the lanes of DNA to allow sizing of fragments. λHindIII markers contain bands of 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56 and 0.13 kb.

2.9.2 Spectrophotometric determination of nucleic acid concentration

Nucleic acid concentrations in solution were calculated from spectrophotometric measurements at 260 and 280 nm. An absorbance of 1.0 at 260 nm corresponds to approximately 50 μg/ml of double-stranded DNA, 40 μg/ml single-stranded DNA and RNA and 20 μg/ml for single-stranded oligonucleotides. The A_{260}/A_{280} ratio was used to determine the purity of the nucleic acid sample. Pure DNA and RNA preparations, free from protein and/or phenol, have ratios of approximately 1.8 and 2.0 respectively. Measurements were taken using a Unicam Heλios-α spectrophotometer (Unicam Ltd, UK).

2.9.3 Direct sequencing of plasmid DNA

Sequencing of DNA from plasmid preparations was carried out manually by the method of Sanger et al. (1977), using a Sequenase™ Version 2.0 DNA sequencing kit (Amersham International plc, UK).

4 μg of plasmid DNA was alkali denatured by the addition of 0.1 volumes 2 M NaOH, 2 mM EDTA at 37°C for 30 mins. Denatured plasmid was then precipitated as outlined in section 2.8.2 and resuspended in 7 μl H_2O. 1 pmol of sequencing primer was then annealed to the denatured plasmid by heating to

65°C in 1x annealing buffer (supplied in sequencing kit) and allowing to cool slowly to 30°C. Sequencing reactions were then performed as outlined in the manufacturers instructions.

Sequencing reactions were subjected to denaturing gel electrophoresis at 2000 V through a 0.8x TBE, 6% polyacrylamide (Scotlab, UK) gel using a Bio-Rad Sequi-gen Nucleic Acid Sequencing System (Bio-Rad Laboratories Ltd, UK). Denaturing gel solution contained:-

Urea	7 M
10x TBE	80 ml/l
40% Acrylamide	150 ml/l

Gel solution was polymerised with 750 µl/l 25% (w/v) ammonium persulphate and 750 µl/l TEMED

Sequencing gels were dried using a Bio-Rad 538 Gel Dryer (Bio-Rad Laboratories Ltd, UK) and sequence data visualised by exposing the gel to Kodak X-OMAT AR scientific imaging film (Kodak Ltd, UK) for an appropriate time period and developing in a Fuji RG II X-ray film processor (Fuji Photographic Film Co. Ltd, Japan).

2.9.4 Computer analysis of DNA sequences

Computer analysis of DNA and translated protein sequences was carried out using the GCG (Genetics Computer Group, Daresbury Laboratory-http://www.seqnet.dl.ac.uk) programs COMPARE, CLUSTALV, PHYLIP, MAP, MOTIFS, PEPTIDESORT, PEPTIDESTRUCTURE, CODONFREQUENCY, PUBLISH, PILEUP and SEQED (Devereux et al. 1984). BLAST nucleotide and amino acid sequence homology searches (Altschul et al. 1990) were carried out at NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.) via the World Wide Web (http://www.ncbi.nlm.nih.gov/) using Genbank, Swissprot, PIR and PROSITE (Bairoch 1993) databases.

2.9.5 Isolation of genomic DNA from A. thaliana tissue

Genomic DNA was extracted from *A. thaliana* tissue using the method described by Dellaporter et al. (1983). 10 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 70 mM SDS, 250 μM PVP 40000, 0.1% (v/v) β-mercaptoethanol), prewarmed to 65°C, was added to 2 g of tissue ground to a powder in liquid nitrogen. The tissue and buffer were gently mixed and incubated at 65°C for 10 mins. 2.6 ml of 5 M KAc was then added and incubated on ice for 5 mins before centrifugation at 15000 xg for 30 mins at 4°C in a Sorvall RC5C centrifuge with an SS-34 rotor (Du Pont Ltd, UK). The supernatant was then removed to a clean tube and the nucleic acids precipitated by the addition of 12.8 ml isopropanol and 1.2 ml 3 M sodium acetate. The tube contents were gently swirled and incubated at -20°C for 1 hour before centrifuging at 15000 xg for 25 mins at 4°C in a Sorvall RC5C centrifuge with an SS-34 rotor (Du Pont Ltd, UK). The pellet was resuspended in 4 ml of TE, pH 8.0 (50 mM Tris/10 mM EDTA), reprecipitated and dried as outlined in section 2.8.2 and dissolved in 500 μl Tris-HCl, pH 7.5. RNA in the final DNA extract was hydrolysed by adding RNaseA (DNase-free) to a final concentration of 50 μg/ml. The volumes in this method were scaled down 20 times for genomic minipreps.

2.10 RNA Analysis

2.10.1 Isolation of total RNA from A. thaliana tissue

Total RNA was isolated from *A. thaliana* tissue using the method of Verwoerd et al. (1989). Tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar and 100-200 mg (aproximately 500 µl of powder) was transferred to a microfuge tube. 500 µl of extraction buffer (0.1 M LiCl, 0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.1% (w/v) SDS in 50% (v/v) phenol) preheated to 85°C was added and the tube vortexed for 30 seconds. 250 µl of chloroform/IAA (24:1 ratio) was then added to the tube before vortexing for a further 30 seconds. The tubes were centrifuged at 10000 xg for 5 mins in a bench-top microfuge at 4°C and the aqueous phase transferred to a clean tube. To minimise the risk of RNA hydrolysis by contaminating RNases all the following steps were carried out at 4°C, using plasticware which had been autoclaved at 121°C for 25 mins and touched only with gloved hands. RNA was then precipitated by adding an equal volume of 4 M LiCl, mixing and incubating over night at 4°C. Next day tubes were centrifuged at 10000 xg for 15 mins, the supernatant removed and the pellet allowed to air dry before resuspending in 250 µl sterile distilled water treated with DEPC (mixing distilled water with 0.1% DEPC before autoclaving inactivates RNases). RNA was then precipitated by the method described in section 2.8.2. The final RNA pellet was resuspended in 21 µl of DEPC-treated water and 1 µl of this used for spectrophotometric determination of concentration as outlined in section 2.9.2.

2.10.2 Primer extension analysis

Analysis of mRNA size in base-pairs was carried out by primer extension analysis based on the method described by Kingston (1987). From the cDNA sequence corresponding to the mRNA to be analysed, a 30mer oligonucleotide

primer was designed (complementary to the sense strand of the cDNA sequence) approximately 100 bases from the 5' end. The primer was then 'end-labelled' in the following reaction mixture which was incubated for 30 mins at 37°C:-

1 µM Primer	0.5 µl
2 µCi/µl [γ–^{32}P]dATP	8 µl
10x kinase buffer	1 µl
T4 polynucleotide kinase (8 U/µl)	0.5 µl

The kinase was inactivated by heating to 65°C for 5 mins and the primer was then precipitated by adding 10 µl 4 M ammonium acetate, 100 µl ethanol and incubating on dry ice for 30 mins. After spinning down at 10000 xg for 10 mins in a bench-top microfuge the pellet was resuspended in 8 µl of DEPC-treated water. The labelled primer was then added to 10 µl of 5 µg/µl total RNA and the primer annealed to the RNA by heating to 65°C for 2 mins and allowing to cool slowly to room temperature. Primer extension was then performed by reverse transcriptase (RT) in the following reaction at 42°C for 90 mins:-

Annealed primer	18 µl
4 mM dNTPs	3 µl
10x RT buffer	2.5 µl
40 U/µl RNase Inhibitor	1.25 µl
200 U/µl Reverse Transcriptase	2 µl
1 mg/ml Actinomycin D	0.5 µl

1 µl of 0.5 M EDTA, pH 8.0 and 1 µl DNase-free pancreatic RNase A (5 µg/ml) were added to the completed RT reaction and incubated at 37°C for 30 mins to hydrolyse the RNA. The remaining labelled first strand cDNA was purified by precipitation as described in section 2.8.2 and resuspended in 30 µl of sterile distilled water. 1 µl of this was then loaded on a DNA sequencing gel as described in section 2.9.3 next to a known sequence to allow calculation of the original mRNA length.

2.11 Southern Blotting of Genomic DNA

2.11.1 Digestion of genomic DNA

10 µg of genomic DNA was made up to 100 µl in sterile distilled water. This was then digested in the following reaction mixture:-

DNA sample	100 µl
10x restriction buffer	20 µl
1 mg/ml Bovine serum albumin	2 µl
0.1 M spermidine	4 µl
Restriction endonuclease (50 U/µl)	1 µl
H_2O	73 µl

The tube was gently mixed and pulsed in a bench-top microfuge before incubation over night at the optimum temperature for the enzyme concerned. The following day the sample was precipitated as outlined in section 2.8.2, resuspended in 16 µl of Tris-HCl, pH 7.5 and 4 µl of DNA loading dye (80% (v/v) glycerol, 0.02% (v/v) bromophenol blue in 1x TBE). The sample was then heated to 65°C for 3 mins to make sure the DNA was fully dissolved.

2.11.2 Electrophoresis of digested genomic DNA

Digested genomic DNA samples were separated over night on 1x TBE, 1% agarose gels in 1x TBE running buffer at 20 V. A lane of λHindIII markers was also loaded to allow later sizing of hybridisation bands (see section 2.9.1). When the dye-front had reached the required distance, the gel was removed from the tank and stained by immersing in 1 µg/ml ethidium bromide, washed in distilled water for 15 mins and photographed on a UV transilluminator using a Polaroid MP-4 camera and Polaroid Type 667 black and white instant film (Polaroid Corp., USA).

2.11.3 Denaturation of digested genomic DNA in agarose gels

Prior to blotting, the separated DNA was denatured by submerging the agarose gel in a suitable volume of 1.5 M NaCl, 0.5 M NaOH for 45 mins with gentle agitation. The gel was then rinsed several times in distilled water before neutralising in a solution of 1 M Tris, pH 7.4, 1 M NaCl, again with agitation, for 30 mins. The solution was then replaced and neutralising continued for a further 15 mins.

2.11.4 Blotting digested genomic DNA

DNA was transfered from the gel to Hybond-N™ nylon membrane (Amersham International plc, UK) following the manufacturers protocol. Two sheets of Whatman 3MM paper (Whatman, UK) were soaked in 20x SSC buffer and laid over a glass plate suspended on top of a shallow dish containing 20x SSC, the ends of the paper forming a wick in the buffer reservoir. The gel was then placed face-down on the paper and all bubbles beneath squeezed out using a glass rod. A piece of Hybond-N™ membrane, precut to the size of the gel, was then gently rolled onto the gel making sure no bubbles were trapped underneath. Three pieces of Whatman 3MM paper, again cut to the size of the gel, were soaked in 20x SSC and placed on top of the membrane. A stack of absorbent paper hand-towels were placed on the 3MM papers and the whole system was weighed down by 500-1000g on a glass plate allowing the DNA to transfer to the membrane over night by capillary flow. Next day the transfer system was dismantled and the position of the wells marked on the membrane with a soft pencil. The gel was then peeled off the membrane which was left to dry at room temperature for 1 hour. DNA was fixed to the membrane by cross-linking with 120 mJ/cm^2 of 254 nm UV light in a XL-1500 Spectrolinker (Spectronics corp., USA).

2.12 Northern Blotting of RNA

2.12.1 Electrophoresis of RNA

Total RNA was extracted from *A. thaliana* tissue and concentrations of samples spectrophotometrically determined as outlined in sections 2.10.1 and 2.9.2. 15 µg of each sample was made up to 10 µl with DEPC-treated water and added to 10 µl of 2x sample buffer (50% (v/v) formamide, 16.5% (v/v) formaldehyde, 10 mM EDTA pH 7.5, 40 mM NaH_2PO_4, 0.2 µg/µl ethidium bromide). 3 µl of RNA ladder markers (Gibco BRL Ltd, UK) were similarly prepared to allow later sizing of hybridisation bands. All RNA samples were incubated at 65°C for 15 mins, quenched on ice for 5 mins and 5 µl of loading buffer (50 mM EDTA pH 8.0, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue) was added to each.

RNA was separated through 1x MOPS, 3% (v/v) formaldehyde, 1% (w/v) agarose gels for 3 hours at 70 V in 1x MOPS reservoir buffer. Gels were then rinsed twice in DEPC-treated water for 5 mins and visualised on a UV transilluminator using a Polaroid MP-4 camera and Polaroid Type 667 black and white instant film (Polaroid Corp., USA).

2.12.2 Blotting RNA

RNA was blotted onto Hybond-N™ (Amersham International plc, UK) nylon membrane using the same capillary transfer method used for genomic Southern blots as outlined in section 2.11.4.

2.13 Hybridisation

2.13.1 Radioactive labelling of DNA probes

DNA probes for Southern and northern hybridisation were radiolabelled with [α-^{32}P]dCTP as described by Feinberg and Vogelstein (1983). 25 ng of double-stranded DNA probe was denatured by boiling for 3 mins and then quenched on ice to prevent re-annealing. Radiolabelling was then carried out using Amersham Multiprime DNA labelling kit (Amersham International plc, UK) following the manufcturers instructions. The reaction was incubated at 37°C for 30 mins and contained:-

Denatured probe	28 µl
Labelling buffer	10 µl
Random primer mix	5 µl
α-^{32}P-dCTP (0.4 MBq/µl)	5 µl
Klenow DNA polymerase (1 U/µl)	2µl
H_2O	to 50µl

Labelled probe was then separated from unincorporated nucleotides by passing through a Sephadex G-50 Nick column (Pharmacia Biotech, UK) using TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) as the eluting agent. The labelled probe was boiled for 6 mins, quenched on ice and used in hybridisation reactions.

2.13.2 Hybridisation of radiolabelled probes

Labelled probe was hybridised to immobilised nucleic acids as outlined in Sambrook et al. (1989) using Techne hybridisation tubes and oven (SLS, UK). Nylon filters were pre-hybridised for 2-5 hours at 42°C in 25 ml prehybridisation solution (40% (v/v) formamide, 5x SSPE, 0.5% (w/v) SDS, 5x Denhardt's reagent, 0.25 mg/ml sheared, denatured herring testes DNA). The

radiolabelled probe was added and incubated over night at 42°C. Next day the filters were washed in an SSPE, 0.1%SDS solution appropriate to the stringency required. For low stringency washing, 2x SSPE, 0.1% SDS solution was used at 42°C for 15 mins. For high stringency washing, lower ionic strengths and higher temperatures were used (such as 0.1x SSPE, 0.1% SDS at 50°C for 15 mins).

Filters were sealed in plastic and exposed to Kodak X-OMAT AR scientific imaging film (Kodak Ltd, UK) or a Fuji BAS IIIs Phosphoimaging screen (Fuji Photographic Film Co. Ltd, Japan) for an appropriate time before developing in a Fuji RGII X-ray film processor or Fujix Bioimaging Analyser IPR1000 (Fuji Photographic Film Co. Ltd, Japan).

2.14 *In Situ* Hybridisation

2.14.1 In vitro transcription of riboprobes

Riboprobes were transcribed as outlined in Cox and Goldberg (1988) from gene-specific cDNA probes. Probes subcloned into pBluescript (Stratagene, USA) or pGEM (Promega UK Ltd) were amplified by PCR using universal M13 primers (section 2.7.1).

Transcription was performed by incubating the following reaction mixture at room temperature for 30 mins.

0.2 µg/µl template DNA	7 µl
200 mM DTT	1 µl
10 mM dATP	1 µl
10 mM dGTP	1 µl
10 mM dCTP	1 µl
10x RNA polymerase buffer	2 µl
40 U/µl RNase inhibitor	1 µl
10 µCi/µl [α–^{35}S]dUTP	5 µl
20 U/µl SP6, T3 or T7	
RNA polymerase	1 µl

The reaction mixture was then treated with 20 U of RNase-free DNaseI in 29 µl DEPC-treated water for 15 mins at 37°C. The riboprobe was purified by vortexing with an equal volume of phenol before centrifugation for 1 min at 10000 xg in a bench-top microfuge. The aqueous phase was removed, vortexed with an equal volume of chloroform/IAA (24:1 ratio) and centrifuged as before. The aqueous layer was finally centrifuged through a Sephadex G-50 minicolumn at 2000 xg for 2 mins and the purified riboprobe collected in a 0.5 ml microfuge tube.

To allow the probe to penetrate the fixed tissue sections more easily riboprobes were hydrolysed to 0.15 kb fragments by incubating in an equal

volume of carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3) at 60°C for a time period *t* calculated using the following equation:-

$$\text{incubation period, } t = \frac{(L_o - L_f)}{k \cdot L_o \cdot L_f}$$

t = incubation period

L_o = initial probe length

L_f = required probe length

k = rate constant (0.11 kb/min)

The hydrolysis reaction was neutralised by adding 5 µl 10% (v/v) acetic acid, the probe precipitated by the method outlined in section 2.8.2 and resuspended in 11 µl DEPC-treated water. 1 µl of this was added to 4 ml Ready Solv HP scintillation cocktail (Beckman Instruments Inc., USA) and the level of [α-^{35}S]dUTP incorporation calculated using a Beckman LS6000 scintillation counter (Beckman Instruments Inc., USA).

2.14.2 Tissue fixation and embedding

Tissue for *in situ* hybridisation was fixed, as described by Cejudo et al. (1992), in FAE solution (3.7% (w/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol). Plant material was submerged in this solution, held under the surface with gauze and subjected to vacuum in a desiccator attached to an oil pump for 15 to 20 mins, making sure the formaldehyde did not boil. Samples were stored over night at 4°C in fresh FAE solution then dehydrated through an ethanol series as outlined on the next page.

Ethanol series (v/v percentages)

50% ethanol, 50% 150 mM NaCl	30 mins at 4°C
60% ethanol, 40% 150 mM NaCl	30 mins at 4°C
70% ethanol, 30% 150 mM NaCl	30 mins at 4°C
85% ethanol, 15% 150 mM NaCl	30 mins at room temperature
95% ethanol, 5% 150 mM NaCl	30 mins at room temperature

The tissue was then incubated over night at 4°C in 95% ethanol, 5% 150 mM NaCl, 0.1% (w/v) Eosin dye. The following day excess dye was washed away by incubating in several changes of 100% ethanol for 1 hour at 4°C. Ethanol was next replaced with Xylol solvent (Merck & Co., USA) by incubating for 1 hour at room temperature in 60% ethanol/40% Xylol, then 40% ethanol/60% Xylol followed by 3x 1 hour in 100% Xylol. To embed the fixed tissue 15 chips of Paraplast wax (Sigma-Aldrich Ltd, UK) per 10 ml of Xylol were added and left over night at room temperature. Samples were then incubated at 42°C and five chips were added every 4 hours for 12 hours. The solution was then carefully poured away and replaced with embedding solution (80% liquid Paraplast wax, 20% Xylol molten at 58°C) and incubated at 60°C. The embedding solution was then changed twice daily for the next 4 days before positioning the tissue with a needle and allowing the wax to set.

2.14.3 Tissue sectioning and mounting

Wax containing fixed tissue was cut into small trapezoid blocks, leaving 2 mm of wax around the sample, and attached to a Reichert-Jung 2040 (R. Jung GmbH, Germany) microtome mounting. 5-10 µm sections were then cut through the tissue and floated on sterile water on microscope slides which had been cleaned in acetone, baked at 180°C for 2 hours and coated with a film of 1 mg/ml poly-L-lysine. The water was carefully removed with the corner of a tissue and the slides allowed to dry on a 42°C hotplate over night.

2.14.4 Tissue pretreatments

Tissue sections were pretreated as described by Cox and Goldberg (1988). Slide mounted tissue sections were rehydrated by passing through an ethanol series in the reverse order to that mentioned in section 2.14.2 and equilibrated in 1x PBS for 2 mins. Proteins in the fixed tissues were then digested by 0.125 mg/ml Pronase in 1x PBS for 10 mins at room temperature before blocking with 0.2% (w/v) glycine in 1x PBS. Tissues were then refixed for 10 mins in 4% (v/v) formaldehyde in 1x PBS before acetylation with 0.5% (v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 mins. After washing for 2 mins in 1x PBS, tissues were dehydrated through the ethanol series described in section 2.14.2 and the slides stored at 4°C.

2.14.5 Hybridisation and washing

The required amount of riboprobe (0.1 µg or 0.3 µg) was diluted to 4 µl with DEPC-treated water and added to 4 µl formamide. The probe was then added to 32 µl of hybridisation solution (80 mM DTT, 300 mM NaCl, 10 mM Tris-HCl pH 6.8, 5 mM EDTA, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 50% (v/v) formamide, 12.5% (w/v) dextran sulphate, 1 mg/ml tRNA, 1x Denhardt's reagent), heated to 80°C for 3 mins and quenched on ice. The probe was then carefully pipetted onto the sample slide and a coverslip lowered on top making sure no bubbles were trapped underneath which would prevent the probe coming into contact with the tissue sections. Hybridisation took place at 50°C over night in a sealed plastic box containing tissues soaked in water to maintain high humidity. Next day slides were washed by immersing and gently agitating for 30 mins in wash buffer (2x SSC, 50% formamide, 5 mM DTT) at 50°C allowing the coverslips to fall off. The wash buffer was replaced and the slides incubated at 50°C for a further 90 mins. Slides were then washed twice for 5 mins at 37°C in NTE

buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA), for 30 mins at 37°C in NTE containing 20 μg/ml RNaseA, twice more for 5 mins at 37°C in NTE and for 2 mins at room temperature in 1x SSC. Slides were then dehydrated through an ethanol series of 30 seconds in 30%, 60%, 80%, 95% (made up with 0.3 M ammonium acetate) and 100% ethanol before air drying.

2.14.6 Exposure and developing

In situ slides were exposed by dipping in Kodak NTB-2 (Kodak Ltd, UK) liquid emulsion. This had been melted at 45°C for 45 mins and diluted with 1.4 volumes of 45°C water in a photographic darkroom. Each dipped slide was left to set for 1 hour, was wrapped in three layers of aluminium foil and stored at 4°C in a light-tight box containing silica gel dessicant for the required exposure period.

The *in situs* were developed by allowing to equilibrate to room temperature, placing in slide racks and submerging in Kodak D19 developer (Kodak Ltd, UK) and leaving to stand for 2 mins, dipping in 1% (v/v) acetic acid stop solution for 30 seconds and fixing in 30% (w/v) sodium thiosulphate for 5 mins. All these steps were carried out in a darkroom using solutions pre-chilled to 14°C. Slides were washed in several changes of distilled water, the tissues stained for 30 seconds in 0.02% (w/v) toluidine blue before dehydrating for 30 seconds in each step of the ethanol series outlined in section 2.14.2. Slides were finally dipped in Histo-Clear (National Diagnostics, USA) and two drops of Depex (Hopkin & Williams, UK) applied to the tissues before covering with a coverslip and allowing to dry in a fume hood for 24 hours.

Slides were viewed using a Leitz Laborlux 12 microscope (Ernst Leitz Wetzlar GmbH, Germany) under dark-field and light-field illumination.

2.15 Protein Analysis

2.15.1 Isolation of protein from A. thaliana tissue

Tissue was ground in 10 ml/g of extraction buffer (0.1 M Tris-HCl pH 8.5, 1 mM EDTA, 4 mM DTT, 250 μM PVP 40000) on ice using a pestle and mortar. Cell debris was then separated by centrifuging at 21000 xg for 20 mins at 4°C in a Sorvall RC5C centrifuge with an HB4 rotor (Du Pont Ltd, UK). The supernatant containing soluble protein was kept on ice for immediate use or stored at -40°C.

2.15.2 Quantitation of protein concentration

Protein concentration in solution was determined according to Bradford (1976). A known volume of solution was made up to 100 μl with 0.15 M NaCl. 1 ml of Bradford's reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 5% (v/v) ethanol, 10% (v/v) phosphoric acid) was added, vortexed for 10 seconds and incubated at room temperature for 2 mins. Protein concentration was then determined by measuring the A_{595} and comparing to a standard curve of known protein concentrations against A_{595}. The standard curve (Figure 2.2) was plotted by performing the above assay with freshly made dilutions of 0, 20, 40, 60, 80 and 100 μg/ml BSA in 0.15 M NaCl. If the unknown protein concentration was outside this range a diluted sample was assayed.

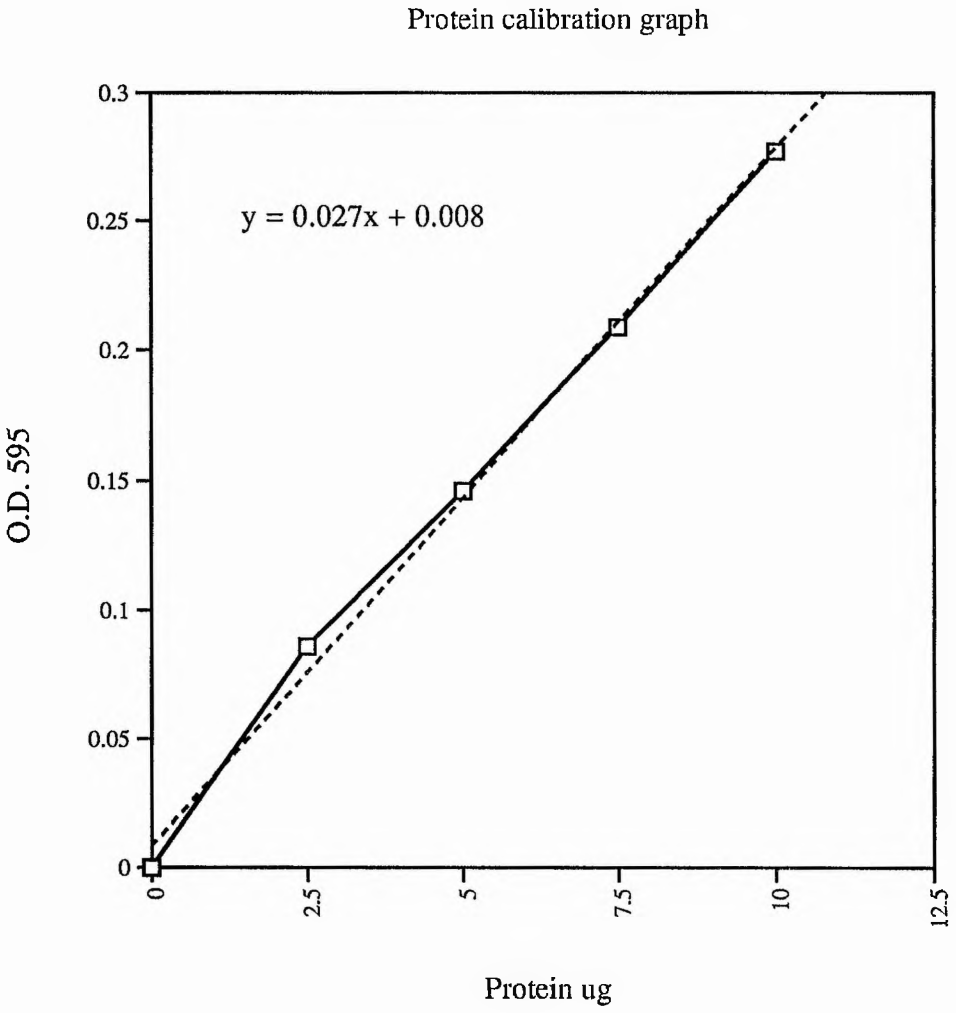


Figure 2.2 Example standard curve from a Bradford's protein quantitation assay (Bradford 1976). BSA was used as the protein standard.

2.15.3 Serine acetyltransferase enzyme assay from crude bacterial extracts

Serine acetyltransferase (SAT) activity in *E. coli* crude extracts was assayed by the method of Kredich and Tompkins (1966). A 5 ml bacterial culture was grown over night in LB medium containing relevant antibiotics at 37°C and 250 rpm in an orbital incubator. 0.5 ml of this culture was used to inoculate 200 ml of LB medium, containing 0.5 mM IPTG, and the culture grown to an OD_{420} of 0.8. The bacterial cells were then pelleted by centrifuging the culture at 15000 xg for 20 mins at 4°C in a Sorvall RC5C centrifuge with a GS-3 rotor (Du Pont Ltd, UK). The pellet was washed with 50 mM Na_2HPO_4 and recentrifuged before grinding in a chilled pestle and mortar with a small amount of alumina and 2.5 ml sodium phosphate buffer, pH 7.2 (68.4 mM Na_2HPO_4, 31.6 mM NaH_2PO_4) per gram of pellet. The slurry was centrifuged at 15000 xg for 20 mins at 4°C and the supernatant retained for enzyme assays.

Serine acetyltransferase activity was determined in the following assay conditions:-

Enzyme extract	150 µl
10 mMTris-HCl, pH 7.6	800 µl
4 mM acetyl coenzyme A	25 µl

Activity was determined by monitoring the decrease in A_{232} (due to cleavage of the acetyl group of acetyl CoA by SAT) on the addition of 25 µl 400 mM *L*-serine. In order to calculate SAT activity per µg protein, aliquots of enzyme extract were retained for protein concentration determination as outlined in section 2.15.2. Acetyl CoA concentrations were calculated using the following equation.

$$\text{acetyl CoA concentration (M)} = \frac{A_{232}}{\varepsilon \cdot L}$$

ε = extinction coefficient (6.5×10^3 l/mol/cm)

L = lightpath length (cm)

2.15.4 Electrophoresis of proteins by SDS-PAGE

Polyacrylamide gel electrophoresis of proteins was carried out using a Mini-PROTEAN II gel system (Bio-Rad Laboratories Ltd, UK) according to the manufacturers guidelines. Each 10% acrylamide gel consisting of a lower resolving gel, which was overlaid with water-saturated butanol and allowed to polymerise for 30 mins, and an upper stacking gel in which the wells were formed which was poured on top of the lower gel. Gels were made from stock solutions as outlined in Table 2.2.

Protein samples were prepared by adding 0.2 volumes of 5x SDS sample buffer (50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.5% (v/v) β-mercaptoethanol) and boiling for 10 mins prior to loading. Gels were run at 150 V for approximately 1 hour in 25 mM Tris pH 8.3 192 mM glycine, 1% (w/v) SDS running buffer. To allow approximate sizing of protein bands a lane of prestained SDS-PAGE molecular weight markers was also loaded which had apparent molecular weights of 126, 102, 81, 53.5, 37 and 31.4 kD (Sigma-Aldrich Ltd, UK) or 202, 133, 71, 42, 31, 18 and 7 kD (Bio-Rad Laboratories Ltd, UK).

To view electrophoresed protein samples, gels were stained by gentle agitation for 1 hour in 40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R-250 and destained by gentle agitation for 4 hours in 40% (v/v) methanol, 10% (v/v) acetic acid. Gels were then dried in a Bio-Rad GelAir Dryer (Bio-Rad Laboratories Ltd, UK).

2.15.5 Western analysis of proteins

For western analysis, proteins in unstained SDS-PAGE gels were electro-transferred to nitrocellulose membranes (Bio-Rad laboratories Ltd, UK) using a Hoefer Mighty Small™ Transfor Tank (Hoefer Scientific Instruments, USA). Transfer took place at 100 V for 90 mins in 1x Towbin buffer (192 mM glycine, 25 mM Tris pH 8.3, 0.1% (w/v) SDS, 20% (v/v) methanol (Towbin et al. 1979)).

Blotted nitrocellulose membranes were allowed to dry at room temperature before blocking by gently agitating in blocking buffer (1x PBS, 5% (w/v) non-fat dry milk powder (Marvel, Premier Beverages, UK), 0.02% (v/v) Tween 20) for a minimum of 3 hours at room temperature. Membranes were then incubated with a suitable dilution of primary antibody in blocking buffer for a further 3 hours before washing for 10 mins in several changes of 1x PBS.

Stock Solution	Resolving gel	Stacking gel
Acrylamide/bisacrylamide (30%/0.8%)	3.32 ml	1.3 ml
0.5 M Tris-HCl, pH 6.8	-	2.5ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	-
10% (w/v) SDS	100 µl	100 µl
10% (w/v) ammonium persulphate	50 µl	50 µl
water	3.98 ml	6.05 ml
TEMED	10 µl	10 µl

Table 2.2 Composition of polyacrylamide gels for protein analysis

This was followed by incubation in a 1:7000 dilution of alkaline phosphatase conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich Ltd, UK) in blocking buffer for 3 hours. After rinsing twice for 5 mins in 1x PBS and once for 10 mins in alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), primary antibodies with affinity to the membrane-bound proteins were visualised by chromogenic detection of conjugated alkaline phosphatase by washing the membrane in 0.4 µM NBT, 0.4 µM BCIP in alkaline phosphatase buffer. The chromogenic reaction was stopped with 500 mM EDTA, pH 8.0 in 1x PBS.

2.15.6 Recombinant Protein Expression in E. coli

Recombinant proteins were expressed and purified using two fusion protein purification systems (GST Purification Module (Pharmacia Biotech., UK) and IMPACT™ I: One Step Purification Module (New England Biolabs Ltd, UK)). cDNA encoding the open reading frame of the desired protein was amplified by PCR using high efficiency Pfu polymerase (section 2.7.2). The PCR product was ligated into the multicloning sites of either pGEX-4T-1(GST Purification Module) or pCYB4 (IMPACT™ Purification Module) by the use of specific PCR primers incorporating the required endonuclease restriction sites. To ensure inserts were cloned in frame with the specific fusion protein, constructs were checked by direct DNA sequencing (section 2.9.3).

Expression constructs were then transformed into competent *E.coli* strain BL21 (section 2.6.2) and transformants selected on LB agar plates containing 50 µg/ml ampicillin. A single transformed colony was inoculated into 5 ml of LB medium containing 50 µg/ml ampicillin and was incubated at 37°C over night at 250 rpm in an orbital incubator. 500 µl of this was used to inoculate 250 ml of LB medium and the culture was grown to an OD_{600} of 0.6. Recombinant protein expression was then induced by 0.5 mM IPTG and cultures grown over night at 20°C. Next day cells were harvested by

centrifugation at 5000 xg for 10 mins at 4°C and resuspended in either 1x PBS (GST Purification Module) or IMPACT Column Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% (v/v) Triton X-100; IMPACT™ Purification Module). Cells were kept on ice and lysed by sonication. After spinning down cell debris by centrifugation twice at 12000 xg for 30 mins at 4°C, the supernatant containing soluble fusion protein was applied to the relevant affinity chromatography column. Columns were washed and the recombinant protein cleaved and eluted as directed by the manufacturer.

The purity and size of the recombinant protein was checked on an SDS-PAGE gel (section 2.15.4).

2.16 Preparation of Polyclonal Antibodies to Recombinant Proteins

2.16.1 Immunisation of rabbit

Polyclonal antibodies were prepared from rabbits immunised with the required recombinant protein (antigen) following protocols outlined in Harlow and Lane (1988). For primary and secondary injections 200 µg of antigen was made up to 500 µl in 1x PBS and emulsified in an equal volume of mineral oil by passing the mixture repeatedly through a 22 gauge syringe needle for 5 to 10 mins. The mineral oil and antigen solution are fully emulsified when no oil is seen to disperse when dropped into distilled water. The emulsion was then injected into the rabbit subcutaneously on the back of the neck at several sites making sure the needle was not inserted into muscle tissue by moving it gently from side to side. After 4 weeks the secondary injection was made following the same process. Subsequent injections were made intravenously to the marginal ear vein. For these inoculations 100 µg of antigen in up to 200 µl of 1x PBS was used directly without the use of an adjuvant. The rabbit was gently restrained and its marginal ear vein dilated by pressing lightly on the base of the ear before injection of the antigen using a 25 gauge needle. The injection site was then

held tightly for a few seconds to prevent bleeding. These intravenous boosts were performed four more times at 4 week intervals. Small blood samples were taken from the rabbit 7 days after each boost and the antiserum tested against western blots (section 2.15.5) of the recombinant antigen to determine the level of the immune response.

2.16.2 Bleeding

As antibody titre is at its highest level 7 days after injection, a 5 ml blood sample was taken at this stage when the rabbit had been fasted for several hours. The rabbit was wrapped in a towel and a patch of fur around the marginal ear vein shaved. A cut was then made to the vein at a 45° angle using a sterile scalpel making sure the vein was not severed. Blood was collected in a sterile tube and bleeding was stopped by applying pressure to the wound with a sterile piece of gauze. For the final, terminal bleed the same procedure was followed but 50 to 100 ml of blood was collected.

2.16.3 Antiserum preparation

Blood samples were allowed to clot at room temperature for several hours. A sealed Pasteur pipette was then used to "ring" the clot by loosening the blood sample from the wall of the tube. After over night incubation at 4°C the clot had reduced to half its original size and was separated from the antiserum. The antiserum was then transferred to a sterile tube and centrifuged at 1500 xg for 10 mins at room temperature to remove any contaminating red blood cells. Antiserum was then stored in 0.02% (w/v) sodium azide at -20°C in 500 µl aliquots. The amount of antiserum to be used in immunoblotting experiments was calculated by testing a range of dilutions (between 1:50 and 1:5000 in 1x PBS) against 20 µg of total plant protein on nitrocellulose membranes.

2.17 Production of Transgenic *A. thaliana* Plants

2.17.1 Construction of transformation vectors

Sense and antisense binary vectors were constructed using plasmids pSLJ4K1 and pSLJ438A2 (Jones et al. 1992) and pBI121 (Clontech Laboratories Inc., USA.). Subcloning was performed as outlined in section 2.8 and constructs checked by PCR using specific primers (section 2.7.1) and restriction analysis (digestion by one or more restriction endonucleases and sizing restriction fragments on an agarose gel). Transformation vector construction is shown diagrammatically in Figures 5.3 and 5.6.

2.17.2 Triparental mating

Transformation vectors were introduced to *Agrobacterium tumefaciens* strain GV3101 by triparental mating. *E.coli* strain HB101 containing helper plasmid pRK2013, *E.coli* strain DH5α containing required transformation vector and *A. tumefaciens* strain GV3101 were plated on separate LB agar plates containing the relevant selective antibiotics. A single colony from each plate was used to inoculate 5 ml of LB medium in separate tubes. The cultures were incubated over night at 37°C with shaking at 250 rpm for the *E.coli* strains and 28°C with shaking at 250 rpm for the *A. tumefaciens* strain. 100 µl of each culture were mixed together and the cells pelleted by spinning for 1 min in a bench-top microfuge at 10000 xg. The pellet was resuspended in 10 µl of 10 mM $MgSO_4$, spread on an LB agar plate containing no antibiotics and incubated over night at 28°C. At this stage the helper plasmid pRK2013 is transferred from the HB101 cells to the *E.coli* strain containing the transformation vector. The helper plasmid then effects the transfer of the transformation vector into *A. tumefaciens*. A loop of the resulting bacterial lawn was then spread onto an LB agar plate containing antibiotics for the selection of both *A. tumefaciens* and

the transformation vector. Colonies were restreaked on selection plates and used for *in planta* transformation.

2.17.3 In planta transformation of A. thaliana

In planta transformation of *A. thaliana* by vacuum infiltration was adapted from the method of Bechtold et al. (1993). 10 cm diameter plant pots were slightly over-filled with two parts Levington M3 compost (Fisons plc, UK) to one part horticultural grade vermiculite (Silvaperl, UK) and covered with fine nylon mesh secured with an elastic band. These were watered and sown with 10 *A. thaliana* seeds per pot. Plants were grown to a stage where the emerging inflorescences reached approximately 5 to 10 cm in length and these were removed. Infiltration was performed 4 days later.

A single colony of *A. tumefaciens* (carrying the appropriate construct) from an LB agar selection plate was used to inoculate 1 litre of LB medium containing appropriate anibiotics and was grown at 28°C over night with shaking at 250 rpm. Cells were harvested when the OD_{600} reached 0.6 by centrifugation at 5000 xg for 15 mins at room temperature and the pellet resuspended to an OD_{600} of 0.8 in infiltration medium (0.5x MS salts, 0.5x Gamborg's B5 vitamins (Gamborg et al. 1968), 2.5 mM MES pH 5.7, 0.15 M sucrose, 0.044 µM benzylaminopurine, 0.005% Triton X-100). Pots of *A. thaliana* plants, grown as outlined above, were then inverted and submerged in 200 ml of *A. tumefaciens* suspension and placed inside a desiccator connected to an oil pump. A vacuum of 650 mmHg was applied for 5 to 10 mins then quickly released and the pots drained, placed on their sides in large bowls and covered with cling film to maintain humidity. Next day pots were uncovered and set upright in a growth chamber. When plants had finished flowering watering was stopped and the plants allowed to dry before harvesting seeds from each individual pot ready for plating on selection medium (see Figure 5.2).

2.17.4 Selection of transformants

Seed from infiltrated plants were sterilised by agitation for 2 mins in 70% ethanol followed by 30 mins in 50% household bleach containing 0.05% Tween 20. After rinsing in 4 changes of sterile distilled water seeds were sown on 0.8% agar selection plates (1x MS salts, 1x Gambourg's B5 vitamins (Gambourg et al. 1968), 2.5 mM MES pH 5.7, 30 mM sucrose, 30 mg/l kanamycin). Positive transformants were identified after 1 to 2 weeks as dark green plants with long roots growing into the agar. Non-transformants grew poorly with pale green leaves and very short roots before dying during this period. Transformants were potted in a mixture of two parts Levington M3 compost (Fisons plc, UK) to one part horticultural grade vermiculite (Silvaperl, UK) and were confirmed as transgenic by PCR amplification of the *nptII* gene from genomic DNA of leaf discs which were excised from the leaf with the tip of a 1 ml pipette and added directly to the PCR reaction mixture described in section 2.7.1.

Chapter 3: Isolation and Characterisation of Novel cDNA Clones Encoding Serine Acetyltransferase from *Arabidopsis thaliana*

3.1 Introduction

Several studies have located SAT (serine acetyltransferase) and *O*ASTL (*O*-acetylserine (thiol) lyase) activity in the chloroplast, cytoplasm and mitochondrion of the higher plant cell (Brunold & Suter 1982; Lunn et al. 1990; Rolland et al. 1992; Ruffet et al. 1994, 1995). As discussed in Chapter 1, the reason why each of these locations requires SAT and *O*ASTL activity is unclear, especially as the reduction of sulphate to sulphide required for cysteine biosynthesis is thought to be exclusively located in the chloroplast. However, it can be assumed that there is an essential role for each enzyme within the chloroplast, cytoplasm and mitochondrion. It has been hypothesised that cysteine may not readily be transported across organellar membranes and therefore cysteine biosynthesis would be required in all compartments in which proteins are produced (Lunn et al. 1990). Molecular approaches will be important in determining the role of SAT and *O*ASTL in the various subcellular compartments, but whilst the enzymatic activity of SAT in plant tissues has been well documented, prior to the outset of this PhD no gene sequences encoding SAT had been published. Compartmentalised activity within the cell suggests the presence of a small gene-family encoding proteins targeted to the various cellular locations. Isolation and characterisation of individual members of such a gene-family would open up areas of research previously unavailable, allowing detailed molecular analysis of the role of SAT, and its product *O*AS, in the regulation of sulphate reduction and cysteine biosynthesis in higher plants.

The availability of bacterial and yeast mutants, deficient at a single locus of known function, allows the cloning of genes from other organisms by an approach known as functional complementation. This approach was used to clone SAT cDNAs from *A. thaliana* (MA Roberts, PhD thesis, University of St. Andrews). Functional complementation is a powerful technique for cloning genes from higher eukaryotes, in which production and characterisation of mutants is difficult

and the introduction and expression of large numbers of cDNA clones is impossible. Functional complementation of bacterial mutants is achieved in two steps:-

(1) Mutant bacterial cells are transfected with an expression vector-based cDNA library of the organism from which the genes are to be cloned.

(2) Mutants which have been complemented to a prototrophic phenotype (wild-type) are isolated on a suitable selection medium.

The cDNA whose expression has complemented the genetic defect of the mutant can be purified from host bacterial cells and sequenced.

A previous student in this laboratory used a functional complementation approach to clone SAT cDNAs from *A. thaliana* (MA Roberts, PhD thesis; Roberts & Wray 1996). The *E. coli* strain JM15, mutated in the *cysE* locus which encodes SAT activity (Jones-Mortimer 1968), was transfected with an *A. thaliana* cDNA library in the expression vector λYES (Elledge et al. 1990). 110 bacterial colonies were isolated which had regained the ability to grow on a selection medium containing sulphate as the sole sulphur source. The λYES multifunctional expression system combines the effective *E. coli* transfection properties of λ bacteriophage with ampicillin-resistance selectivity and the manipulation properties of a bacterial plasmid. Once inside the host bacterial cell λYES automatically forms a 'circular' plasmid, pYES, by cre-*lox* site specific recombination (Figure 3.1; Elledge et al. 1990).

Analysis of one of the SAT clones, Sat-1 (Roberts & Wray 1996), revealed a 1.28 kilobase (kb) cDNA with substantial homology to a range of previously cloned bacterial SAT genes (Denk & Böck 1987; Jacobson et al. 1989; Evans et al. 1991; Lai & Baumann 1992; Gagnon et al. 1994). Sequencing and primer extension analysis of Sat-1 revealed a full length cDNA of 1.52 kb encoding a protein of 391 amino acids. This protein (SAT1) included an N-terminal extension

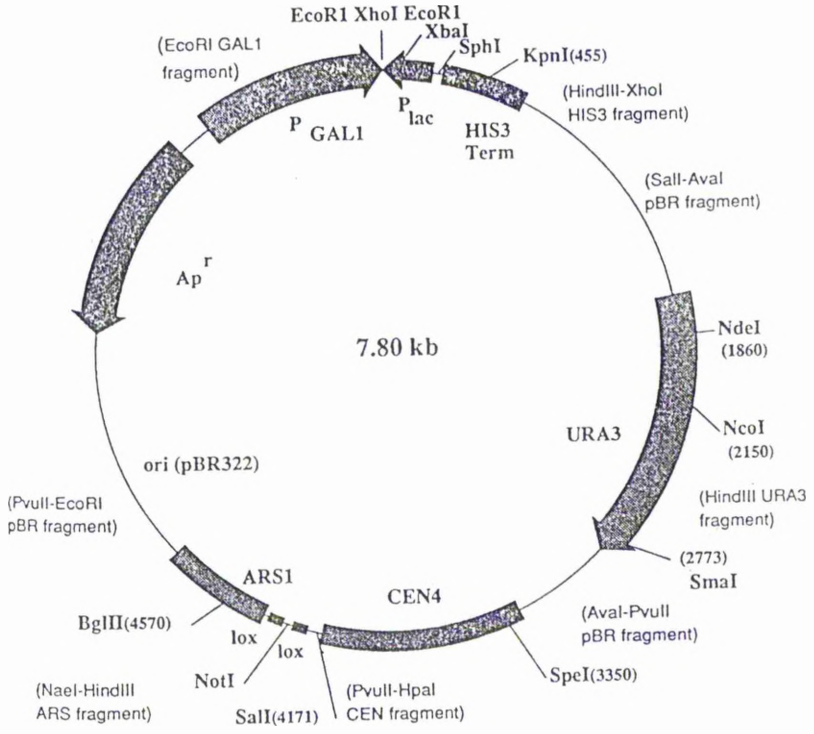


Figure 3.1 Plasmid map of the pYES expression vector (Elledge et al. 1991). The unique *Xho*I site in which the cDNA inserts are cloned is at position 0. All other unique restriction sites, with nucleotide positions in brackets, are also shown.

of 120 to 130 amino acids, when compared to the known bacterial SAT sequences. The N-terminal extension contained a high proportion of hydroxylated amino acids (23%), a high serine fraction when compared to arginine content and had no tendencies to form α or β-sheets when the protein structure was predicted using the GCG software package (Devereux et al. 1984). These are all characteristics of chloroplast targeting peptides (von Heijne et al. 1989; von Heijne & Nishikawa 1991; von Heijne 1992) and although no obvious consensus cleavage site was present in the sequence, a putative plastidic location was suggested for this enzyme in *A. thaliana* (Roberts & Wray 1996). A cDNA encoding an isoform homologous to SAT1, but truncated by 87 amino acids (SAT1-6), was also cloned from *A. thaliana* at approximately the same time (Bogdanova et al. 1995).

The first aim of this study was to examine the remaining uncharacterised SAT cDNAs, which were cloned in the functional complementation experiment outlined above, for cDNAs encoding novel SAT isoforms. This chapter describes the isolation and characterisation of three new, previously unpublished cDNAs representing SAT from *A. thaliana*.

3.2 Results

3.2.1 Sizing and preliminary sequence analysis of SAT cDNA clones

The complemented JM15 colonies described in section 3.1 putatively contained cDNAs encoding proteins with SAT activity, and were stored in 20% glycerol stocks at -80°C. The JM15 cells containing the complementing SAT cDNAs were catalogued as strains JM15/SAT1 to JM15/SAT110 (MA Roberts, PhD thesis, University of St. Andrews).

A streak of each complemented SAT strain was plated on selective M9 minimal medium containing 50 μg/ml ampicillin to reconfirm prototrophy on sulphate as a sole sulphur source. A single colony of each SAT strain was then

cultured in 5 ml of M9 liquid selection medium over night and minipreparations of each of the plasmids were performed as outlined in the Materials and Methods chapter. The isolated plasmids were correspondingly numbered pSAT1 to pSAT110.

The cDNA cloning site of the pYES vector consists of an *Xho*I restriction site (position 0 in Figure 3.1) between two *Eco*RI sites (*Eco*RI-*Xho*I-*Eco*RI: GAATTCCTCGAGGAATTC). The cDNA inserts were excised from all 110 plasmids by digestion with *Eco*RI. Digestion mixtures were then separated by electrophoresis through agarose gels and the cDNA inserts sized by their relative distances of migration when compared to λ*Hin*dIII base-pair markers. Most of the complementing cDNAs were found to range between 0.92 and 1.4 kb although one cDNA of 1.57 kb (Sat-11) and one of 4.93 kb (Sat-8) were also detected. Table 3.1 shows the approximate sizes of each of the complementing SAT clones as calculated from migration distances in the agarose gels.

Ten of the longest complementing clones, and therefore most likely to represent full-length cDNAs, were initially analysed by sequencing approximately 400 nucleotides from the 5' and 3' ends using oligonucleotide primers derived from the λYES vector sequence (Elledge et al. 1990). These clones were found to form three distinct groups. The Sat-7, Sat-21, Sat-62, Sat-83 and Sat-97 cDNAs showed 100% homology to the Sat-1 sequence previously described (Roberts & Wray 1996). The Sat-52 and Sat-71 cDNAs represented a second form of SAT and Sat-53 represented a third member of the gene-family. The Sat-52 (which was 78 bp longer than Sat-71) and Sat-53 cDNAs, being the longest of each of the two novel forms of SAT from *A. thaliana,* were chosen for further analysis. A third novel type of SAT cDNA, Sat-106, was later isolated from a dot-blot hybridisation experiment using Sat-1, Sat-52 and Sat-53 gene-specific probes as described in section 3.2.6. Whilst Sat-52, Sat-53 and Sat-106 were being analysed, a gene from *A. thaliana*, *sat5*, was cloned by Ruffet et al. (1995). The Sat-53 cDNA showed 100% nucleotide homology to the *sat5* gene.

Table 3.1 cDNA insert sizes of the JM15-complementing plasmids pSAT1 to pSAT110. Clones chosen for preliminary sequence analysis are marked by asterisks (*).

Clone	Size (kb)	Clone	Size (kb)	Clone	Size (kb)
Sat-1	1.12	Sat-38	1.17	Sat-75	1.02
" 2	1.14	" 39	1.07	" 76	1.12
" 3	1.08	" 40	1.14	" 77	0.99
" 4	1.10	" 41	1.12	" 78	1.10
" 5	1.13	" 42	1.12	" 79	0.99
" 6	1.15	" 43	1.01	" 80	1.11
" 7	1.38*	" 44	1.08	" 81	1.13
" 8	4.93*	" 45	1.11	" 82	1.05
" 9	1.10	" 46	1.10	" 83	1.33*
" 10	1.05	" 47	1.09	" 84	0.97
" 11	1.57*	" 48	1.10	" 85	0.94
" 12	1.12	" 49	0.99	" 86	1.12
" 13	1.05	" 50	1.14	" 87	1.03
" 14	1.05	" 51	1.19	" 88	1.12
" 15	1.00	" 52	1.29*	" 89	1.10
" 16	1.10	" 53	1.20*	" 90	0.98
" 17	0.99	" 54	1.25	" 91	1.04
" 18	1.13	" 55	1.06	" 92	1.13
" 19	1.10	" 56	1.12	" 93	0.92
" 20	1.07	" 57	1.01	" 94	0.98
" 21	1.40*	" 58	1.08	" 95	1.06
" 22	1.10	" 59	1.21	" 96	1.12
" 23	0.96	" 60	1.04	" 97	1.34*
" 24	1.08	" 61	1.10	" 98	1.12
" 25	1.10	" 62	1.26*	" 99	1.16
" 26	1.07	" 63	1.04	" 100	1.05
" 27	1.11	" 64	1.00	" 101	1.07
" 28	1.05	" 65	0.98	" 102	1.05
" 29	1.07	" 66	1.17	" 103	0.94
" 30	0.98	" 67	0.99	" 104	1.00
" 31	1.06	" 68	0.97	" 105	0.98
" 32	1.10	" 69	0.97	" 106	1.19
" 33	1.11	" 70	0.99	" 107	0.99
" 34	1.12	" 71	1.30*	" 108	1.01
" 35	1.02	" 72	1.05	" 109	0.96
" 36	1.07	" 73	1.04	" 110	1.13
" 37	1.11	" 74	1.06		

The first *ca* 400 nucleotides from the 5' and 3' ends of the 1.57 kb Sat-11 cDNA were found to have 97% homology to the *A. thaliana* actin gene ACT2 and 90% homology to the *A. thaliana* actin gene ACT8 (An et al. 1996). Actin is unlikely to exhibit any activity which would be able to complement an *E. coli cysE* mutant to prototrophy. It is assumed that this clone was isolated as an artifact of the cloning procedure. The growth of the JM15/SAT11 colony may have been permitted by a small contaminating reduced sulphur source in either the medium or the solidifying agar of the selection plate, which would allow survival of the bacteria in the absence of SAT activity.

The 4.93 kb Sat-8 cDNA showed no homology to SAT or any other DNA sequence at either the 3' or 5' end when compared against all the available sequences deposited in the GenBank database (using BLAST search software (Altschul et al. 1990)). As the initial sequencing of Sat-8 did not detect an open reading frame and the Sat-8 cDNA was almost five times the length expected for SAT cDNAs, this clone was also assumed to be an artifact of the cloning procedure.

3.2.2 Retransformation of E. coli JM15 mutant implies that Sat-52, Sat-53 and Sat-106 confer SAT activity

The *E.coli cysE* mutant JM15, used in the functional complementation experiments previously described, was retransformed with purified pSAT52, pSAT53 and pSAT106 plasmid to demonstrate the requirement of the cDNA for prototrophic growth. The retransformed JM15 cells (JM15/pSAT52 etc.), JM15 transformed with empty pYES vector (SAT/YES) and JM15 containing no plasmid (JM15) were plated on three selection media to confirm the SAT activity of the proteins encoded by the SAT cDNAs. Medium 1 contained M9 minimal medium with sulphate as the sole sulphur source, medium 2 contained M9 minimal medium supplemented with 0.5 mM cystine as a reduced sulphur source (eliminating the

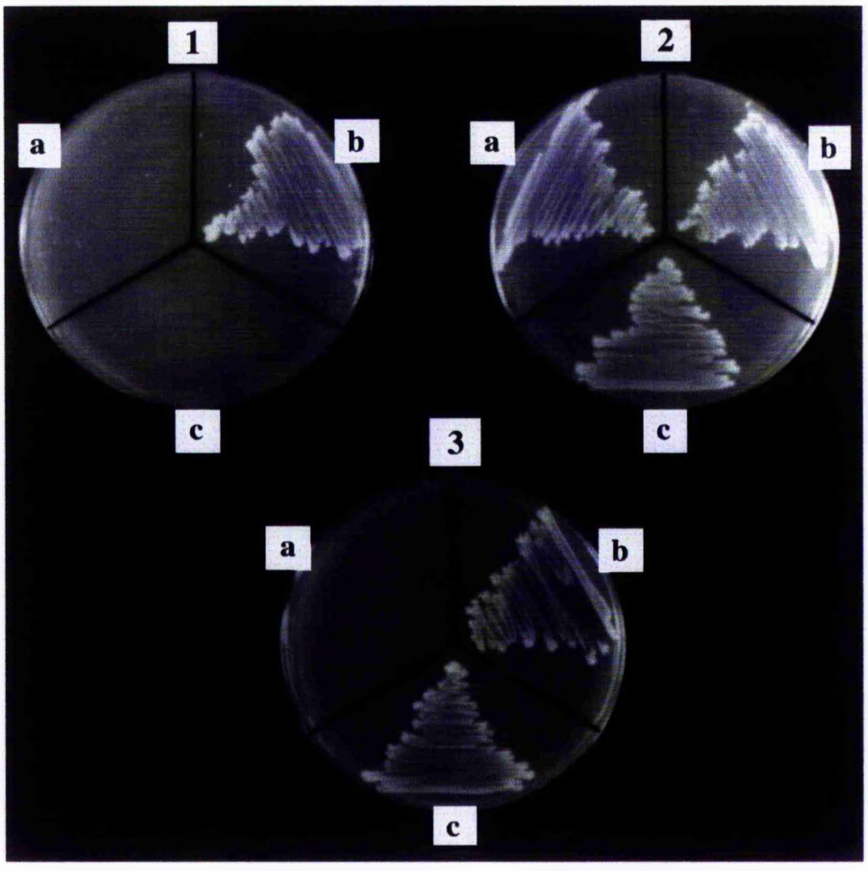


Figure 3.2 *E. coli cysE* mutant JM15 (a), strain JM15/pSAT52 (b) and JM15 containing the empty pYES vector (c) plated on three selection media. **1** is M9 minimal medium with sulphate as the sole sulphur source, **2** M9 minimal medium supplemented with 0.5 mM cystine as a reduced sulphur source (eliminating the requirement of SAT activity for growth), and **3** M9 minimal medium containing 0.5 mM cystine and 0.5 mg/ml ampicillin. Identical growth characteristics were observed with strains JM15/pSAT53 and JM15/pSAT106 (not shown).

requirement of SAT activity for growth) and medium 3 was M9 minimal medium containing 0.5 mM cystine and 0.5 mg/ml ampicillin (Figure 3.2). Only growth of JM15 containing pSAT52, pSAT53 or pSAT106 was observed on medium 1. All strains grew on medium 2 and only JM15 containing pSAT52, pSAT53, pSAT106 and the empty pYES vector grew on medium 3. These growth characteristics indicate that a) all three tested strains can grow when a reduced sulphur source is provided, eliminating the requirement for SAT activity, b) the empty pYES vector provides resistance to ampicillin but does not permit growth on sulphate as a sole sulphur source and c) the SAT cDNA is required to complement the *cysE* mutation, restoring prototrophy to the JM15 mutant on medium containing sulphate as the sole sulphur source. The results shown in Figure 3.2 are for strain JM15/pSAT52, but identical growth characteristics were obtained with strains JM15/pSAT53 and JM15/pSAT106 (not shown).

3.2.3 Assay of serine acetyltransferase activity in crude extracts from JM15/pSAT52

The activity of enzymes encoded by cDNAs cloned in expression vector systems can be measured in crude protein extracts from the mutant bacterial strains carrying them. SAT activity in such extracts is calculated spectrophotometrically by monitoring the *L*-serine-dependent cleavage of acetyl CoA at OD_{232} (Kredich & Tomkins 1966). The rate of decrease in OD_{232} on the addition of *L*-serine was used to calculate SAT activity as outlined in the Materials and Methods. The SAT activity of SAT1 protein in crude bacterial extract was measured at approximately 225 nmol acetyl CoA cleaved/min/mg protein (Roberts & Wray 1996) and SAT5, the protein identical to SAT53, was measured at 64 nmol/min/mg protein by Ruffet et al. (1995).

Activity of the SAT52 protein was calculated from the mean of 30 replicate SAT assays in the presence of 0.125 mM acetyl CoA and 10 mM *L*-serine

Table 3.2 SAT activity assay data on crude JM15/pSAT52 protein extracts

Assay components	SAT activity (nmol/min/mg protein)
JM15/pSAT52	157 ± 19.5
JM15/pSAT52 no *L*-serine	0
JM15	0
Boiled JM15/pSAT52	0

(concentrations above the calculated K_m of these substrates for SAT1 protein (Roberts & Wray 1996)). Control assays on JM15 extract, JM15/pSAT52 extract in the absence of *L*-serine and boiled extract were also performed. The results of these assays are shown in Table 3.2.

Direct comparisons of activities of the different SAT isoforms cannot be made from these assays as the concentration of recombinant SAT protein in the crude extracts is not known. The assays do however confirm that the proteins encoded by the complementing cDNAs confer SAT activity which is dependent on the substrates acetyl CoA and *L*-serine.

3.2.4 Sequencing of two novel cDNAs representing SATs

Both strands of the Sat-52 cDNA and one strand of the Sat-53 cDNA were fully sequenced. In the case of Sat-52, oligonucleotide primers were designed from sequence towards the 3' end of overlapping sequencing reactions. In the case of Sat-53, oligonucleotide primers were designed from the *sat5* gene from *A. thaliana* (Ruffet et al. 1995) which shared 100% homology to the first *ca* 400 nucleotides at the 5' and 3' ends of Sat-53. Primers used for sequencing Sat-52 and Sat-53 are shown in Table 3.3. The complete nucleotide sequences of Sat-52 and Sat-53 cDNAs with their deduced amino acid translations are shown in Figures 3.3 and 3.4 respectively.

The Sat-52 cDNA consists of 1158 nucleotides with an open reading frame (ORF) of 936 nucleotides, a 56 nucleotide 5' untranslated region and a 147 nucleotide non-coding 3' region followed by a 19 nucleotide polyadenylation sequence. A nucleotide sequence resembling the consensus plant polyadenylation signal (Hunt 1994; Wu et al. 1995) is found 45 nucleotides upstream from the polyadenylation sequence. The ORF encodes a 312 amino acid polypeptide with a predicted molecular weight of 32.77 kDa and an isoelectric point of 7.16. An in-

Table 3.3 Oligonucleotide primers used in Chapter 3

Primer	Used for:-	Primer sequence (5'→3')
52.1	Sat-52 5'→3' internal primer 1	GCCGGATTATGGACACAG
52.2	Sat-52 5'→3' internal primer 2	CTCCGATCCTTCTCTTCG
52.3	Sat-52 5'→3' internal primer 3	GATACTTCTAGACCACG
52.4	Sat-52 5'→3' internal primer 4	GTGCTAAAGTAGGAGCTG
52.5	Sat-52 3'→5' internal primer 1	GATCCATCGATTCTCCAG
52.6	Sat-52 3'→5' internal primer 2	CAATCACCGCTGTTTCTCC
52.7	Sat-52 3'→5' internal primer 3. Also used as 3'→5' primer in preparation of the Sat-52 gene-specific probe	GTGGCGTTACGAAGAGAAG
52.8	Sat-52 3'→5' internal primer 4	GATCTGTGTCCATAATCC
53.1	Sat-53 5'→3' internal primer 1	CACCACACCCAAATCG
53.2	Sat-53 5'→3' internal primer 2	GACCCAGCTTGTATAAGC
53.3	Sat-53 5'→3' internal primer 3	GAACAGGGAAACAGAGTGG
53.gspp	Used as 3'→5' primer in Sat-53 gene-specific probepreparation	CTTTCTTCTAAAACGCTTATG
106.4	Sat-106 5'→3' internal primer 1	GTAGCTTCTTGTATGCTGG
106.5	Sat-106 5'→3' internal primer 2	CGTTACAAGCATATAGG
106.6	Sat-106 5'→3' internal primer 3	GAAAGGAAACTGGCGATCG
106.7	Sat-106 5'→3' internal primer 4	CTCTAGCAATGAAACACG
106.1	Sat-106 3'→5' internal primer 1	CTAGAGACGGGTCTTGC
106.2	Sat-106 3'→5' internal primer 2	CCGTTGCCTATCACAGC
106.3	Sat-106 3'→5' internal primer 3	GCAGAACTATACGACAG
P1	pYES derived primer for sequencing inserts in a 3'→5' direction	TGTGGAATTGTGAGCGG
P2	pYES derived primer for sequencing inserts in a 5'→3' direction	ACTTTAACGTCAAGGAG
52Pr.Ext.	Sat-52 primer extension	GCTGCCGCAGATATCGCTGC
P9	Sat-1 derived primer. Used as 3'→5' primer in Sat-1 gene-specific probe preparation	TAAGCGGAAACAATAGG

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AAATTAACTTTTATAGGTAGGAGGAGATAGAAATCGAATCTTATCGCCGCGTTAATATGC      60
                  *                                          M  P      2

CACCGGCCGGAGAACTCCGACATCAATCTCCATCAAAGGAGAAACTATCTTCCGTTACCC     120
  P  A  G  E  L  R  H  Q  S  P  S  K  E  K  L  S  S  V  T  Q      22

AATCCGATGAAGCAGAAGCAGCGTCAGCAGCGATATCTGCGGCAGCTGCAGATGCGGAAG     180
  S  D  E  A  E  A  A  S  A  A  I  S  A  A  A  A  D  A  E  A      42

CTGCCGGATTATGGACACAGATCAAGGCGGAAGCTCGCCGTGATGCTGAGGCGGAGCCAG     240
  A  G  L  W  T  Q  I  K  A  E  A  R  R  D  A  E  A  E  P  A      62

CTTTAGCTAGCTATCTATATTCGACGATTCTTTCTCATTCGTCTCTTGAACGATCTATCT     300
  L  A  S  Y  L  Y  S  T  I  L  S  H  S  S  L  E  R  S  I  S      82

CGTTTCATCTAGGAAACAAGCTTTGTTCCTCAACGCTTTTATCCACACTTTTATACGATC     360
  F  H  L  G  N  K  L  C  S  S  T  L  L  S  T  L  L  Y  D  L     102

TGTTCTTAAACACTTTTTCCTCCGATCCTTCTCTTCGTAACGCCACCGTCGCAGATCTAC     420
  F  L  N  T  F  S  S  D  P  S  L  R  N  A  T  V  A  D  L  R     122

GCGCTGCTCGTGTTCGTGATCCTGCTTGTATCTCGTTCTCTCATTGTCTCCTCAATTACA     480
  A  A  R  V  R  D  P  A  C  I  S  F  S  H  C  L  L  N  Y  K     142

AAGGCTTCTTAGCTATTCAGGCGCATCGTGTATCACACAAGCTATGGACACAATCACGGA     540
  G  F  L  A  I  Q  A  H  R  V  S  H  K  L  W  T  Q  S  R  K     162

AGCCATTAGCATTAGCTCTACACTCAAGAATCTCCGATGTATTCGCTGTTGATATCCATC     600
  P  L  A  L  A  L  H  S  R  I  S  D  V  F  A  V  D  I  H  P     182

CAGCAGCGAAGATCGGAAAAGGGATACTTCTAGACCACGCAACCGGAGTTGTAGTCGGAG     660
  A  A  K  I  G  K  G  I  L  L  D  H  A  T  G  V  V  V  G  E     202

AAACAGCGGTGATTGGGAACAATGTTTCAATCCTTCACCATGTGACACTAGGTGGAACAG     720
  T  A  V  I  G  N  N  V  S  I  L  H  H  V  T  L  G  G  T  G     222

GTAAAGCTTGTGGAGATAGACATCCGAAGATCGGTGACGGTTGTTTGATTGGAGCTGGAG     780
  K  A  C  G  D  R  H  P  K  I  G  D  G  C  L  I  G  A  G  A     242

CGACTATTCTTGGAAATGTGAAGATTGGTGCAGGTGCTAAAGTAGGAGCTGGTTCTGTTG     840
  T  I  L  G  N  V  K  I  G  A  G  A  K  V  G  A  G  S  V  V     262

TGCTGATTGACGTGCCTTGTCGAGGTACTGCGGTTGGGAATCCGGCGAGACTTGTCGGAG     900
  L  I  D  V  P  C  R  G  T  A  V  G  N  P  A  R  L  V  G  G     282

GGAAAGAGAAGCCAACGATTCATGATGAGGAATGTCCTGGAGAATCGATGGATCATACTT     960
  K  E  K  P  T  I  H  D  E  E  C  P  G  E  S  M  D  H  T  S     302

CATTCATCTCGGAATGGTCAGATTACATCATATAAAGTTGTTGTTATAATTGTTTCTGTG    1020
  F  I  S  E  W  S  D  Y  I  I  *                               312

GTTTTGAAAGTGAAAAACTTTCTGACCATTCGCTTATAGAGAGGTGCTGAGGTAAGTGAG    1080
CTTTGTTGTATTTGTAATTTACAAAACATCTTACGATGTTCAATACAAGAAATGGCCTTA    1140
AAAAAAAAAAAAAAAAAA                                              1158
```

Figure 3.3 Nucleotide sequence of the Sat-52 cDNA with deduced amino acid translation. In-frame stop codons at the 5' and 3' ends of the open reading frame (ORF) are marked with asterisks (*). Putative polyadenylation motif sequences are underlined 134 and 44 nucleotides from the poly-A tail. The sequence used to design the primer for primer extension experiments is also underlined.

```
tctcttctcttctttatctcaaactctttcttcttccaaatcataaaccatggCAACATG      60
                                                 *     M  A  T  C       4

CATAGACACATGCCGAACCGGTAATACCCAAGACGATGATTCCCGGTTCTGTTGCATCAA     120
 I  D  T  C  R  T  G  N  T  Q  D  D  D  S  R  F  C  C  I  K      24

GAATTTCTTTCGACCCGGTTTCTCTGTAAACCGGAAGATTCACCACACCCAAATCGAAGA     180
 N  F  F  R  P  G  F  S  V  N  R  K  I  H  H  T  Q  I  E  D      44

TGACGATGATGTCTGGATCAAGATGCTTGAAGAAGCCAAATCCGATGTTAAACAAGAACC     240
 D  D  D  V  W  I  K  M  L  E  E  A  K  S  D  V  K  Q  E  P      64

CATTTTATCAAACTACTACTACGCTTCGATCACATCTCATCGATCTTTAGAGTCTGCTTT     300
 I  L  S  N  Y  Y  Y  A  S  I  T  S  H  R  S  L  E  S  A  L      84

AGCTCACATCCTCTCCGTAAAGCTCAGCAATTTAAACCTACCAAGCAACACACTCTTCGA     360
 A  H  I  L  S  V  K  L  S  N  L  N  L  P  S  N  T  L  F  E     104

ACTGTTCATAAGCGTTTTAGAAGAAAGCCCTGAGATCATCGAATCCACGAAGCAAGATCT     420
 L  F  I  S  V  L  E  E  S  P  E  I  I  E  S  T  K  Q  D  L     124

TATAGCAGTCAAAGAAAGAGACCCAGCTTGTATAAGCTACGTTCATTGCTTCTTGGGCTT     480
 I  A  V  K  E  R  D  P  A  C  I  S  Y  V  H  C  F  L  G  F     144

CAAAGGCTTCCTCGCTTGTCAAGCTCATCGAATAGCTCATACCCTCTGGAAACAGAACAG     540
 K  G  F  L  A  C  Q  A  H  R  I  A  H  T  L  W  K  Q  N  R     164

AAAAATCGTAGCTTTATTGATCCAAAACAGAGTATCAGAATCTTTCGCCGTCGATATTCA     600
 K  I  V  A  L  L  I  Q  N  R  V  S  E  S  F  A  V  D  I  H     184

TCCCGGAGCGAAGATCGGAAAAGGGATTCTTTTAGACCATGCGACGGGCGTGGTGATCGG     660
 P  G  A  K  I  G  K  G  I  L  L  D  H  A  T  G  V  V  I  G     204

AGAGACGGCGGTGGTTGGAGACAATGTTTCGATTCTACACGGAGTGACCTTGGGAGGAAC     720
 E  T  A  V  V  G  D  N  V  S  I  L  H  G  V  T  L  G  G  T     224

AGGGAAACAGAGTGGTGATCGGCATCCGAAGATTGGTGATGGTGTGTTGATTGGAGCTGG     780
 G  K  Q  S  G  D  R  H  P  K  I  G  D  G  V  L  I  G  A  G     244

GAGTTGTATATTGGGGAATATAACAATCGGTGAGGGAGCTAAGATTGGATCAGGGTCGGT     840
 S  C  I  L  G  N  I  T  I  G  E  G  A  K  I  G  S  G  S  V     264

GGTGGTTAAGGATGTGCCGGCGCGTACGACGGCGGTTGGAAATCCGGCGAGGTTGATTGG     900
 V  V  K  D  V  P  A  R  T  T  A  V  G  N  P  A  R  L  I  G     284

TGGGAAAGAGAATCCGAGAAAACATGATAAGATTCCTTGTCTGACTATGGACCAGACATC     960
 G  K  E  N  P  R  K  H  D  K  I  P  C  L  T  M  D  Q  T  S     304

GTATTTAACCGAGTGGTCTGATTATGTGATTTAACACAAATGTGTATTTCTTTCTTTCTT    1020
 Y  L  T  E  W  S  D  Y  V  I  *                                314

GTAACTGATGATGATGAAACAAGTCTTGTCTATTTCTTAATATTTTACTATGTACTAATC    1084
AAACAAGTCTTGAAATCAAAAAAAAAAAAAAAAAAAAAA                        1123
```

Figure 3.4 The nucleotide sequence of the Sat-53 cDNA with deduced amino acid translation. In-frame stop codons at the 5' and 3' ends of the ORF are marked with asterisks (*). A putative polyadenylation motif sequence is underlined 39 nucleotides from the poly-A tail. The Sat-53-homologous genomic clone (GenBank accession AC002304) indicates that the Sat-53 cDNA ORF is 4 nucleotides short at the 5' end. Additional 5' nucleotides from the genomic sequence are shown in lower case letters.

frame stop codon at the 5' end of the Sat-52 ORF indicates that the deduced amino acid sequence of the SAT52 protein is full-length.

The Sat-53 cDNA consists of 1066 nucleotides with an ORF of 938 nucleotides, a 106 nucleotide non-coding 3' region followed by a 22 nucleotide polyadenylation sequence. A putative polyadenylation signal is found 39 nucleotides upstream from the polyadenylation sequence. A comparison of the Sat-53 cDNA sequence to the *sat5* genomic sequence (Ruffet et al. 1995), which share 100% nucleotide homology in their overlapping region, shows that the Sat-53 coding region is 4 nucleotides short of full-length at the 5' end. The Sat-53 ORF encodes a 312 amino acid polypeptide with a predicted molecular weight of 34.01 kDa and an isoelectric point of 6.92. The full length SAT53-type protein is a 314 amino acid polypeptide with a predicted molecular weight of 34.25 kDa and an isoelectric point of 6.92.

The predicted molecular weights of SAT52 and SAT53 proteins are within the ranges of other reported SAT proteins (Nakamura & Tamura 1990; Ruffet et al. 1995; Bogdanova et al. 1995; Roberts & Wray 1996).

3.2.5 Design of gene-specific probes for SAT isogenes

In order to study gene expression and transcript abundance of the different SAT isogenes from *A. thaliana*, specific DNA probes are required. Oligonucleotide primers were designed to amplify probes from the Sat-1, Sat-52 and Sat-53 cDNAs by PCR. As observed in section 3.2.9 and Figure 3.5, the 5' ends of the three SAT cDNAs show little homology, whereas at the 3' ends there is a relatively high level of nucleotide sequence consensus. The 5' regions of the 3 cDNAs were therefore thought most likely to be gene-specific if used as a probe in expression studies. Amplification of DNA from the 5' end of the cDNAs in pSAT1, pSAT52 and pSAT53 was carried out using pYES primer 1 and a reverse oligonucleotide primer specific to each SAT isoform as detailed in Table 3.3. The final putative gene-

Figure 3.5 Nucleotide alignment of the four SAT cDNA sequences from *A. thaliana* calculated using the PILEUP and PRETTY programs of the GCG software package (Devereux et al. 1984). The consensus sequence, of nucleotides homologous between all four sequences, is also shown.

```
                1
Sat-1           atgttgccgg tcacaagtcg ccgccacttc acaatgtccc tatatatgct ccgttcatct tctccacaca tcaatcatca ctctttcctt cttcttctt
Sat-52          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sat-53          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sat-106         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

              101
Sat-1           ttgtttcctc caaattcaaa caccatactt tatctcctcc tccttctcct cctcctcctc ctcctatggc tgcgtgcatc gacacctgcc gcactggtaa
Sat-52          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sat-53          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Sat-106         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

              201
Sat-1           aaaccccaga tttctcctcg cgattcttct aaacaccacg acgatgaatc tggctttcgt tacatgaact acttccgtta tcctgatcga tcttccttca
sat-52          .......... .......... .......... .......... ..atgccacc ggccggagaa ctccgacatc aatctccatc aaaggagaaa ctatcttccg
sat-53          .......... .......... .......... ...atggcaa catgcataga cacatgccga accggtaata cccaagacga tgattcccgg ttctgttgca
sat-106         .......... .......... .......... .......... .......... .......... .........a tgaatggcga tgagcttcct ttcgagagtg
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

              301
Sat-1           atggaaccca gaccaaaacc ctccatactc gtcctttGct tgaagatctc gatcgcgacg ctgaagtcGa tgatgTtTGG gccaaaATcc gaGaaGAgGC
sat-52          ttacccaatc cgatgaagca gaagcagcgt cagcagcGat atctgcggca gctgcagatg cggaagctGc cggatTaTGG acacagATca agGcgGAaGC
sat-53          tcaagaattt ctttcgaccc ggtttctctg taaaccgGaa gattcaccac acccaaatcg aagatgacGa tgatgTcTGG atcaagATgc ttGaaGAaGC
sat-106         gtttcgaggt ttacgctaag ggaactcata agtcagaGtt tgactcgaat ttgcttgatc ctcgttctGa tcctaTtTGG gatgctATaa gaGaaGAaGC
Consensus       ---------- ---------- ---------- -------G-- ---------- ---------- --------G- -----T-TGG ------AT-- --G--GA-GC

              401
Sat-1           taaatctGAt atcgccaaaG AaCCtatTgT ttccgctTat tatcAcgCtt cgATtgtttC tCAgcgtTcg tTgGAagctg CgtTggcgaa tacttTatct
Sat-52          tcgccgtGAt gctgaggcgG AgCCagcTtT agctagcTat ctatAttCga cgATtctttC tCAttcgTct cTtGAacgat CtaTctcgtt tcatcTagga
Sat-53          caaatccGAt gttaaacaaG AaCCcatTtT atcaaacTac tactAcgCtt cgATcacatC tCAtcgaTct tTaGAgtctg CttTagctca catccTctcc
Sat-106         taaacttGAg gcagagaaaG AgCCtatTtT gagtagcTtc ttgtAtgCtg gtATcttagC aCAtgatTgt tTaGAgcaag CttTagggtt tgttcTagcc
Consensus       -------GA- ---------G A-CC---T-T -------T-- ----A--C-- --AT-----C -CA----T-- -T-GA----- C--T------ -----T----

              501
Sat-1           gttaaaCTca gcaatttgAa tcTtccaagc AacacgcTtT tcGAttTgTT ctctggtgtT cTtcaaggaa acccagataT tgttgaatCt gtcaagctaG
Sat-52          aacaagCTtt gttcctcaAc gcTtttatcc AcactttTaT acGAtcTgTT cttaaacacT tTttcctccg atccttctcT tcgtaacgCc accgtcgcaG
Sat-53          gtaaagCTca gcaatttaAa ccTaccaagc AacacacTcT tcGAacTgTT cataagcgtT tTagaagaaa gccctgagaT catcgaatCc acgaagcaaG
Sat-106         aaccgtCTcc aaaacccaAc ctTgttggca AcacaacTcT tgGAtaTaTT ttatggtgtT aTgatgcatg acaaaggtaT tcagagttCg attcgccatG
Consensus       ------CT-- --------A- --T------- A------T-T --GA--T-TT ---------T -T-------- ---------T --------C- ---------G
```

```
                601
Sat-1           ATCTtttaGC tgttaagGag aGaGAtCCtG CTTGTaTaag cTacgtTcaT tgttTccTtc actttAAaGG cTtcctcGCt tgtCAaGCgc ATcGtaTtgC
Sat-52          ATCTacgcGC tgctcgtGtt cGtGAtCCtG CTTGTaTctc gTtctcTcaT tgtcTccTca attacAAaGG cTtcttaGCt attCAgGCgc ATcGtgTatC
Sat-53          ATCTtataGC agtcaaaGaa aGaGAcCCaG CTTGTaTaag cTacgtTcaT tgctTctTgg gcttcAAaGG cTtcctcGCt tgtCAaGCtc ATcGaaTagC
Sat-106         ATCTccagGC atttaaaGat cGtGAtCCtG CTTGTcTgtc gTatagTtcT gctaTttTac atctgAAgGG tTatcatGCg ttaCAaGCat ATaGggTtgC
Consensus       ATCT----GC -------G-- -G-GA-CC-G CTTGT-T--- -T----T--T ----T--T-- -----AA-GG -T-----GC- ---CA-GC-- AT-G--T--C

                701
Sat-1           tCAtgagCTt TGGActcAgg acaGaAAaat ccTAGCttTg ttgaTcCAga acaGAgTctc tGAagccTTc GctgTtGAtt TcCAcCCtGg aGCtAaaATc
Sat-52          aCAcaagCTa TGGAcacAat cacGgAAgcc atTAGCatTa gctcTaCAct caaGAaTctc cGAtgtaTTc GctgTtGAta TcCAtCCaGc aGCgAagATc
Sat-53          tCAtacccTc TGGAaacAga acaGaAAaat cgTAGCttTa ttgaTcCAaa acaGAgTatc aGAatctTTc GccgTcGAta TtCAtCCcGg aGCgAagATc
Sat-106         gCAtaaaCTg TGGAatgAag ggaGgAAact atTAGCtcTt gcatTgCAaa gccGAaTaag cGAggttTTt GgcaTtGAca TaCAtCCaGc gGCaAgaATt
Consensus       -CA----CT- TGGA---A-- ---G-AA--- --TAGC--T- ----T-CA-- ---GA-T--- -GA----TT- G---T-GA-- T-CA-CC-G- -GC-A--AT-

                801
Sat-1           GGtaccGGgA TttTgcTaGA cCAtGctACg GctaTtGTga TcGGtGAgAC gGCgGTtgTg GGgaACaaTG TtTCgATtcT cCAtaacGTt ACgcTtGGaG
Sat-52          GGaaaaGGgA TacTtcTaGA cCAcGcaACc GgagTtGTag TcGGaGAaAC aGCgGTgaTt GGgaACaaTG TtTCaATccT tCAccatGTg ACacTaGGtG
Sat-53          GGaaaaGGgA TtcTttTaGA cCAtGcgACg GgcgTgGTga TcGGaGAgAC gGCgGTggTt GGagACaaTG TtTCgATtcT aCAcggaGTg ACctTgGGaG
Sat-106         GGggagGGaA TatTgtTgGA tCAtGgaACt GgagTgGTca TtGGtGAgAC cGCtGTgaTa GGcaACggTG TcTCgATctT aCAtggtGTg ACttTaGGaG
Consensus       GG----GG-A T--T--T-GA -CA-G--AC- G---T-GT-- T-GG-GA-AC -GC-GT--T- GG--AC--TG T-TC-AT--T -CA----GT- AC--T-GG-G

                901
Sat-1           GAACgGGgAA acagtgTGGa GATaGgCAcC CgAAGATtGG cGAtGGggtt TTGaTTGGAG CTgGgacttg TATttTgGGg AAtaTcAcgA TtGGTGaaGG
Sat-52          GAACaGGtAA agcttgTGGa GATaGaCAtC CgAAGATcGG tGAcGGttgt TTGaTTGGAG CTgGagcgac TATtcTtGGa AAtgTgAagA TtGGTGcaGG
Sat-53          GAACaGGgAA acagagTGGt GATcGgCAtC CgAAGATtGG tGAtGGtgtg TTGaTTGGAG CTgGgagttg TATatTgGGg AAtaTaAcaA TcGGTGagGG
Sat-106         GAACcGGaAA ggaaacTGGc GATcGcCAcC CaAAGATaGG tGAaGGtgca TTGcTTGGAG CTtGtgtgac TATacTtGGt AAcaTaAgcA TaGGTGctGG
Consensus       GAAC-GG-AA ------TGG- GAT-G-CA-C C-AAGAT-GG -GA-GG---- TTG-TTGGAG CT-G------ TAT--T-GG- AA--T-A--A T-GGTG--GG

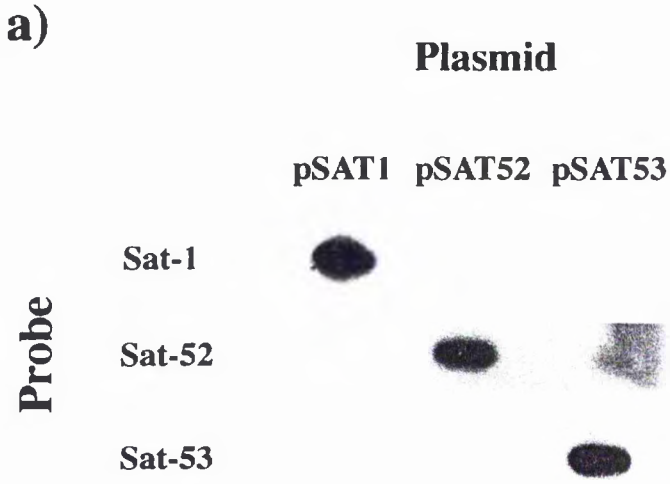
                1001
Sat-1           aGCtAagaTt GgtgCgGGgT CggTgGTGtT gAaaGAcGTg CCgccgCgta cgacgGctGt TGGaAATCCg GCgAggtTgc TtgGtGgtAa aGAtaAtCcg
Sat-52          tGCtAaagTa GgagCtGGtT CtgTtGTGcT gAttGAcGTg CCttgtCgag gtactGcgGt TGGgAATCCg GCgAgacTtg TcgGaGggAa aGAgaAgCca
Sat-53          aGCtAagaTt GgatCaGGgT CggTgGTGgT tAagGAtGTg CCggcgCgta cgacgGcgGt TGGaAATCCg GCgAggtTga TtgGtGggAa aGAgaAtCcg
Sat-106         aGCaAtggTa GctgCaGGtT CacTtGTGtT aAaaGAcGTt CCttcgCata gtgtgGtgGc TGGaAATCCt GCaAaacTga TcaGgGtcAt gGAagAgCaa
Consensus       -GC-A---T- G---C-GG-T C--T-GTG-T -A--GA-GT- CC----C--- -----G--G- TGG-AATCC- GC-A---T-- T--G-G--A- -GA--A-C--

                1101
Sat-1           aaaacgcaTg acaag...at tCctggtttg aCtAtgGAcc agacgtcgca taTatCcGAg tGgTcggAtt atgtaattTg a......... .........
Sat-52          acgattcaTg atgaggaatg tCctggagaa tCgAtgGAtc atacttcatt caTctCgGAa tGgTcagAtt acatcataTa a......... .........
Sat-53          agaaaacaTg ataag...at tCcttgtctg aCtAtgGAcc agacatcgta ttTaaCcGAg tGgTctgAtt atgtgattTa a......... .........
Sat-106         gacccgtcTc tagcaatgaa aCacgatgct aCtAaaGAgt tctttcgaca tgTagCtGAt gGtTacaAag gggcacaaTc taactttcag caggagatac
Consensus       --------T- ---------- -C-------- -C-A--GA-- ---------- --T--C-GA- -G-T---A-- --------T- ---------- ----------

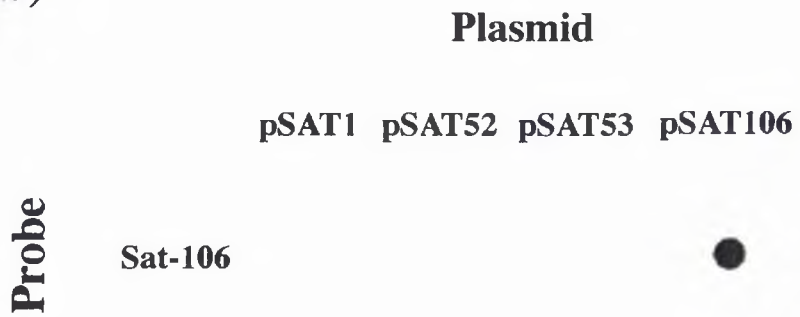
                1201                                                                  1268
Sat-1           ......... ......... ......... ......... ......... ......... ........
Sat-52          ......... ......... ......... ......... ......... ......... ........
Sat-53          ......... ......... ......... ......... ......... ......... ........
Sat-106         agagaaagga cacactaaca gcacatcatg agatctttgt atgcaaattt cttaccatca taagctaa
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- --------
```

Figure 3.6 (a) Slot-blots to test the cross-reactivity of each SAT gene-specific probe. 0.5 ng of denatured pSAT1, pSAT52 and pSAT53 was blotted onto 3 replicate nylon membranes. These were hybridised with the Sat-1, Sat-52 and Sat-53 gene-specific probes. Filters were washed at medium stringency (1x SSPE, 0.1% SDS, 42°C). (b) Slot-blot to test the cross-reactivity of the Sat-106 gene-specific probe. 0.5 ng of denatured pSAT1, pSAT52, pSAT53 and pSAT106 was blotted onto a nylon membrane and hybridised with the Sat-106 gene-specific probe before washing at medium stringency (1x SSPE, 0.1% SDS, 50°C).

a)


Plasmid
pSAT1 pSAT52 pSAT53
Probe
Sat-1
Sat-52
Sat-53

b)


Plasmid
pSAT1 pSAT52 pSAT53 pSAT106
Probe
Sat-106

specific probes consisted of the nucleotides 1-457 of the 1278 bp Sat-1 cDNA, nucleotides 1-406 of Sat-52 and nucleotides 1-471 of Sat-53. These probes were radiolabelled and tested for gene-specificity by probing the 'slot-blots' shown in Figure 3.6a. As shown in Figure 3.6a, no cross-reaction between the three probes was observed, each being specific for the SAT isogene from which they were prepared, even under low stringency washing conditions.

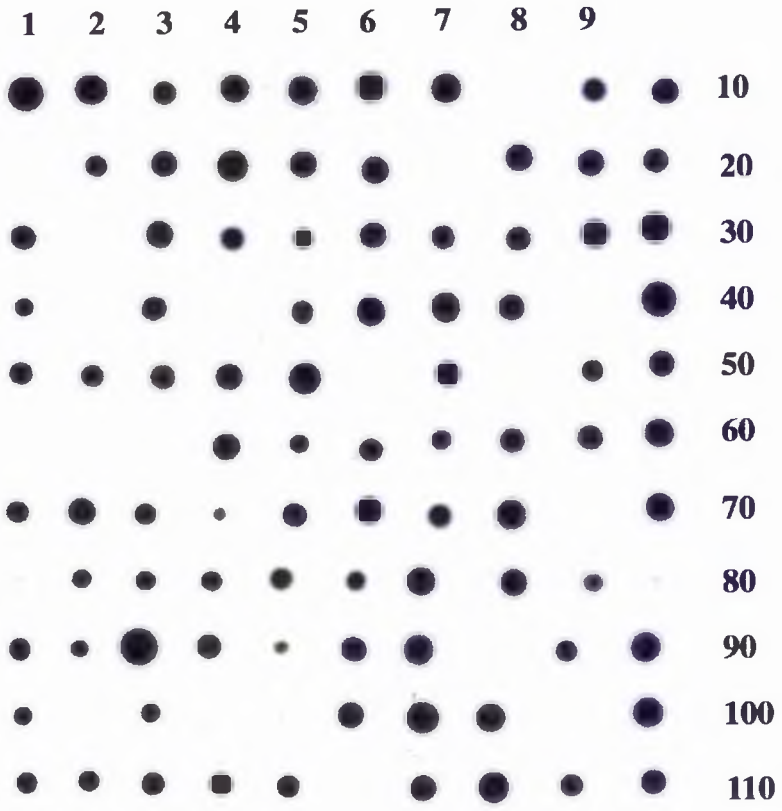
3.2.6 Use of SAT gene-specific probes to analyse the remaining JM15-complementing cDNAs

The three SAT gene-family members represented by cDNAs Sat-1, Sat-52 and Sat-53 were isolated by the characterisation of only 10 clones from the 110 cDNAs isolated by functional complementation. The SAT gene-specific probes amplified and tested as outlined in section 3.2.5 were used to detect any further members of the SAT gene-family. Approximately 0.5 ng of plasmids pSAT1 to pSAT110 were 'dot-blotted' onto Hybond-N (Amersham International plc, UK) nylon membrane which was sequentially probed with each of the SAT gene-specific probes as shown in Figure 3.7. The hybridisation patterns observed show that the Sat-1-type cDNA was by far the most common form among the JM15-complementing cDNAs, hybridising to 88 of the 110 SAT cDNAs. The Sat-52 gene-specific probe hybridised to 11 cDNAs and the Sat-53 gene-specific probe to 6 cDNAs.

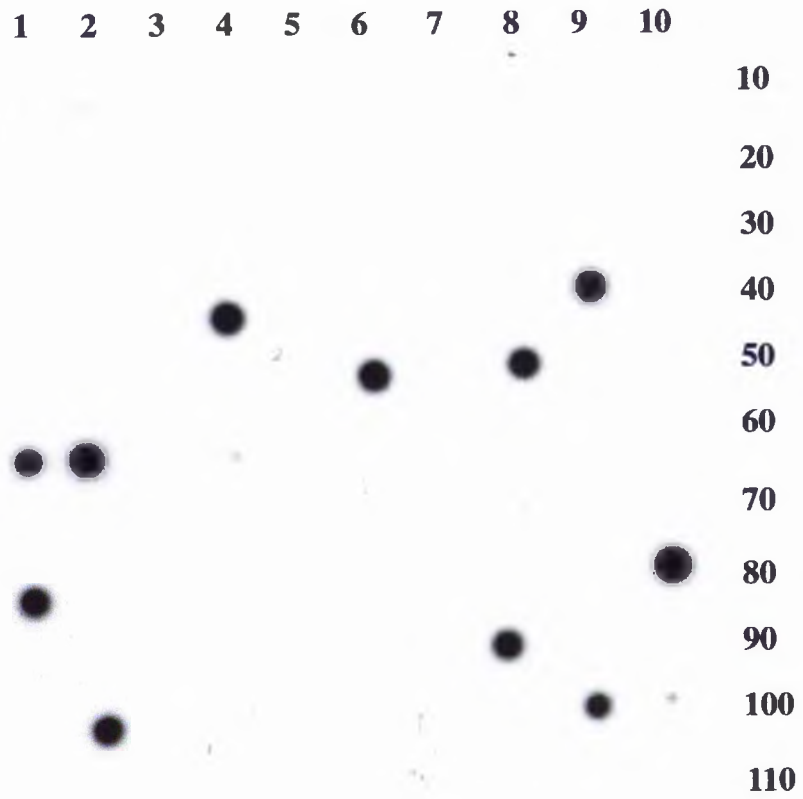
Of the remaining 5 cDNAs (Sat-8, Sat-11, Sat-17, Sat-64 and Sat-106) Sat-11 had previously been shown to encode an actin protein and Sat-8 consisted of DNA with no known function (see section 3.2.1). Sequencing of *ca* 400 bp from the 5' and 3' ends of Sat-17, Sat-64 and Sat-106 was carried out using pYES derived primers. Sat-17 was found to be homologous to Sat-52, and Sat-64 was found to be homologous to Sat-1. cDNAs Sat-17 and Sat-64 were two of the shortest isolated, as shown in Table 3.1, with approximate sizes of 0.99 and 1.00 kb respectively. These cDNAs, truncated at the 5' ends, were not long enough to

Figure 3.7 Dot-blot of the 110 plasmids obtained by functional complementation of the *E. coli cysE* mutant JM15. 0.5 ng of each plasmid, pSAT1 to pSAT110, was blotted onto a nylon membrane which was then sequentially probed with (a) Sat-1, (b) Sat-52 and (c) Sat-53 gene-specific probes.

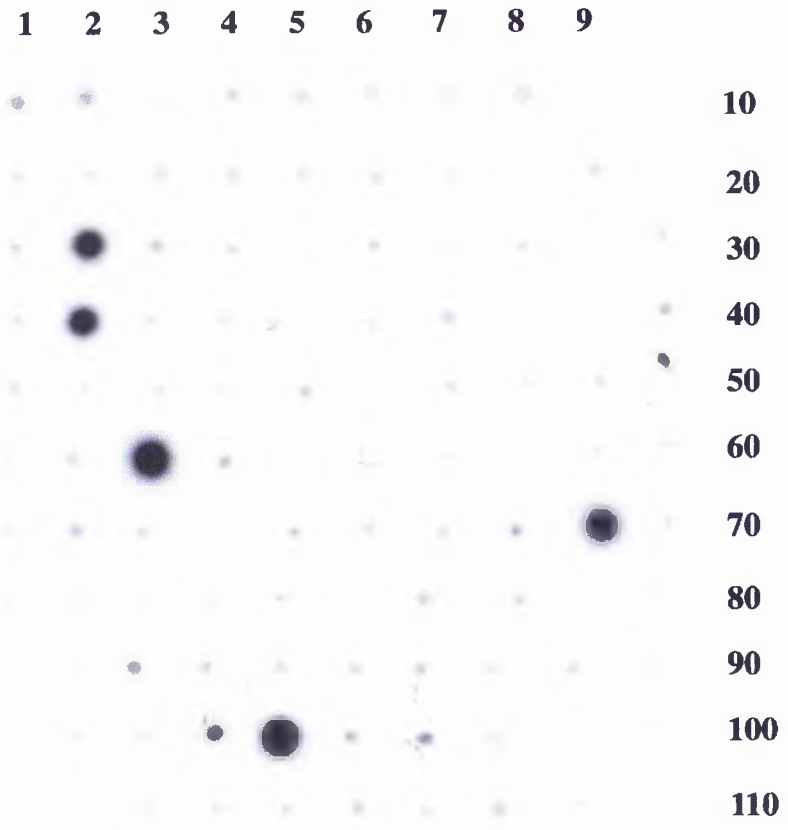
(a) Sat-1



(b) Sat-52



(c) Sat-53



hybridise with their respective gene-specific probes, which were designed from the 5' ends of the full length sequences. However, Sat-106 was found to be the sole representative of a fourth novel form of SAT and was sequenced in the pYES plasmid.

3.2.7 Sequencing and analysis of cDNA clone Sat-106

The Sat-106 cDNA sequence and its deduced amino acid translation are shown in Figure 3.8. The cDNA is 1114 nucleotides long with an open reading frame (ORF) of 969 nucleotides, a 23 nucleotide 5' untranslated region and a 123 nucleotide non-coding 3' region. Although no poly-A tail was found at the 3' end of the cDNA, the presence of consensus polyadenylation signals 21 and 33 nucleotides from the 3' end suggest that the cDNA is close to full-length. The ORF encodes a 323 amino acid polypeptide with a predicted molecular weight of 34.53 kDa and an isoelectric point of 6.23. An in-frame stop codon at the 5' end of the Sat-106 ORF indicates that the deduced amino acid sequence of the SAT106 protein is full-length.

As shown in Figure 3.5 the nucleotide homology between Sat-106 and the other *A. thaliana* SAT cDNAs is highest towards their 3' ends. Nucleotides 1 to 406 from the 5' end of the Sat-106 cDNA were amplified by PCR using primers P2 and 106.3 (Table 3.3). This DNA fragment was tested for cross-reactivity with the pSAT1, pSAT52, pSAT53 plasmids as shown in Figure 3.6b. No cross-hybridisation was observed therefore the amplified fragment can be used as a Sat-106 gene-specific probe.

3.2.8 Primer extension analysis of Sat-52 mRNA

Primer extension analysis is a technique which can be used to accurately measure the length of RNA molecules and can be used to determine the full length of the

```
CTTTATCGTCTCCTTGGAGAGATATGAATGGCGATGAGCTTCCTTTCGAGAGTGGTTTCG      60
                          M  N  G  D  E  L  P  F  E  S  G  F  E      13

AGGTTTACGCTAAGGGAACTCATAAGTCAGAGTTTGACTCGAATTTGCTTGATCCTCGTT     120
  V  Y  A  K  G  T  H  K  S  E  F  D  S  N  L  L  D  P  R  S      33

CTGATCCTATTTGGGATGCTATAAGAGAAGAAGCTAAACTTGAGGCAGAGAAAGAGCCTA     180
  D  P  I  W  D  A  I  R  E  E  A  K  L  E  A  E  K  E  P  I      53

TTTTGAGTAGCTTCTTGTATGCTGGTATCTTAGCACATGATTGTTTAGAGCAAGCTTTAG     240
  L  S  S  F  L  Y  A  G  I  L  A  H  D  C  L  E  Q  A  L  G      73

GGTTTGTTCTAGCCAACCGTCTCCAAAACCCAACCTTGTTGGCAACACAACTCTTGGATA     300
  F  V  L  A  N  R  L  Q  N  P  T  L  L  A  T  Q  L  L  D  I      93

TATTTTATGGTGTTATGATGCATGACAAAGGTATTCAGAGTTCGATTCGCCATGATCTCC     360
  F  Y  G  V  M  M  H  D  K  G  I  Q  S  S  I  R  H  D  L  Q     113

AGGCATTTAAAGATCGTGATCCTGCTTGTCTGTCGTATAGTTCTGCTATTTTACATCTGA     420
  A  F  K  D  R  D  P  A  C  L  S  Y  S  S  A  I  L  H  L  K     133

AGGGTTATCATGCGTTACAAGCATATAGGGTTGCGCATAAACTGTGGAATGAAGGGAGGA     480
  G  Y  H  A  L  Q  A  Y  R  V  A  H  K  L  W  N  E  G  R  K     153

AACTATTAGCTCTTGCATTGCAAAGCCGAATAAGCGAGGTTTTTGGCATTGACATACATC     540
  L  L  A  L  A  L  Q  S  R  I  S  E  V  F  G  I  D  I  H  P     173

CAGCGGCAAGAATTGGGGAGGGAATATTGTTGGATCATGGAACTGGAGTGGTCATTGGTG     600
  A  A  R  I  G  E  G  I  L  L  D  H  G  T  G  V  V  I  G  E     193

AGACCGCTGTGATAGGCAACGGTGTCTCGATCTTACATGGTGTGACTTTAGGAGGAACCG     660
  T  A  V  I  G  N  G  V  S  I  L  H  G  V  T  L  G  G  T  G     213

GAAAGGAAACTGGCGATCGCCACCCAAAGATAGGTGAAGGTGCATTGCTTGGAGCTTGTG     720
  K  E  T  G  D  R  H  P  K  I  G  E  G  A  L  L  G  A  C  V     233

TGACTATACTTGGTAACATAAGCATAGGTGCTGGAGCAATGGTAGCTGCAGGTTCACTTG     780
  T  I  L  G  N  I  S  I  G  A  G  A  M  V  A  A  G  S  L  V     253

TGTTAAAAGACGTTCCTTCGCATAGTGTGGTGGCTGGAAATCCTGCAAAACTGATCAGGG     840
  L  K  D  V  P  S  H  S  V  V  A  G  N  P  A  K  L  I  R  V     273

TCATGGAAGAGCAAGACCCGTCTCTAGCAATGAAACACGATGCTACTAAAGAGTTCTTTC     900
  M  E  E  Q  D  P  S  L  A  M  K  H  D  A  T  K  E  F  F  R     293

GACATGTAGCTGATGGTTACAAAGGGGCACAATCTAACGGACCATCACTTTCAGCAGGAG     960
  H  V  A  D  G  Y  K  G  A  Q  S  N  G  P  S  L  S  A  G  D     313

ATACAGAGAAAGGACACACTAACAGCACATCATGAGATCTTTGTATGCAAATTTCTTACC    1020
  T  E  K  G  H  T  N  S  T  S  *                                 332

ATCATAAGCTAATTCTTGTATGCTCTTGCTTATCATCATAAGCTAATCTCAGCATTTCCT    1080
CAATAAGTTGTCTAATAAAAGATTTTGTTCACTG                               1114
```

Figure 3.8 The nucleotide sequence of the Sat-106 cDNA with deduced amino acid translation. The 3' in-frame stop codon is marked with an asterisk (*). Putative polyadenylation motif sequences are underlined 32 and 20 nucleotides from the cDNA 3' end.

mRNA for which a cDNA is available. A complementary oligonucleotide primer is designed from a region approximately 100 bp downstream of the 5' end of a known cDNA sequence. This primer is then radioactively end-labelled, annealed to its corresponding mRNA in a total RNA sample and extended using reverse transcriptase. Transcription stops when the extending cDNA strand reaches the 5' end of the mRNA molecule. The exact length of the newly transcribed, radiolabelled cDNA can then be calculated by separating the cDNA strand on a sequencing gel next to a known reference sequence. The *Sat-1* mRNA was sized to 1515 nucleotides using this method (Roberts & Wray 1996). Primer extension analysis was carried out to determine the exact size of the full-length *Sat-52* mRNA.

A 20mer oligonucleotide primer was designed, complementary to the Sat-52 cDNA sequence between bases 148 and 167 (see Table 3.3), and primer extension was carried out as outlined in the Materials and Methods chapter, using RNA samples from roots, leaves and stems of *A. thaliana*. The extension reaction product was then separated on a sequencing gel next to a reference of Sat-52 in λYES, sequenced using the primer extension primer. The autoradiogram of the primer extension products next to the Sat-52 reference sequence is shown in Figure 3.9.

Primer extension analysis indicated the transcription of two predominant sizes of *Sat-52* mRNAs in root, leaf and stem tissue of *A. thaliana* both of which were shorter than the Sat-52 cDNA. As no band the size of the Sat-52 cDNA was detected by primer extension, the *Sat-52* mRNA would appear to be transcribed from a transcription start site further upstream from those most commonly utilised. Of the *Sat-52*-type mRNAs detected by primer extension, Type 1 is 4 nucleotides shorter than mRNA Type 2 and is the predominant mRNA transcript in all tissues. This was deduced from the greater intensity of the band representing the Type 1 transcript, indicating a greater abundance of this mRNA species in the template RNA to which the extension primer was annealed. This primer extension analysis

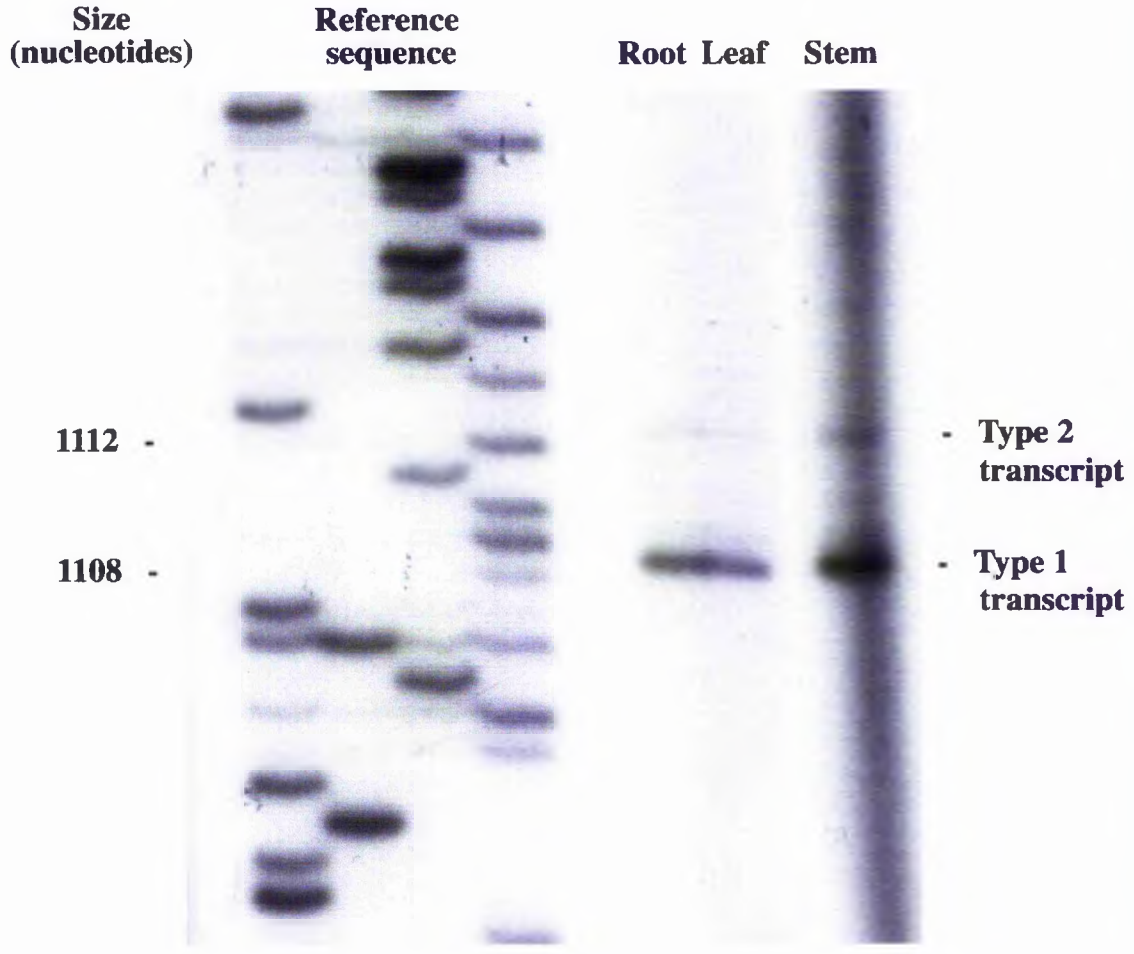


Figure 3.9 Sat-52 primer extension products transcribed from root, leaf and stem total RNA samples, separated next to the pSAT52 reference sequence. Band sizes shown represent nucleotides from the polyadenylation sequence. Type 1 and Type 2 extension products are indicated.

results suggests either that several transcription start sites are present, and operative, in the *Sat-52* gene or that the gene is represented by several copies in the *A. thaliana* genome. Southern analysis suggests that *Sat-52* is a single copy gene (section 3.2.11), therefore it is most likely that there are multiple transcription start sites within a single gene. Several transcription initiation sequences are present in the promoter region of the *Sat-52* gene, as shown in Figure 3.10. A comparison of the primer extension products to the reference sequence indicates that Type 1 and Type 2 *Sat-52* mRNAs are 1108 and 1112 nucleotides in length respectively to the polyadenylation sequence (Figure 3.9).

3.2.9 Computer analysis of nucleotide sequences

Nucleotide homology between the SAT cDNAs were calculated using the PILEUP program of the GCG software package (Devereux et al. 1984) at Daresbury Laboratory (http://www.seqnet.dl.ac.uk). Table 3.4 shows the percentage cDNA homologies between Sat-1, Sat-52, Sat-53 and Sat-106. Figure 3.5 shows the nucleotide alignment of the four SAT isoforms from which these homologies were calculated. The conserved nucleotide homologies between all four cDNAs are shown as a consensus sequence below. The region of highest nucleotide homology is found towards the 3' ends of the cDNA coding regions. This suggests that the 3' region may encode the active site and/or a structurally important domain of the SAT enzyme.

Nucleotide codon usages within the coding regions of the four SAT genes represented by the Sat-1, Sat-52, Sat-53 and Sat-106 cDNAs were calculated using the CODONFREQUENCY program of the GCG software package (Devereux et al. 1984). Table 3.5 shows the codon usage for each amino acid in the SAT protein sequences. Preferences for the codons AGA, AGG and CGU for arginine, CUC, CUU and UUG for leucine, GGA and GGU for glycine, AUC and AUU for isoleucine, GCU for alanine and GUU for valine are particularly predominant in *A.*

Table 3.4 Nucleotide homologies between the Sat-1, Sat-52, Sat-53 and Sat-106 cDNAs from *A. thaliana*. Coding region nucleotide homologies are shown in brackets.

Sat-1	100% (100%)			
Sat-52	52% (56%)	100% (100%)		
Sat-53	67% (72%)	49% (55%)	100% (100%)	
Sat-106	47% (46%)	49% (51%)	46% (49%)	100% (100%)
	Sat-1	Sat-52	Sat-53	Sat-106

Table 3.5 Open reading frame codon-usages of each SAT cDNA from *A. thaliana*

Amino Acid	Codon	Sat-1	Sat-52	Sat-53	Sat-106
Arginine (R)	CGU	7	5	1	3
	CGC	5	2	0	2
	CGA	2	3	4	1
	CGG	0	1	3	0
	AGA	3	3	4	2
	AGG	2	0	1	3
Leucine (L)	CUU	11	10	3	9
	CUC	6	3	5	3
	CUA	4	8	2	3
	CUG	0	2	2	5
	UUA	3	8	8	9
	UUG	14	1	6	10
Serine (S)	UCU	17	9	6	3
	UCC	6	7	4	0
	UCA	1	9	3	4
	UCG	6	6	4	6
	AGU	1	0	2	5
	AGC	3	1	5	4
Threonine (U)	ACU	7	4	1	5
	ACC	5	3	6	3
	ACA	2	6	7	1
	ACG	10	3	5	0
Proline (P)	CCU	17	4	2	7
	CCC	1	0	3	0
	CCA	3	6	2	3
	CCG	6	3	4	1
Alanine (A)	GCU	18	16	10	14
	GCC	3	3	2	1
	GCA	0	10	2	13
	GCG	7	12	6	3
Glycine (G)	GGU	9	8	7	13
	GGC	3	1	3	3
	GGA	9	14	11	10
	GGG	7	4	6	3
Valine (V)	GUU	15	7	7	7
	GUC	4	3	3	3
	GUA	1	4	4	3
	GUG	5	5	9	7

Table 3.5 continued overleaf

Table 3.5 continued

Amino Acid	Codon	Sat-1	Sat-52	Sat-53	Sat-106
Asparagine (N)	AAU	16	6	10	4
	AAC	5	9	10	4
Lysine (K)	AAA	16	6	10	10
	AAG	5	9	10	5
Aspartate (D)	GAU	17	13	13	12
	GAC	8	3	7	4
Glutamate (E)	GAA	9	10	10	6
	GAG	5	5	6	12
Histidine (H)	CAU	10	10	8	10
	CAC	10	4	4	2
Glutamine (Q)	CAA	2	3	6	6
	CAG	7	2	3	4
Tyrosine (Y)	UAU	5	2	2	5
	UAC	3	3	4	2
Cysteine (C)	UGU	5	7	5	3
	UGC	2	0	4	0
Phenylalanine (F)	UUU	3	2	1	6
	UUC	11	5	9	4
Isoleucine (I)	AUU	15	8	11	8
	AUC	9	10	12	3
	AUA	2	3	7	9
Methionine (M)	AUG	6	2	3	6
Tryptophan (W)	UGG	3	3	3	2
Stop Codons	UAA	0	1	1	1
	UAG	0	0	0	0
	UGA	1	0	0	0

thaliana genes (Wada et al. 1992). These preferences were generally observed in the ORFs of Sat-1, Sat-52, Sat-53 and Sat-106, as shown in Table 3.5.

3.2.10 Analysis of the Sat-52, Sat-53 and Sat-106 genomic sequences

Genomic DNA sequences, including promoter and coding regions, are invaluable to studies of molecular regulation of gene expression. Whilst individual genes can be cloned conventionally, from genomic libraries screened with cDNA probes, a number of projects are currently underway to sequence the entire genomes of several model biological research species, including the *A. thaliana* Columbia ecotype. These projects will eventually provide all the genomic information required for molecular research without the laborious conventional processes of gene cloning. To date, workers from several laboratories involved in the AGI (Arabidopsis Genome Initiative. Internet web-site: http://genome-www.stanford.edu/Arabidopsis/AGI) have sequenced 33.06 Mb of the 100-120 Mb *Arabidopsis* genome. This sequencing is performed on large overlapping chromosome fragments contained in a variety of vectors, such as BACs (Bacterial Artificial Chromosomes) and YACs (Yeast Artificial Chromosomes), which allow the manipulation of several hundred kilobases of DNA at a time. The Sat-1, Sat-52, Sat-53 and Sat-106 cDNA sequences were used to search the AGI databases for their equivalent genomic clones. Genes homologous to the Sat-52, Sat-53 and Sat-106 cDNAs were detected by BLAST homology searches (Altschul et al. 1990). The *Sat-1* gene sequence is not yet available. The three SAT genes sequenced so far by the AGI are located on different chromosomes. The chromosomal loci of the *Sat-52*, *Sat-53* and *Sat-106* genes are shown in Figure 3.13.

3.2.10.1 Analysis of the Sat-52 genomic sequence

The coding region of the *Sat-52* gene (Figure 3.10) was found between 59703 and 60787 bp on BAC MIK19 (GenBank accession AB013392) which

was sequenced at the Kazusa DNA Research Institute, Chiba, Japan (Nakamura et al. 1998, unpublished). The *A. thaliana* DNA of BAC MIK19 originates from a locus at 115.7 centiMorgans (cM) on chromosome V (Figure 3.13). The *Sat-52* gene contains one intron of 146 bp and the exons share 100% nucleotide sequence homology to the Sat-52 cDNA.

Two thousand nucleotides upstream from the initiating methionine codon were analysed for promoter elements which may be involved in the function or regulation of expression of the *Sat-52* gene. As shown on Figure 3.10, TATA-box sequences, which bind the transcription factor responsible for the induction of RNA polymerase activity, are found at positions -297 and -457 as well as at several sites further upstream. This may indicate several start sites for transcription of the *Sat-52* gene. Several CAAT-box sequences, also involved in transcription initiation, are found in the promoter region. A sequence resembling the SEF 4 transcription factor recognition site (A/G-TTTTT-A/G) is found at position -1616 on Figure 3.10. The SEF 4 transcription factor is known to induce transcription of the β-conglycinin gene in soybean in response to sulphur-deficiency (Lessard et al. 1991) and may similarly be implicated in the expression of the *Sat-52* gene. A MYB-like transcription factor recognition sequence (AAACTG) was also present in the *Sat-52* promoter region, at position -1542 on Figure 3.10. The ATMYB2 protein from *A. thaliana* is accumulated under salt and drought stress and binds to MYB promoter elements (TAACTG), inducing gene expression (Urao et al. 1993). The presence of a MYB-like recognition sequence in the *Sat-52* gene promoter may lead to the induction of *Sat-52* expression under salt and drought stress.

The position of the Sat-52 cDNA poly-A tail is indicated by a # on Figure 3.10. Polyadenylation signals are usually located 6 to 40 nucleotides upstream from the poly-A tail (Hunt 1994) of an mRNA molecule. No ATAAA consensus was found in this region of Sat-52. However, sequences similar to the polyadenylation consensus are found at positions 1096 (ATAAT), 1186 (AATTTA) and 1212 (AATA) on Figure 3.10.

Figure 3.10 *Sat-52* genomic nucleotide sequence (from BAC MIK19, GenBank accession AB013392). The 2 exons encoding the ORF are shown in uppercase letters. The initiating ATG methionine codon of the *SAT52* protein and the 3' stop codon are marked with arrows (⇓). # marks the position of the poly-A tail of the Sat-52 cDNA. TATA and CAAT-box sequences in the promoter region and polyadenylation signal sequences in the 3' untranslated region of the gene are underlined. Putative transcription factor recognition sequences are named above their recognition sequence, which are also shown in uppercase lettering.


10 20 30 40 50 60 70 80 90
SBF 4
MYB
exon 1
intron 1
exon 2

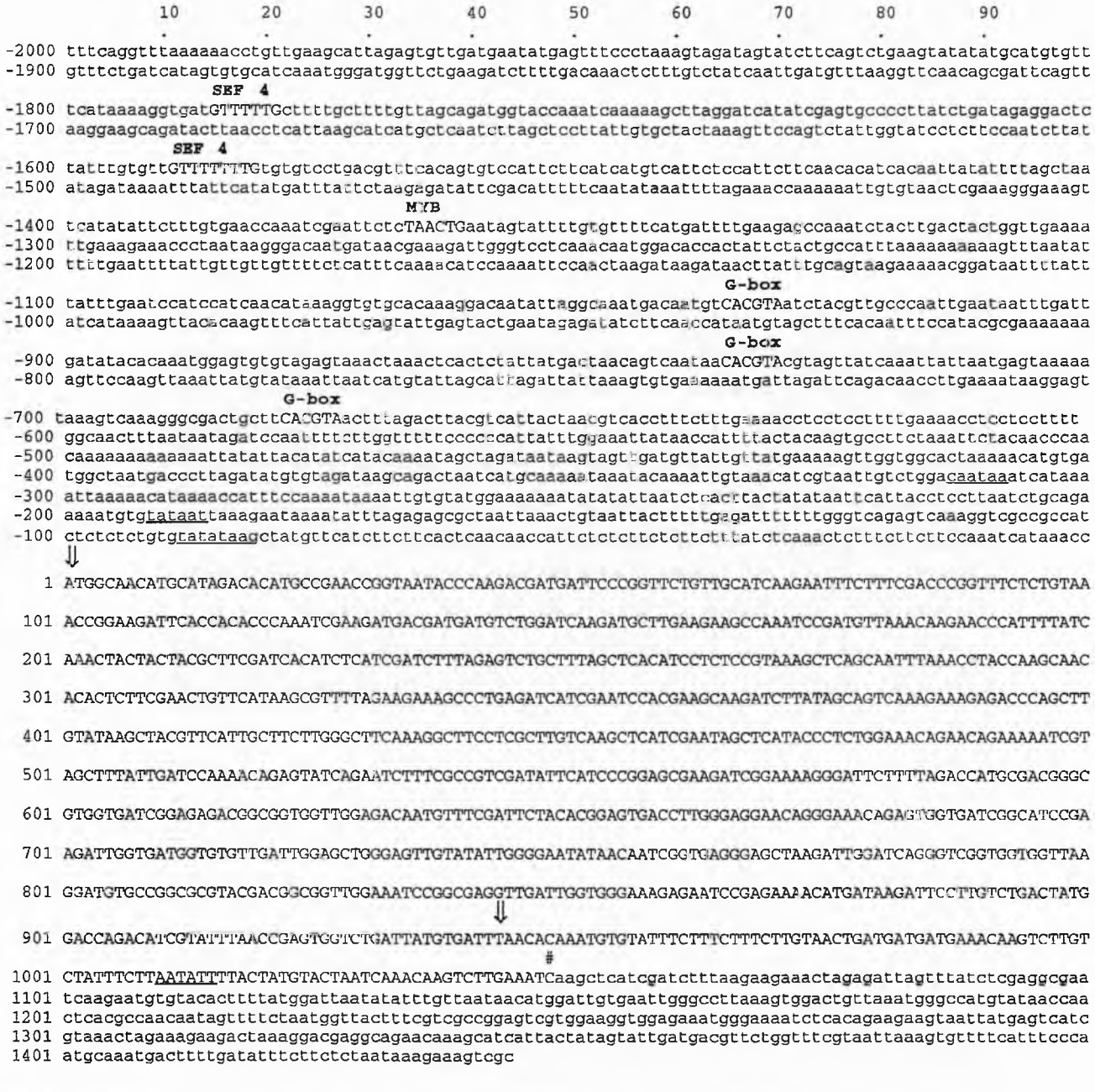
3.2.10.2 Analysis of the Sat-53 genomic sequence

The coding region of the *Sat-53* gene (Figure 3.11) was found between 44401 and 45342 bp of BAC F14J16 (GenBank accession AC002304) which was sequenced at the University of Pennsylvania, USA (Ecker et al. 1997 unpublished). The *A. thaliana* DNA of BAC F14J16 originates from a locus at 81.6 centiMorgans (cM) on chromosome I (Figure 3.13). The *Sat-53* gene shows 100% sequence homology to the Sat-53 cDNA and contains no introns.

Again, two thousand nucleotides upstream from the initiating methionine codon were analysed for promoter elements. A TATA-box sequence is found at position -89 in Figure 3.11 with several other TATA motifs located further upstream from the coding region. A CAAT-box sequence is found at position -314. As found in the *Sat-52* promoter region, SEF 4-like transcription factor recognition sites are present at positions -1589 and -1785 of the *Sat-53* genomic sequence. The MYB- transcription factor recognition sequence, (TAACTG) described in section 3.2.10.1 was also found in the *Sat-53* promoter sequence at position -1366. The ATMYB2 protein may bind to this recognition sequence inducing *Sat-53* expression under salt and drought stress. An experiment to study the effects of salt and drought stress on expression of the *Sat-53* gene are described in Chapter 4 of this thesis. G-box-like promoter elements (CACGTA) were also detected in the *Sat-53* promoter at positions -677, -835 and -1035. The G-box promoter element (CACGTG) is known to be involved in induction of gene expression by abscisic acid (ABA) (Giraudat et al. 1994 and references therein). The G-box-like motifs detected in the promoter sequence in Figure 3.11 may be involved in regulation of *Sat-53* expression by ABA.

Following the *Sat-53* coding region, an AATATT nucleotide sequence at position 1010 resembles the consensus polyadenylation signal AATAAA (Hunt 1994; Wu et al. 1995). The poly-A tail of the Sat-53 cDNA was positioned 34 nucleotides downstream from this sequence as indicated in Figure 3.11.

Figure 3.11 *Sat-53* genomic nucleotide sequence (from BAC F14J16, GenBank accession AC002304). The ORF is shown in uppercase letters. The initiating ATG methionine codon of the *SAT53* protein and the 3' stop codon are marked with arrows (⇓). # marks the position of the poly-A tail of the Sat-53 cDNA. TATA and CAAT-box sequences in the promoter region and polyadenylation signal sequences in the 3' untranslated region of the gene are underlined. Putative transcription factor recognition sequences are named above their recognition sequence, which are also shown in uppercase lettering.


SEF 4
SEF 4
MYB
G-box
G-box
G-box

3.2.10.3 Analysis of the Sat-106 genomic sequence

The Sat-106 cDNA sequence was also used to search the GenBank nucleotide sequence database for genomic clones. The *Sat-106* gene (Figure 3.12) was found between 75979 and 77758 bp on BAC T17A5 (GenBank accession AF024504), which originates from a locus at approximately 34.1 cM on *A. thaliana* chromosome II (Figure 3.13). BAC T17A5 was sequenced at the Cold Spring Harbour Laboratory, USA (Parnell et al. 1997 unpublished). *The Sat-106* gene has 10 exons and 9 introns and the exons show 100% nucleotide homology to the Sat-106 cDNA sequence described in section 3.2.7. Eight TATA-box motifs are found between nucleotides -271 and -620 in Figure 3.12. 6 CAAT-box sequences are also found in this region. This may indicate several possible transcription start sites in the *Sat-106* gene.

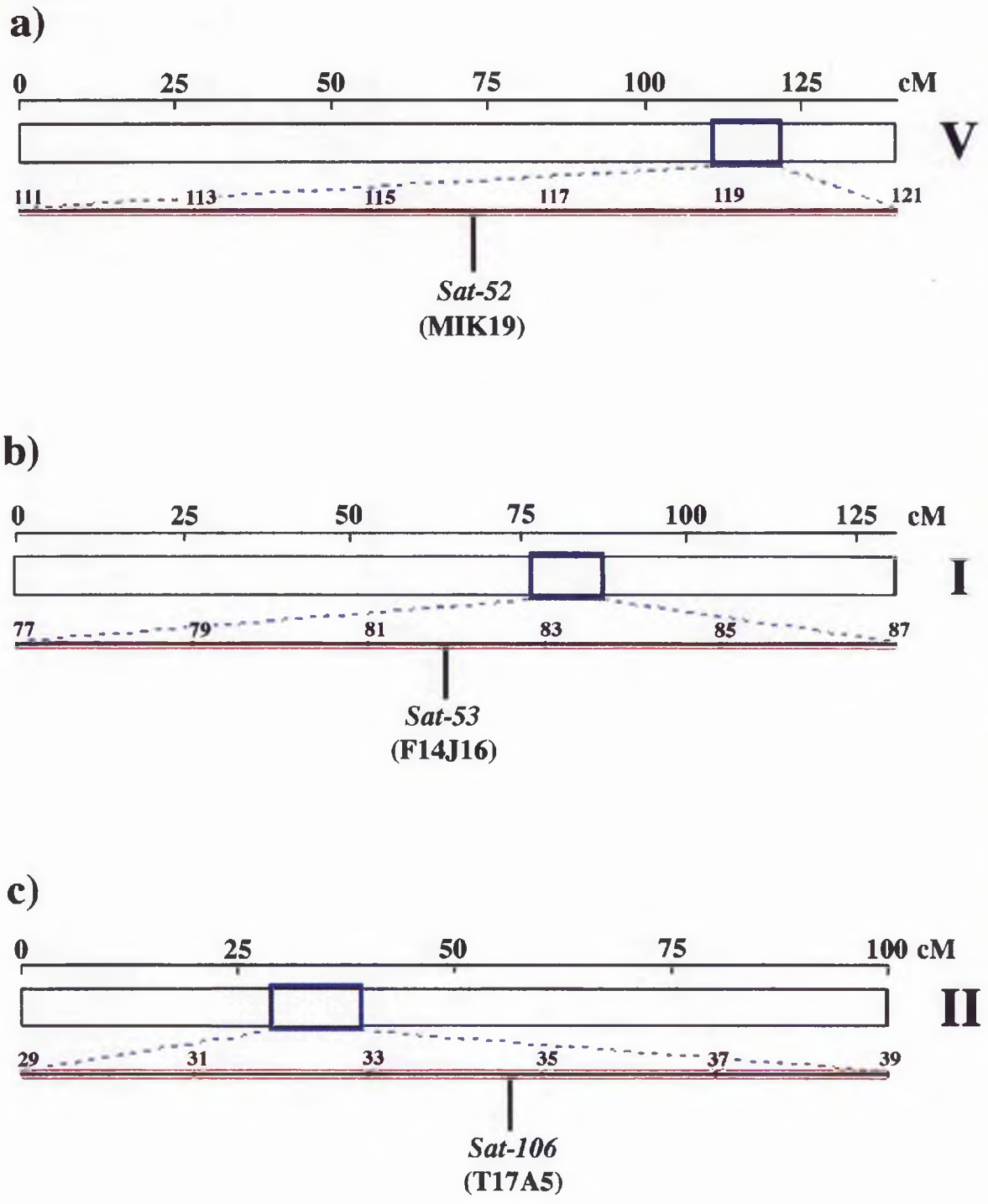
The sequence AACCGACAA at position -1353 on Figure 3.12 shows remarkable similarity to the DRE (**D**rought **R**esponsive **E**lement) transcription factor recognition sequence (TACCGACAT). This promoter element is involved in regulation of the dehydration-induced *A. thaliana* gene *rd29a* (Yamaguchi-Shinozaki & Shinozaki 1994). The AACCGACAA sequence in the promoter region of *Sat-106* may therefore be involved in expression of the *Sat-106* gene under drought-stress. As observed previously in the *Sat-52* and *Sat-53* promoter sequences, a MYB transcription factor recognition sequence (TAACTG) was also found in the promoter of the *Sat-106* gene at position -1402 on Figure 3.12.

Consensus polyadenylation signals AATAA and AATAAAA (Hunt 1994; Wu et al. 1995) at positions 2076 and 2088 are likely to be involved in polyadenylation of the *Sat-106* mRNA, although no poly-A tail was present on the Sat-106 cDNA described in section 3.2.7.

Figure 3.12 *Sat-106* genomic nucleotide sequence (from BAC T17A5, GenBank accession AF024504). The 8 exons encoding the ORF are shown in uppercase letters. Untranslated flanking regions and the 7 introns are shown in lowercase letters. The initiating ATG methionine codon of the *SAT106* protein and the 3' stop codon are marked with arrows (⇓). The 3' end of the Sat-106 cDNA is marked by a #. TATA and CAAT-box sequences in the promoter region and polyadenylation signal sequences in the 3' untranslated region of the gene are underlined. Putative transcription factor recognition sequences are named above their recognition sequence, which are also shown in uppercase lettering.


10 20 30 40 50 60 70 80 90
MYB
DRE
exon 1
intron 1
exon 2
intron 2
exon 3
intron 3
exon 4
intron 4
exon 5
intron 5
exon 6
intron 6
exon 7
intron 7
exon 8
intron 8
exon 9
intron 9
exon 10

Figure 3.13 Chromosomal locations of the *Sat-52*, *Sat-53* and *Sat-106* genes. White bars represent entire *A. thaliana* chromosomes with chromosome number at the right. Blue boxes are amplified below each chromosome to show the location of the SAT genes. Scale bars show chromosome length in centiMorgans (cM). a) Chromosome V locus of BAC clone MIK19 (GenBank accession AB013392) which contains the *Sat-52* gene. b) Chromosome I locus of BAC clone F14J6 (GenBank accession AC002304) which contains the *Sat-53* gene. c) Chromosome II locus of BAC clone T17A05 (GenBank accession AF024504) which contains the *Sat-106* gene.


a)
0
25
50
75
100
125
cM
V
111
113
115
117
119
121
Sat-52
(MIK19)
b)
0
25
50
75
100
125
cM
I
77
79
81
83
85
87
Sat-53
(F14J16)
c)
0
25
50
75
100 cM
II
29
31
33
35
37
39
Sat-106
(T17A5)

3.2.11 Southern analysis: genomic organisation of the SAT gene-family from A. thaliana

Figure 3.14 shows the Southern blots of digested genomic DNA probed with Sat-1, Sat-52 and Sat-53 cDNAs. Southern hybridisation using the Sat-52 probe was performed against 10µg samples of *A. thaliana* genomic DNA digested with the restriction endonucleases *Bam*HI, *Ban*II, *Bst*EII, *Hin*dIII and *Pst*I. Sat-1 and Sat-53 cDNA probes were hybridised to 10µg of *A. thaliana* genomic DNA digested with *Ban*II, *Bst*EII, *Eco*RI and *Pst*I.

*Bam*HI, *Ban*II and *Bst*EII do not cut within the Sat-52 cDNA sequence. Single bands were observed when the Sat-52 probe was hybridised against DNA digested with these enzymes. *Hin*dIII and *Pst*I cut twice and once respectively within the Sat-52 cDNA sequence. *Hin*dIII and *Pst*I-digested DNA showed three and two bands respectively when hybridised with the Sat-52 probe (Figure 3.14b).

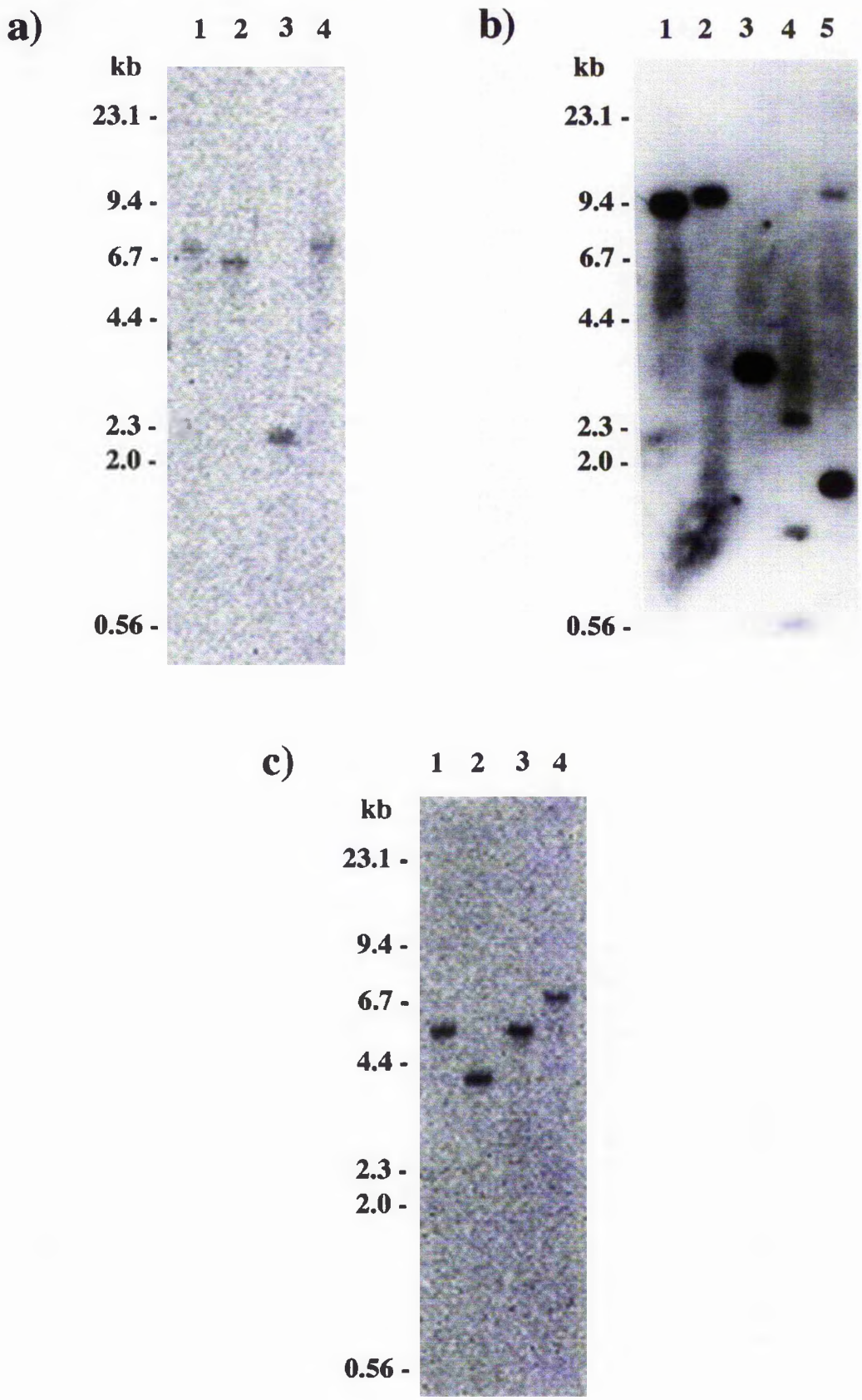
*Ban*II, *Bst*EII, *Eco*RI and *Pst*I do not cut within the Sat-1 or Sat-53 cDNA sequences. Single bands were observed when the Sat-1 and Sat-53 probes were hybridised against DNA digested with these enzymes (Figures 3.14a and 3.14c).

These Southern blot studies on the genomic organisation of SAT suggest that *Sat-1*, *Sat-52* and *Sat-53* genes are represented by single copies in the *A. thaliana* genome.

3.2.12 Computer analysis of deduced amino acid sequences

Conceptual translation of amino acid sequences of the *A. thaliana* SAT isoforms from nucleotide sequence data, and the analysis of these protein sequences, was carried out using the MAP, PILEUP, MOTIFS, CLUSTALV and PHYLIP programs of the GCG software package (Devereux et al. 1984) at Daresbury Laboratory (http://www.seqnet.dl.ac.uk). Figure 3.15 shows the alignment of the deduced

Figure 3.14 Southern blots of digested genomic DNA from *A. thaliana* probed with a) Sat-1, b) Sat-52 and c) Sat-53 cDNAs. Lanes 1 to 4 in a) and c) contain genomic DNA digested with *Ban*II, *Bst*EII, *Eco*RI and *Pst*I respectively. Lanes 1 to 5 in b) contain genomic DNA digested with *Bam*HI, *Ban*II, *Bst*EII, *Hin*dIII and *Pst*I respectively. The positions of λ*Hin*dIII base-pair markers are shown to the left of each blot.


a)
1 2 3 4
kb
23.1 -
9.4 -
6.7 -
4.4 -
2.3 -
2.0 -
0.56 -
b)
1 2 3 4 5
kb
23.1 -
9.4 -
6.7 -
4.4 -
2.3 -
2.0 -
0.56 -
c)
1 2 3 4
kb
23.1 -
9.4 -
6.7 -
4.4 -
2.3 -
2.0 -
0.56 -

SAT amino acid sequences from *A. thaliana* with all the other known plant SATs. The other plant SATs include SAT1-6 (Bogdanova et al. 1995), which is a truncated SAT1-type SAT from *A. thaliana*, SAT2 (Saito et al. 1995), a putative cytoplasmic isoform from *Citrullus vulgaris* (watermelon) and two SAT53-type SATs from *A. thaliana*, SAT5 (Ruffet et al. 1995) and SAT1 (Murillo et al. 1995: not to be confused with the putatively plastidic SAT1 (Roberts & Wray 1996)). Whilst SAT5 shares 100% sequence identity with SAT53, SAT1 (Murillo et al. 1995) has eight non-identical residues at positions 61, 89, 135, 138, 166, 325, 326 and 327 on Figure 3.15. These non-identical residues suggest that SAT1 (Murillo et al. 1995) is encoded by a separate gene from SAT53 and SAT5 (Ruffet et al. 1995). As Southern blots suggest that SAT53 is encoded by a single copy gene, sequencing errors in the SAT1 cDNA (Murillo et al. 1995) may be the cause of the differences from the Sat-53 and *sat5* sequences. Table 3.6 shows the amino acid similarities and identities between the SAT1, SAT52, SAT53 and SAT106 proteins from *A. thaliana*.

Figure 3.16 shows the alignment of the SAT amino acid sequences from *A. thaliana* with those of bacterial SATs. Both Figure 3.15 and 3.16 reveal a region of high amino acid sequence homology towards the C-termini of the plant SAT proteins. The C-terminal region of the SAT proteins from *A. thaliana* is encoded by the region of high nucleotide homology between the cDNA sequences shown in Figure 3.5. The region of high amino acid sequence homology begins approximately at residue 205 in Figures 3.15 and 3.16, extending to the C-terminus of the proteins. Many amino acids in this region are found to be conserved between most of the known SAT proteins. These regions are therefore likely to have an essential catalytic or structural function in the SAT enzyme.

Analysis of the *A. thaliana* SAT protein sequences using the GCG program MOTIF (Devereux et al. 1984) showed a consensus motif in the region between residues 322 and 358 in Figure 3.15. This motif (Accn. PS00101 in release11.1 of the PROSITE database (Bairoch 1993)) has the sequence [IV]-G-X(2)-[S/T/A/V]-X-

Table 3.6 Amino acid similarities between the SAT1, SAT52, SAT53 and SAT106 proteins from *A. thaliana*. The percentage of identical amino acids are shown in brackets.

SAT1	100% (100%)			
SAT52	70% (55%)	100% (100%)		
SAT53	87% (76%)	68% (52%)	100% (100%)	
SAT106	68% (50%)	69% (52%)	67% (49%)	100% (100%)
	SAT1	SAT52	SAT53	SAT106

Figure 3.15 Amino acid sequence alignment of all known SAT proteins from plants. All sequences are compared to SAT52 from *A. thaliana*. Gaps in the alignment are shown by dots (.). Identical amino acid residues to the SAT52 sequence are shown by dashes (-). Represented in the alignment are SAT52 (GenBank U30298; Howarth et al. 1997), SAT1 (U22964; Roberts & Wray 1996), SAT1-6 (X82888; Bogdanova et al. 1995), SAT53 (this thesis), SAT5 (L34076; Ruffet et al. 1995), SAT1 (L42212; Murillo et al. 1995) and SAT106 (this thesis) from *A. thaliana*, SAT2 from *Citrullus vulgaris* (D85624; Saito et al. 1997) and an unpublished SAT from *Spinacea oleracea* (D88530).

```
                    1
A.thal. SAT52                                                                                                  MPPAGE LRHQSPSKEK
A.thal. SAT1        MLPVTSRRHF TMSLYMLRSS SPHINHHSFL LPSFVSSKFK HHTLSPPPSP PPPPPMAACI DTCRTGKPQI SPRDSSKHHD DESGFRYMNY F-YPDR-...
A.thal. SAT1-6                                                                                                 MNY F-YPDR-...
A.thal. SAT53                                                                  MATCI DTCRTGNTQ. .........D DDSRFCCIKN FFRPGF-...
A.thal. SAT5                                                                   MATCI DTCRTGNTQ. .........D DDSRFCCIKN FFRPGF-...
A.thal. SAT1                                                                   MATCI HTCRTGNTQ. .........D DDSRFCCINK FFRPGF-...
A.thal. SAT106                                                                                                 MNGD- -PFE-GF.-V
C.vulg. SAT2                                                                                                   M-V-- --FS-Q-...
S.oler.                                                                  ML AVSYAIPSCC INRFYIREFS SSLQPALHHP WYKKRFGCCS TNQENC-LGS

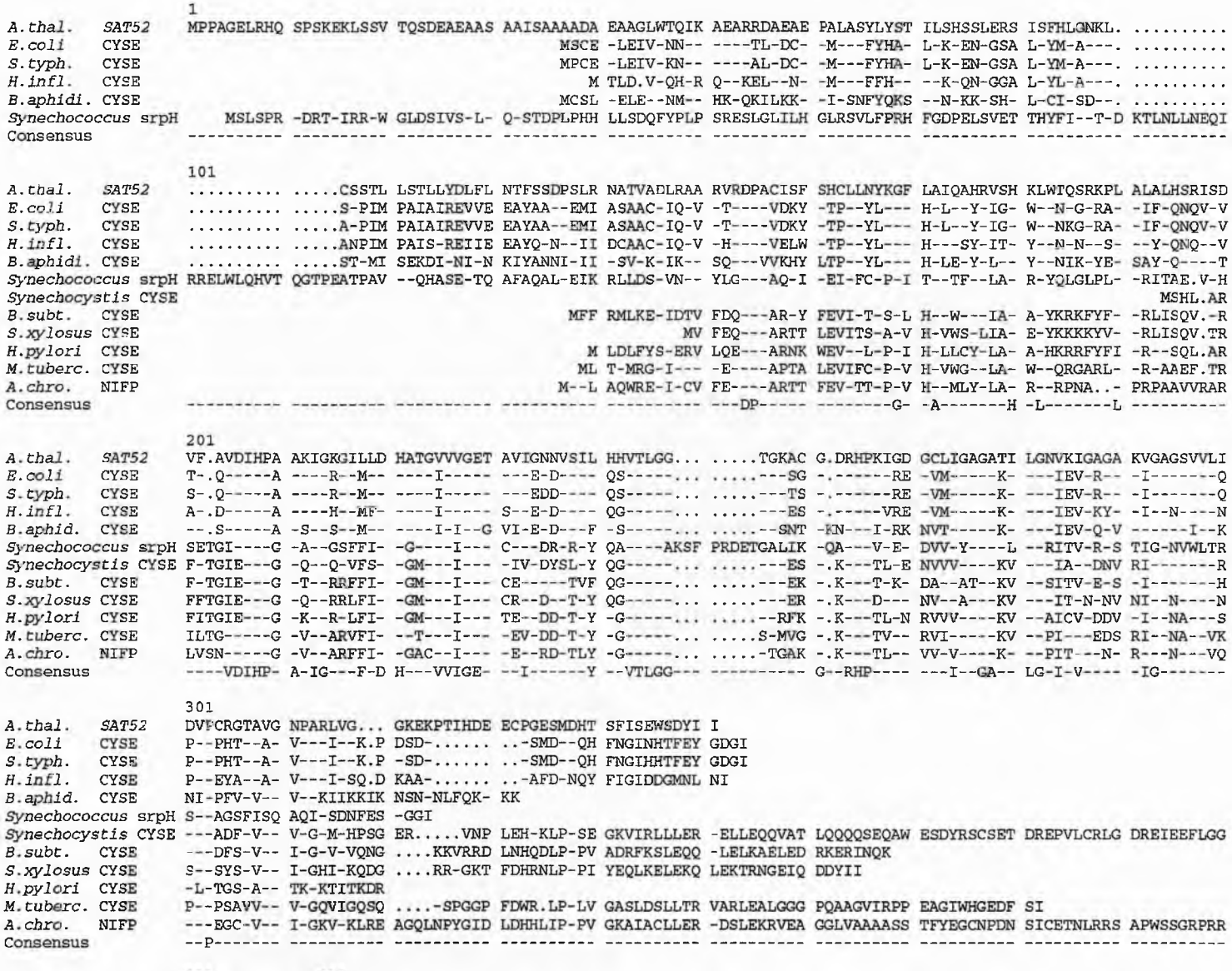
                    101
A.thal. SAT52       LSSVTQSDEA EAASAAISAA AADAEAAGLW TQIKAEARRD AEAEPALASY LYSTILSHSS LERSISFHLG NKLCSSTLLS TLLYDLFLNT FSSDPSLRNA
A.thal. SAT1        .-FNGTQTKT LHTRPLLEDL DR---VDDV- AK-RE--KS- IAK--IVSA- YHAS-V-QR- --AALANT-S V--SNLN-P- NT-F---SGV LQGN-DIVES
A.thal. SAT1-6      .-FNGTQTKT LHTRPLLEDL DR---VDDV- AK-RE--KS- IAK--IVSA- YHAS-V-QR- --AALANT-S V--SNLN-P- NT-F---SGV LQGN-DIVES
A.thal. SAT53       .V.....NRK IHHTQIEDD. ......DDV- IKMLE--KS- VKQ--I-SN- Y-AS-T--R- --SALAHI-S V--SNLN-P- NT-FE--ISV LEES-EIIES
A.thal. SAT5        .V.....NRK IHHTQIEDD. ......DDV- IKMLE--KS- VKQ--I-SN- Y-AS-T--R- --SALAHI-S V--SNLN-P- NT-FE--ISV LEES-EIIES
A.thal. SAT1        .V.....NRK IHHTQIEDD. ......DDV- IKMLK--ES- VKQ--I-SN- Y-AS-T--R- --SALGHI-S V--SNLN-P- NT-FE--ISV LEES-EIIES
A.thal. SAT106      YACG-HKS-. ......FDSN LL-PRSDPI- DA-RE--KLE --K--I-S-F --AG--A-DC --QALG-V-A -R-QNP---A -Q-L---YGV MMH-KGIQSS
C.vulg. SAT2        .......... ...-TTVVES TTNNDETW-- G--------- --S------- ---------- ----L----- ---------- ---------A --T-YC--S-
S.oler.             PTHLPRMPGG DLSV-PSVGH LTANNE-W-- D---G----- -DS------- ---------- ----L----- ---------- ---------I L---S--LD-

                    201
A.thal. SAT52       TVADLRAARV RDPACISFSH CLLNYKGFLA IQAHRVSHKL WTQSRKPLAL ALHSRISDVF AVDIHPAAKI GKGILLDHAT GVVVGETAVI GNNVSILHHV
A.thal. SAT1        VKL--L-VKE -------YV- -F-HF----- C----IA-E- ---D--I--- LIQN-V-EA- ---F--G--- -T-------- AI-I-----V --------N-
A.thal. SAT1-6      VKL--L-VKE -------YV- -F-HF----- C----IA-E- ---D--I--- LIQN-V-EA- ---F--G--- -T-------- AI-I-----V --------N-
A.thal. SAT53       -KQ--I-VKE -------YV- -F-GF----- C----IA-T- -K-N--IV-- LIQN-V-ES- ------G--- ---------- ---I-----V -D------G-
A.thal. SAT5        -KQ--I-VKE -------YV- -F-GF----- C----IA-T- -K-N--IV-- LIQN-V-ES- ------G--- ---------- ---I-----V -D------G-
A.thal. SAT1        -KQ--I-VKE -------YV- -F-GF----- C----IA-T- -K-N--IV-- LIQN-V-ES- ------G--- ---------- ---I-----V -D------G-
A.thal. SAT106      IRH--Q-FKD -----L-Y-S AI-HL--YH- L--Y--A--- -NEG--L--- --Q----E-- GI------R- -E------GT ---I------ --G-----G-
C.vulg. SAT2        V----Q---E -----V---- ---------- C-----A--- -N---R---- --Q---A--- --------R- -----F---- ---------- ----------
S.oler.             V--------- -----V---- ---------- C-S---A--- -N-D-R---- ---------- --------R- -----F---- ---I----I- -D-C------

                    301
A.thal. SAT52       TLGGTGKACG DRHPKIGDGC LIGAGATILG NVKIGAGAKV GAGSVVLIDV PCRGTAVGNP ARLVGGKEKP TIHDEECPGE SMDHTSFISE WSDYII
A.thal. SAT1        -------Q-- ---------V -----TC--- -IT--E---I -------K-- -P-T------ ---L---DN- KT-.DKI--L T--Q--H--- ----V-
A.thal. SAT1-6      -------Q-- ---------V -----TC--- -IT--E---I -------K-- -P-T------ ---L---DN- KT-.DKI--L T--Q--H--- ----V-
A.thal. SAT53       -------QS- ---------V -----SC--- -IT--E---I -S----VK-- -A-T------ ---I----N- RK-.DKI-CL T--Q--YLT- ----V-
A.thal. SAT5        -------QS- ---------V -----SC--- -IT--E---I -S----VK-- -A-T------ ---I----N- RK-.DKI-CL T--Q--YLT- ----V-
A.thal. SAT1        -------QS- ---------V ----SCI--- -IT--E---I -S----VK-- -A-T------ ---I----N- RK-.DKI-CL T--Q--YLT- ----V-
A.thal. SAT106      -------ET- -------E-A -L--CV---- -IS-----M- A---L--K-- -SHSVVA--- -K-IRVM-EQ DPSLAMKHDA TKEFFRHVAD GYKGAQSN
C.vulg. SAT2        -------M-- ---------V ---------- -----E---I ---------- -P-T-E---- ---------- S.QL-DI--- ---------- ------
S.oler.             --------G- -----V---V ---------- --R--D---I ---------- -P-T------ ---I------ S.QNSDV--- ---------- ------

                    401             418
A.thal. SAT106      GPSLSAGDTE KGHTNSTS
```

Figure 3.16 Amino acid sequence alignment of all known bacterial SAT proteins. For comparison to plant SAT proteins, all sequences are compared to that of SAT52 from *A. thaliana*. Gaps in the alignment are shown by dots (.). Identical amino acid residues to the SAT52 sequence are shown by dashes (-). Represented in the alignment are SAT proteins from *A. thaliana* (SAT52; GenBank U30298; Howarth et al. 1997), *Escherichia coli* (M15745; Denk & Böck 1987), *Salmonella typhimurium* (X59594; Sivaprasad et al. 1992), *Haemophilus influenzae* (U32743), *Buchanera aphidicola* (M90644; Lai and Bauman 1992), *Synechococcus* sp. (U23436; Nicholson et al. 1995), *Synechocystis* sp. (D90912), *Bacillus subtilis* (L14580), *Staphylococcus xylosus* (Y07614; Fiegler & Bruckner 1997), *Heliobacter pylori* (U43917), *Mycobacterium tuberculosis* (Z84724) and *Azotobacter chroococcum* (M60090; Evans et al. 1991),


1
A.thal. SAT52 MPPAGELRHQ SPSKEKLSSV TQSDEAEAAS AAISAAAADA EAAGLWTQIK AEARRDAEAE PALASYLYST ILSHSSLERS ISFHLGNKL. .....
E.coli CYSE MSCE -LEIV-NN-- ----TL-DC- -M---FYHA- L-K-EN-GSA L-YM-A---. .....
S.typh. CYSE MPCE -LEIV-KN-- ----AL-DC- -M---FYHA- L-K-EN-GSA L-YM-A---. .....
H.infl. CYSE M TLD.V-QH-R Q--KEL--N- -M---FFH-- --K-QN-GGA L-YL-A---. .....
B.aphidi. CYSE MCSL -ELE--NM-- HK-QKILKK- -I-SNFYQKS --N-KK-SH- L-CI-SD--. .....
Synechococcus srpH MSLSPR -DRT-IRR-W GLDSIVS-L- Q-STDPLPHH LLSDQFYPLP SRESLGLILH GLRSVLFPRH FGDPELSVET THYFI--T-D KTLNLLNEQI
Consensus
101
A.thal. SAT52 ....CSSTL LSTLLYDLFL NTFSSDPSLR NATVADLRAA RVRDPACISF SHCLLNYKGF LAIQAHRVSH KLWIQSRKPL ALALHSRISD
E.coli CYSE ....S-PIM PAIAIREVVE EAYAA--EMI ASAAC-IQ-V -T----VDKY -TP--YL--- H-L--Y-IG- W--N-G-RA- -IF-QNQV-V
S.typh. CYSE ....A-PIM PAIAIREVVE EAYAA--EMI ASAAC-IQ-V -T----VDKY -TP--YL--- H-L--Y-IG- W--NKG-RA- -IF-QNQV-V
H.infl. CYSE ....ANPIM PAIS-REIIE EAYQ-N--II DCAAC-IQ-V -H----VELW -TP--YL--- H---SY-IT- Y--N-N--S- --Y-QNQ--V
B.aphidi. CYSE ....ST-MI SEKDI-NI-N KIYANNI-II -SV-K-IK-- SQ---VVKHY LTP--YL--- H-LE-Y-L-- Y--NIK-YE- SAY-Q----T
Synechococcus srpH RRELWLQHVT QGTPEATPAV --QHASE-TQ AFAQAL-EIK RLLDS-VN-- YLG---AQ-I -EI-FC-P-I T--TF--LA- R-YQLGLPL- -RITAE.V-H
Synechocystis CYSE MSHL.AR
B.subt. CYSE MFF RMLKE-IDTV FDQ---AR-Y FEVI-T-S-L H--W---IA- A-YKRKFYF- -RLISQV.-R
S.xylosus CYSE MV FEQ---ARTT LEVITS-A-V H-VWS-LIA- E-YKKKKYV- -RLISQV.TR
H.pylori CYSE M LDLFYS-ERV LQE---ARNK WEV--L-P-I H-LLCY-LA- A-HKRRFYFI -R--SQL.AR
M.tuberc. CYSE ML T-MRG-I--- -E----APTA LEVIFC-P-V H-VWG--LA- W--QRGARL- -R-AAEF.TR
A.chro. NIFP M--L AQWRE-I-CV FE----ARTT FEV-TT-P-V H--MLY-LA- R--RPNA..- PRPAAVVRAR
Consensus ---DP----- --------G- -A-------H -L-------L
201
A.thal. SAT52 VF.AVDIHPA AKIGKGILLD HATGVVVGET AVIGNNVSIL HHVTLGG... .....TGKAC G.DRHPKIGD GCLIGAGATI LGNVKIGAGA KVGAGSVVLI
E.coli CYSE T-.Q-----A ----R--M-- ----I----- ---E-D---- QS-----... .....---SG -.------RE -VM-----K- ---IEV-R-- -I-------Q
S.typh. CYSE S-.Q-----A ----R--M-- ----I----- ---EDD---- QS-----... .....---TS -.------RE -VM-----K- ---IEV-R-- -I-------Q
H.infl. CYSE A-.D-----A ----H--MF- ----I----- S--E-D---- QG-----... .....---ES -.-----VRE -VM-----K- ---IEV-KY- -I--N----N
B.aphid. CYSE --.S-----A -S--S--M-- ----I-I--G VI-E-D---F -S-----... .....-SNT -KN---I-RK NVT-----K- ---IEV-Q-V ------I--K
Synechococcus srpH SETGI----G -A--GSFFI- -G----I--- C---DR-R-Y QA----AKSF PRDETGALIK -QA---V-E- DVV-Y----L --RITV-R-S TIG-NVWLTR
Synechocystis CYSE F-TGIE---G -Q--Q-VFS- -GM---I--- -IV-DYSL-Y QG-----... .....---ES -.K---TL-E NVVV----KV ---IA--DNV RI-------R
B.subt. CYSE F-TGIE---G -T--RRFFI- -GM---I--- CE-----TVF QG-----... .....---EK -.K---T-K- DA--AT--KV --SITV-E-S -I-------H
S.xylosus CYSE FFTGIE---G -Q--RRLFI- -GM---I--- CR--D--T-Y QG-----... .....---ER -.K---D--- NV--A---KV ---IT-N-NV NI--N----N
H.pylori CYSE FITGIE---G -K--R-LFI- -GM---I--- TE--DD-T-Y -G-----... .....--RFK -.K---TL-N RVVV----KV --AICV-DDV -I--NA---S
M.tuberc. CYSE ILTG-----G -V--ARVFI- --T---I--- -EV-DD-T-Y -G-----... .....S-MVG -.K---TV-- RVI-----KV --PI---EDS RI--NA--VK
A.chro. NIFP LVSN-----G -V--ARFFI- -GAC--I--- -E--RD-TLY -G-----... .....-TGAK -.K---TL-- VV-V----K- --PIT--N- R---N---VQ
Consensus ----VDIHP- A-IG---F-D H---VVIGE- --I------Y --VTLGG--- ---------- G--RHP---- ---I--GA-- LG-I-V----- -IG-------
301
A.thal. SAT52 DVFCRGTAVG NPARLVG... GKEKPTIHDE ECPGESMDHT SFISEWSDYI I
E.coli CYSE P--PHT--A- V---I--K.P DSD-..... ..-SMD--QH FNGINHTFEY GDGI
S.typh. CYSE P--PHT--A- V---I--K.P -SD-..... ..-SMD--QH FNGIHHTFEY GDGI
H.infl. CYSE P--EYA--A- V---I-SQ.D KAA-..... ..-AFD-NQY FIGIDDGMNL NI
B.aphid. CYSE NI-PFV-V-- V--KIIKKIK NSN-NLFQK- KK
Synechococcus srpH S--AGSFISQ AQI-SDNFES -GGI
Synechocystis CYSE ---ADF-V-- V-G-M-HPSG ER.....VNP LEH-KLP-SE GKVIRLLLER -ELLEQQVAT LQQQQSEQAW ESDYRSCSET DREPVLCRLG DREIEEFLGG
B.subt. CYSE ---DFS-V-- I-G-V-VQNG ....KKVRRD LNHQDLP-PV ADRFKSLEQQ -LELKAELED RKERINQK
S.xylosus CYSE S--SYS-V-- I-GHI-KQDG ....RR-GKT FDHRNLP-PI YEQLKELEKQ LEKTRNGEIQ DDYII
H.pylori CYSE -L-TGS-A-- TK-KTITKDR
M.tuberc. CYSE P--PSAVV-- V-GQVIGQSQ ....-SPGGP FDWR.LP-LV GASLDSLLTR VARLEALGGG PQAAGVIRPP EAGIWHGEDF SI
A.chro. NIFP ---EGC-V-- I-GKV-KLRE AGQLNPYGID LDHHLIP-PV GKAIACLLER -DSLEKRVEA GGLVAAAASS TFYEGCNPDN SICETNLRRS APWSSGRPRR
Consensus --P-------
401 416
Synechocystis CYSE TL..... .....
A.chro. NIFP PAHAGDRVSG RAKGSD

[I/V]-X(5)-[I/V]-G-X(2)-[S/T/A/V]-X-[I/V]-[G/A]-[G/S/T/A]-X-[S/A]-X-V-X(3)-[I/V]-X(7)-[G/S] and is also found in other acetyltransferases such as thiogalactosidase acetyltransferase (*Lac*A) from *E. coli* (Hediger et al. 1985), UDP-N-acetylglucosamide acyltransferase (*Lpx*A) of enteric bacteria (Vuorio et al. 1991) and the *nod*L acetyltransferase protein of *Rhizobium meliloti* (Baev & Kondorosi 1992). This motif, known as the *Cys*E/*Lac*A/*Lpx*A/*Nod*L signature, was demonstrated in all of the SAT protein sequences from *A. thaliana*.

A second motif, already demonstrated in the SAT1 protein from *A. thaliana* (MA Roberts, PhD thesis), the FirA protein from *E. coli* (Dicker & Seetharam 1992) and LpxA and D proteins from *E. coli, S. typhimurium, Yersinia enterocolitica* and *Ricketsia ricketsii* (Vuorio et al. 1994), was also demonstrated in the SAT52, SAT53 and SAT106 isoforms. This hexapeptide multiple repeat signature has the consensus [(I/V/L)-G-X(4)]$_5$-(I/V/L). This and similar residue patterns are found between residues 270 and 346 in Figures 3.15 and 3.16. Structurally these motifs form β-sheets.

Another feature of note is a conserved methionine (M) residue at positions 382 and 337 in many of the sequences aligned in Figures 3.15 and 3.16 respectively. This methionine, which is usually followed by an aspartate residue (D), has been shown to be essential for feedback inhibition of SAT by cysteine (Denk & Böck 1987).

3.2.13 Do the deduced amino acid sequences of the A. thaliana SATs have organellar targeting peptides?

The aim of this study is to characterise the SAT gene-family from *A. thaliana* at a molecular level, allowing the role of the enzyme in the various intracellular compartments to be studied. It was therefore of interest to identify the putative cellular location of each of the SAT isoforms encoded by the cDNAs Sat-52, Sat-53 and Sat-106. Sequence analysis of the N-terminal region of the SAT1 protein

from *A. thaliana* has already suggested a plastidic location for this isoform (Roberts & Wray 1996). Amino acid homologies between the plant SAT protein sequences begin at residue 130 in Figure 3.15. The N-terminal residues from this point also represent extensions when aligned with the bacterial CYSE proteins. Organellar proteins translated on cytoplasmic ribosomes are targeted to the mitochondrion and chloroplast by N-terminal amino acid sequences which are cleaved during or after transport across their respective organellar membranes (von Heijne et al. 1989). The N-terminal extensions of the plant SAT proteins are therefore most likely to include any sequence involved in organellar targeting, and were assessed for characteristics of targeting-peptides. Figure 3.17 shows targeting peptide analysis of the N-terminal region of each SAT protein.

Chloroplast targeting-peptides are remarkably variable in their amino acid composition, containing no highly conserved residue motifs. This is also true of mitochondrial targeting-peptides. However, there are several structural characteristics which can be used to determine the putative organellar destinations of nuclear encoded proteins. Typically, chloroplast targeting-peptides contain few tyrosines (Y), or acidic residues such as glutamate (E) and aspartate (D). They are enriched in threonine (T) and serine (S) and show no strong tendencies to form α-helices or β-sheets. The first 5 to 10 residues of chloroplast targeting peptides also lack glycine (G), proline (P) and positively charged residues, and usually have an alanine (A) residue in position 2 next to the initial methionine (M) (von Heijne et al. 1989). Although a general cleavage-site motif, [V/I]-X-[A/C]↓A, has been proposed for chloroplast targeting peptides, many chloroplastically targeted proteins do not exhibit this consensus sequence (Gavel & von Heijne 1990).

Mitochondrial targeting sequences tend to be enriched in alanine (A), leucine (L), serine (S) and arginine (R), are deficient in asparagine (N), glutamine (Q), isoleucine (I) and lysine (K) and form helices, particularly towards their N- and C- termini (von Heijne et al. 1989; von Heijne & Nishikawa 1991; von Heijne 1992; Sjöling & Glaser 1998). Mitochondrial targeting-peptides often exhibit a

cleavage-site resembling either an R-2 (R-X↓[A/S]-[T/S]) or R-3 (R-X-[F/Y]↓[A/S]-[T/S]) consensus (Sjöling & Glaser 1998).

Assessment of the first 45 amino acids of the SAT52 protein revealed an absence of structural characteristics of chloroplast targeting peptides possessing seven acidic residues and only one threonine (T). Of the first ten amino acids four are positively charged and one is a glycine (G) residue. SAT52 is therefore highly unlikely to have chloroplastic location. However, secondary structure predictions using the GCG program PEPTIDESTRUCTURE (Devereux et al. 1984) revealed a helical structure, characteristic of the C-terminal 10-15 residues of mitochondrial targeting-peptides (von Heijne et al. 1989; Sjöling & Glaser 1998), between amino acids 22 and 57 of SAT52. Also, the amino acid residues alanine (A), leucine (L), arginine (R) and serine (S), which are extremely predominant in mitochondrial targeting-peptides, represent 24 of the first 45 amino acids of the SAT52 protein. In comparison, A, L, R and S represent only 6 of the first 45 amino acids of SAT53, and 9 of the first 37 amino acids of SAT106. Residues 55 to 58 of the SAT52 sequence, RDAE, resemble the R-2 mitochondrial precursor-protein cleavage site motif, R-X↓[A/S]-[T/S] (Sjöling & Glaser 1998). The average length of plant mitochondrial targeting peptides is 40 to 50 amino acids (Sjöling & Glaser 1998). It is noted that whilst having many characteristics of mitochondrial targeting-peptides, the SAT52 protein is not longer than the putative cytoplasmic SAT53 protein (see below).

The N-terminal 45 amino acid extension of the SAT53 protein is found to be rich in threonine (T) but also rich in the acidic amino acids, aspartate (D) and glutamate (E), and contains only one serine (S). No tendencies to form α-helices were detected in the same 45 amino acids but regions of β-sheets were predicted by the PEPTIDESTRUCTURE program. No characteristics of targeting peptides are therefore observed in the N-terminal region of the SAT53 protein.

Figure 3.17 Targeting-peptide analysis of the four SAT proteins from *A. thaliana*. N-terminal amino acid sequences, from the point at which homology between the aligned SAT proteins begins, were analysed for the presence of a) chloroplastic and b) mitochondrial targeting-peptide features. Characteristic residues are marked +. Uncharacteristic residues are marked -. In b), regions of residues predicted to form a helix by the GCG secondary structure analysis program PEPTIDESTRUCTURE (Devereux et al. 1984) are indicated by **HHH**.

a)

Chloroplast Targeting-Peptide Characteristics

SAT1 (126 residues. 21% +ves, 14% -ves)

```
  - ++     + + -    +++        +     +   ++      + +    +
MLPVTSRRHFTMSLYMLRSSSPHINHHSFLLPSFVSSKFKHHTLSPPPSPPPPP

       -+   +      +   -++    ---+    -   -   - - ++    + + +
PMAACIDTCRTGKPQISPRDSSKHHDDESGFRYMNYFRYPDRSSFNGTQTKTLH

+     -- - - - --
TRPLLEDLDRDAEVDDVW
```

SAT52 (46 residues. 13% +ves, 20% -ves)

```
 --   -     + + -   ++ + +-- -              - -
MPPAGELRHQSPSKEKLSSVTQSDEAEAASAAISAAAADAEAAGLW
```

SAT53 (49 residues. 16% +ves, 18% -ves)

```
 ++   -+   +   + ---+              +        +   -----
MATCIDTCRTGNTQDDDSRFCCIKNFFRPGFSVNRKIHHTQIEDDDDVW
```

SAT106 (37 residues. 14% +ves, 24% -ves)

```
  ---    -+   -      +   +- -+    -   +-
MNGDELPFESGFEVYACGTHKSEFDSNLLDPRSDPIW
```

b)

Mitochondrial Targeting-Peptide Characteristics

SAT1 (126 residues. 31% +ve, 10% -ve)

```
  +    +++      ++   +++++   --   + ++ +   ++- -    ++    +
MLPVTSRRHFTMSLYMLRSSSPHINHHSFLLPSFVSSKFKHHTLSPPPSPPPPP

   ++ -    +   - --+ + ++-       +   +   -        +++ -   - - +
PMAACIDTCRTGKPQISPRDSSKHHDDESGFRYMNYFRYPDRSSFNGTQTKTLH

  + ++   + + +
TRPLLEDLDRDAEVDDVW
     HHHHHHHHHHHHHHH
```

SAT52 (46 residues. 50% +ve, 10% -ve)

```
    +   ++ -+ +- -+++   -+   + ++ ++-+++++ + ++ +
MPPAGELRHQSPSKEKLSSVTQSDEAEAASAAISAAAADAEAAGLW
    HHHHHH                    HHHHHHHHHHHHHHHHHHHHHHH
```

SAT53 (49 residues. 14% +ve, 20% -ve)

```
  +   -    +   - -    ++    ---   +    +   +--    --
MATCIDTCRTGNTQDDDSRFCCIKNFFRPGFSVNRKIHHTQIEDDDDVW
                                                HHHH
```

SAT106 (37 residues. 24% +ve, 11% -ve)

```
  -    +    +      +     -+    +-++   ++   -
MNGDELPFESGFEVYAKGTHKSEFDSNLLDPRSDPIW
        HHHHHHHHHH
```

The SAT106 protein is the shortest *A. thaliana* SAT isoform being only 27 amino acids longer than the *E. coli* CYSE protein at the N-terminus. These 27 residues also show no significant characteristics of targeting-peptides (Figure 3.17).

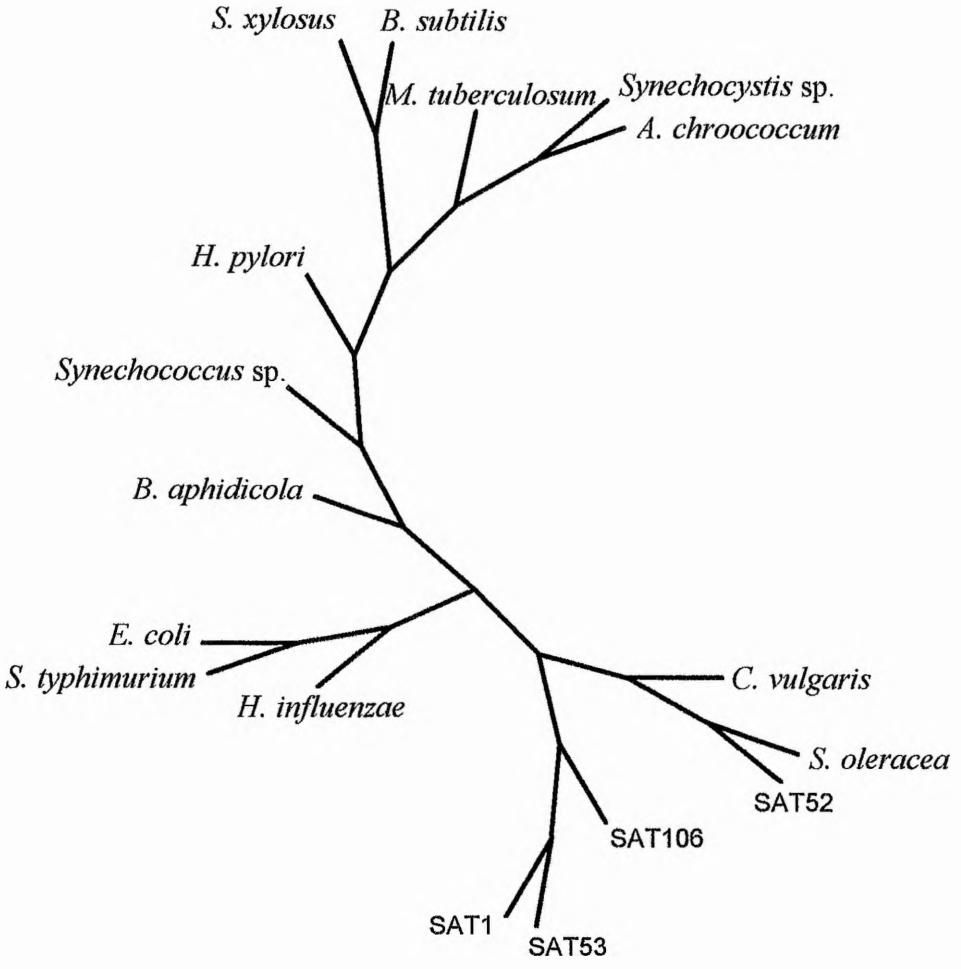
3.2.14 Phylogenetic analysis of available SAT protein sequences

The phylogenetic relationships between all known plant and bacterial SATs were calculated and plotted on a radial phylogenetic tree using the CLUSTAL V alignment (Higgins et al. 1991), and PHYLIP 3.4 (Felsenstein 1993) programs as shown in Figure 3.18. Plant and bacterial SATs are grouped separately on distinct branches of the tree.

Within the bacterial SATs, several species from the same taxonomic subdivisions are found to be phylogenetically grouped together. Bacteria of the Proteobacteria γ-subdivision, which includes *E. coli*, *S. typhimurium* and *Haemophilus influenzae*, are grouped together on a separate branch of the tree. Members of the Firmicutes, *Bacillus subtilis* and *Staphylococcus xylosus*, are also grouped together.

Distinct groupings are also observed within the plant SAT sequences. SAT1 and SAT53 are positioned together indicating a strong phylogenetic relationship between these two isoforms. SAT52 is less closely related to the SAT1 and SAT53 proteins and is phylogenetically grouped with the putatively cytoplasmic SAT2 protein from *C. vulgaris* (Saito et al. 1995) and a putatively mitochondrial isoform of SAT cloned from spinach (GenBank accession D88529). SAT106 is more distantly related to the other known plant SAT proteins being positioned between the bacterial and plant enzymes on the phylogenetic tree shown in Figure 3.18.

Figure 3.18 Radial tree showing the phylogenetic relationships between all known deduced SAT protein sequences. Bacterial SATs included are those from *Azotobacter chroococcum* (Genbank M60090; Evans et al. 1991), *Bacillus subtilis* (GenBank L14580), *Buchanera aphidicola* (Genbank M90644; Lai and Bauman 1992), *Escherichia coli* (Genbank M15745; Denk & Böck 1987), *Haemophilus influenzae* (GenBank U32743), *Heliobacter pylori* (GenBank U43917), *Mycobacterium tuberculosis* (GenBank Z84724), *Salmonella typhimurium* (GenBank X59594; Sivaprasad et al. 1992), *Staphylococcus xylosus* (GenBank Y07614; Fiegler & Bruckner 1997), *Synechococcus* sp. (GenBank U23436; Nicholson et al. 1995) and *Synechocystis* sp. (GenBank D90912). The deduced plant SAT sequences represented are SAT1 (GenBank U22964; Roberts & Wray 1996), SAT52 (GenBank U30298; Howarth et al. 1997), SAT53 (this thesis) and SAT106 (this thesis) from *A. thaliana*, SAT2 from *Citrullus vulgaris* (D85624; Saito et al. 1997) and SAT56 from *Spinacea oleracea* (D88530; Saito et al. 1996 unpublished). Alignments were performed using the CLUSTALV program (Higgins et al. 1991) and the tree was drawn using the PHYLIP 3.4 program (Felsenstein 1993).


S. xylosus
B. subtilis
M. tuberculosum
Synechocystis sp.
A. chroococcum
H. pylori
Synechococcus sp.
B. aphidicola
E. coli
S. typhimurium
H. influenzae
C. vulgaris
S. oleracea
SAT52
SAT106
SAT1
SAT53

3.3 Discussion

3.3.1 The serine acetyltransferase gene-family

The work detailed in this chapter has shown that SAT activity in *A. thaliana* is conferred by a small gene-family consisting of at least four members. cDNAs representing three novel members of this gene-family, Sat-52, Sat-53 and Sat-106, have been isolated and characterised in addition to the single family member, Sat-1, previously cloned in this laboratory (Roberts & Wray 1996).

Southern analysis of the four SAT genes indicates that each is represented by a single copy in the *A. thaliana* genome. As at least three, different sized, mRNA molecules have been shown to encode the SAT52 protein by primer extension analysis, it can be deduced that all are transcribed from the same gene. The *Sat-52* promoter sequence (deposited in the AGI database after the primer extension experiment described in section 3.2.8 was carried out) is found to have multiple putative transcription start sites (Figure 3.10). Multiple transcription start sites are also suggested from the *Sat-106* genomic sequence (Figure 3.12) by several TATA and CAAT-box motifs in the promoter region. Two transcription start sites were detected in the *Sat-53* promoter sequence (Figure 3.11).

Two gene sequences of the *Sat-53*-type were published by other workers in the duration of this PhD as mentioned in section 3.2.12. The *sat5* sequence (Ruffet et al. 1995) shows 100% nucleotide homology to Sat-53, whilst SAT1 (Murillo et al. 1995) differs in 13 nucleotides from the Sat-53 and *sat5* sequences. As Southern blots suggest that *Sat-53* is a single copy gene, sequencing errors are likely to be the cause of the differences in the SAT1 sequence.

3.3.2 SAT protein structure and function

Nucleotide sequence alignments of the four SAT gene-family members from *A. thaliana* (Figure 3.5) show regions of high homology towards the 3' ends. Corresponding homologies are observed in the C-terminal regions of the conceptually translated SAT proteins. Amino acid sequence alignments of all known SAT proteins from plants and bacteria (Figures 3.15 and 3.16) reveal several highly conserved residues and motifs which can be presumed to be essential to the function or structure of the enzyme.

The *Cys*E/*Lac*A/*Lpx*A/*Nod*L signature, between amino acids 322 and 358 of the sequences in Figure 3.15, is found in all the plant and many bacterial SAT proteins as well as a range of other acetyl and acyltransferases such as thiogalactosidase acetyltransferase (*LacA*) (Hediger et al. 1985; Tenigkeit & Matzura 1991), UDP-N-acetylglucosamide acyltransferase (*LpxA*) (Vuorio et al. 1991) and *NodL* acetyltransferase (Baev & Kondorosi 1992). The *Cys*E/*Lac*A/*Lpx*A/*Nod*L signature coincides with a region of the *NIFP* protein from *Azotobacter chroococcum* which was shown to encode an acetyl CoA binding site (Evans et al. 1991). The *Cys*E/*Lac*A/*Lpx*A/*Nod*L signature is therefore likely to represent an essential acetyl CoA binding domain of the SAT proteins.

A second motif detected in the plant SAT proteins, with a general consensus of (I/V/L)-G-X(4), was found extensively throughout the C-terminal 130 amino acids of Figure 3.15. This motif is thought to be involved in dimerisation, heterodimerisation or oligomerisation with other protein molecules (Dicker & Seetharam 1992). The β-sheets formed by these short amino acid sequences interact forming associations with other protein molecules. In the case of SAT these associations presumably occur between other SAT molecules and with *O*ASTL in the formation of the cysteine synthase complex. The cysteine synthase complex consists of a 4 SAT/4 *O*ASTL heterooctamer as described in section 1.3.2. In recent work studying the molecular associations of the cysteine

synthase complex in *A. thaliana* (Bogdanova & Hell 1997), utilising the yeast two-hybrid system (Fields & Song 1989), the SAT53-type isoform failed to complex with *O*ASTL when 50 amino acids were cleaved from the C-terminus of the protein, or when 213 amino acids were cleaved from the N-terminus. Smaller deletions from the N-terminus were not observed to have an effect on cysteine synthase complex formation. The region of the [(I/V/L)-G-X(4)]$_5$-(I/V/L) motif in plant SAT proteins coincides with the region Bogdanova and Hell (1997) found to be essential for cysteine synthase formation. This supports the hypothesis that the motif is involved in protein-protein interactions within the cysteine synthase complex.

The methionine residue at position 382 in Figure 3.15, conserved in all plant and several bacterial SAT sequences, is essential for feedback inhibition of SAT by cysteine. Feedback sensitivity of SAT by cysteine was reduced ten-fold by the localised mutagenesis of this methionine residue to an isoleucine residue in *E. coli* (Denk & Böck 1987). The reduction in cysteine sensitivity caused by the mutation of methionine 382 resulted in a large over-production and excretion of cysteine by the mutant strain. The conserved nature of the methionine 382 residue suggests a feedback control of SAT by cysteine in all proteins containing this residue. Proteins possessing the methionine 382 residue include all known plant SAT isoforms (except SAT106 from *A. thaliana*) and those of the γ-subdivision of the Proteobacteria, *E. coli*, *S. typhimurium* and *H. influenzae*. Biochemical evidence of allosteric feedback inhibition of SAT by cysteine is well documented for many plant and bacterial SAT enzymes (Smith & Thompson 1971; Brunold & Suter 1982; Denk & Böck 1987: Kredich 1987; Saito et al. 1995). The absence of the conserved methionine residue in the SAT106 sequence suggests a differential regulation of the SAT isoforms from *A. thaliana* by cysteine. As described in Chapter 6, the effects of cysteine on the different isoforms can be tested using recombinant proteins and SAT enzyme assays similar to those outlined in section 2.15.3, but incorporating a range of cysteine concentrations.

3.3.3 Cellular targeting of SAT proteins in A. thaliana

Cellular localisation studies have detected SAT activity in the chloroplast, cytoplasm and mitochondrion of plants (Smith & Thompson 1969; Smith 1972; Brunold & Suter 1982; Droux et al. 1992; Ruffet et al. 1994, 1995). Analysis of deduced amino acid sequences of the four SAT cDNAs from *A. thaliana* indicates that the SAT1 and SAT52 proteins may be targeted to the chloroplast and mitochondrion respectively by N-terminal organelle targeting-peptides.

SAT106, which is the shortest of the four SAT proteins at the N-terminus, is likely to represent a cytoplasmic isoform of the enzyme and shows none of the characteristics of an organelle-targeted protein. SAT53 does not have any of the typical features of targeting peptides either, and may represent a second cytoplasmic SAT isoform. However, it is noted that SAT53 is 2 amino acids longer than the putatively mitochondrial SAT52 protein. Therefore, SAT53 may still possess an N-terminal targeting peptide. The high proportion of acidic residues and deficiency of alanine (A), leucine (L), arginine (R) or serine (S) in the N-terminal region of the SAT53 protein make it an unlikely candidate for targeting to either the chloroplast or mitochondrion. However, targeting-peptides have very few highly conserved features. SAT53 may have a targeting-peptide which is not detected by the characteristics outlined in section 3.2.13. Consequently further experimental work will be necessary to unequivocally determine the cellular location of the four SAT isoforms from *A. thaliana.*

Cellular localisation of SAT isoforms could be determined by fusing the cDNA encoding the N-terminal region of each SAT protein to a suitable reporter gene, such as GFP or Luciferase, in a transgenic expression vector. Expressing these SAT/reporter gene fusion proteins in *A. thaliana* cells would allow the cellular location of each of the SAT isoforms to be visualised. Determination of the

cellular localisation of each of the SAT isoforms will be fundamental to the interpretation of studies on the role of the SAT gene-family in plant metabolism.

3.3.4 SAT gene-specific probes will be used to study expression in A. thaliana tissues

The design of DNA probes specific to each of the SAT genes from *A. thaliana* directly led to the detection of the Sat-106 cDNA as a fourth member of the gene-family. The availability of gene-specific probes also allowed the study of SAT gene expression in different tissues and cell types of *A. thaliana*, under various nutritional and stress conditions. The results of these experiments are presented in Chapter 4.

Chapter 4: Expression of the Serine Acetyltransferase Gene-Family in *Arabidopsis thaliana* Tissues

4.1 Introduction

4.1.1 Background

The production of four DNA probes which specifically hybridised to each of the four known SAT cDNAs from *A. thaliana*, even under low stringency washing conditions, was described in sections 3.2.5 and 3.2.7. These gene-specific probes were used to study the expression of the SAT gene-family in *A. thaliana* tissues. The results of these expression studies are presented in this chapter. Work presented in this chapter will provide the basis for further studies on the expression of the various SAT gene-family members, increasing our understanding of their roles in cysteine biosynthesis. As *O*AS supply is known to be a major regulating factor in cysteine biosynthesis (Saito et al. 1994a), determining the expression patterns of SAT isogenes in various cell types and under different nutritional and stress conditions may determine future approaches to the manipulation of cysteine biosynthesis in plants. Certain SAT genes may be pinpointed as candidates for overexpression studies in which levels of cysteine biosynthesis could be increased. This may allow plants requiring increased biosynthesis of reduced-sulphur compounds for adaptation to environmental conditions (such salt stress and osmotic stress; see discussion to this chapter) to benefit from increased levels of cysteine biosynthesis.

As outlined in section 3.2.6, the Sat-106 cDNA was detected as a fourth representative of the SAT gene-family by its inability to hybridise with the probes specific to the Sat-1, Sat-52 or Sat-53 cDNAs. As the Sat-106 clone was isolated and characterised towards the end of this study, the experiments described here do not include results for the abundance of the *Sat-106* mRNA transcript. No hybridisation was observed when the Sat-106 gene-specific probe (described in section 3.2.7; Figure 3.6b) was used in northern blots

against RNA extracted from *A. thaliana* tissues. This suggests that the *Sat-106* gene may be expressed at an extremely low level.

4.1.2 Techniques employed to study expression of the SAT gene-family in A. thaliana tissues

Two techniques were used in this chapter to study SAT gene expression. The first, and most widely employed technique in mRNA transcript studies, was northern blotting/hybridisation. As described in section 2.12, northern blotting is performed by separating RNA extracts by electrophoresis through agarose gels before being 'blotted', by capillary transfer, on to nylon membranes. Once fixed to the nylon, by UV light or baking, the membrane is then incubated with a specific ^{32}P-radiolabelled DNA probe which hybridises to the mRNA being studied. After washing excess and non-specifically bound probe from the membrane, at a predetermined salt concentration and temperature depending on the stringency required (see section 2.13.2), bands of mRNA homologous to the DNA probe can be detected.

Analysis of hybridising bands on northern blots in this chapter is performed either photographically or using a bioimaging analyser (Fuji Photographic Film Co. Ltd, Japan). For the photographic method, the filter is placed against photographic film which, when developed, reveals bands of hybridising mRNA on an autoradiogram. The intensity of these bands is proportional to the abundance of the mRNA transcript in the RNA sample originally separated through the agarose gel. Differences between mRNA transcript levels in separate samples can be deduced from relative band intensities on the autoradiogram, providing equal amounts of RNA were originally fixed to the filter. The relative amounts of each RNA sample fixed to a nylon filter can be determined by hybridising with a probe homologous to a constitutively expressed gene. Several constitutive probes are commonly used

to demonstrate equal loading of RNA on northern blots, such as actin, polyubiquitin, elongation factor I, tubulin, cyclophilin and 18S ribosomal DNA (which hybridises to 18S ribosomal RNA). An 18S ribosomal DNA probe was used in all the expression studies presented in this chapter.

Analysing hybridising-bands on northern blots by the use of a bioimaging analyser is much more accurate than by the photographic method. Whilst estimation of band intensity on autoradiograms is performed by eye and comparisons of relative band-intensity are purely qualitative, the bioimaging analyser accurately measures the phosphorescent light emitted from a phosphorimaging screen which has been exposed to a radioactive source. When the phosphorimaging screen is exposed to northern blots, hybridised with a ^{32}P-radiolabelled probe, radioactivity emitted from each hybridising-band can be accurately quantified.

The second technique used in this chapter to study expression of the SAT gene-family was *in situ* hybridisation. *In situ* hybridisation allows the detection of specific mRNA molecules in the cells of a tissue cross-section. Pieces of tissue are fixed in formaldehyde, embedded in paraffin wax and sectioned on a microtome before mounting on microscope slides. The tissue sections are then pretreated with Pronase (Type XIV protease from *Streptomyces griseus*), to degrade cell proteins which may inhibit probe binding, and acetic anhydride, which acetylates positively charged residues in the tissue and on the slide preventing non-specific probe binding. Tissue sections are hybridised with a [α-^{35}S]dUTP-radiolabelled RNA probe (riboprobe). Riboprobes transcribed from Sat-1, Sat-52 and Sat-53 gene-specific probe DNA were used in this chapter. After washing away excess and unbound probe, the cellular location of the specific mRNA is visualised by exposing the slides to liquid photographic emulsion. During the exposure period, silver grains are deposited in the cells to which the radiolabelled probe bound. These silver grains appear as bright, white dots when the mounted

tissue sections are viewed under dark-field illumination on a phase-contrast microscope.

4.1.3 SAT expression studies in A. thaliana

Four mRNA expression studies are described in this chapter, each using the SAT gene-specific probes described in section 3.2.5. The first study determines the distribution of each SAT mRNA transcript in the major organs of the *A. thaliana* plant. This was carried out to reveal any organ-specific expression of the SAT genes and to indicate whether certain SAT isoforms have roles specific to distinct tissue types. The second study utilises *in situ* hybridisation techniques to determine the spatial distribution of SAT mRNA transcripts at a cellular level within root, leaf and stem tissue. A third study uses northern hybridisations to assess the effects of sulphate and/or nitrate availability on the transcript abundance of each SAT mRNA. Finally, results are presented from a collaborative study on the effects of salt (NaCl) and osmotic-stress on the expression of genes encoding putatively cytoplasmic isoforms of SAT (*Sat-53*) and *O*ASTL (*Atcys-3A*). Expression of the *Sat-53* and *Atcys-3A* genes in plants treated with abscisic acid (ABA), a signalling compound often involved in plants' response to environmental stress (Giraudat et al. 1994), was also studied. The effects of salt, osmotic-stress and ABA treatments on *Sat-53* transcript abundance were performed in St. Andrews, and the results are presented in sections 4.2.4 and 4.2.5. Expression of the *O*ASTL mRNA *Atcys-3A* under the same conditions was performed by C. Gotor and co-workers at the University of Seville, Spain. The results of the *Atcys-3A* experiments are shown in Figures 4.6b and 4.7b for comparison with the *Sat-53* data.

4.2 Results

4.2.1 Organ-specific expression of SAT in A. thaliana

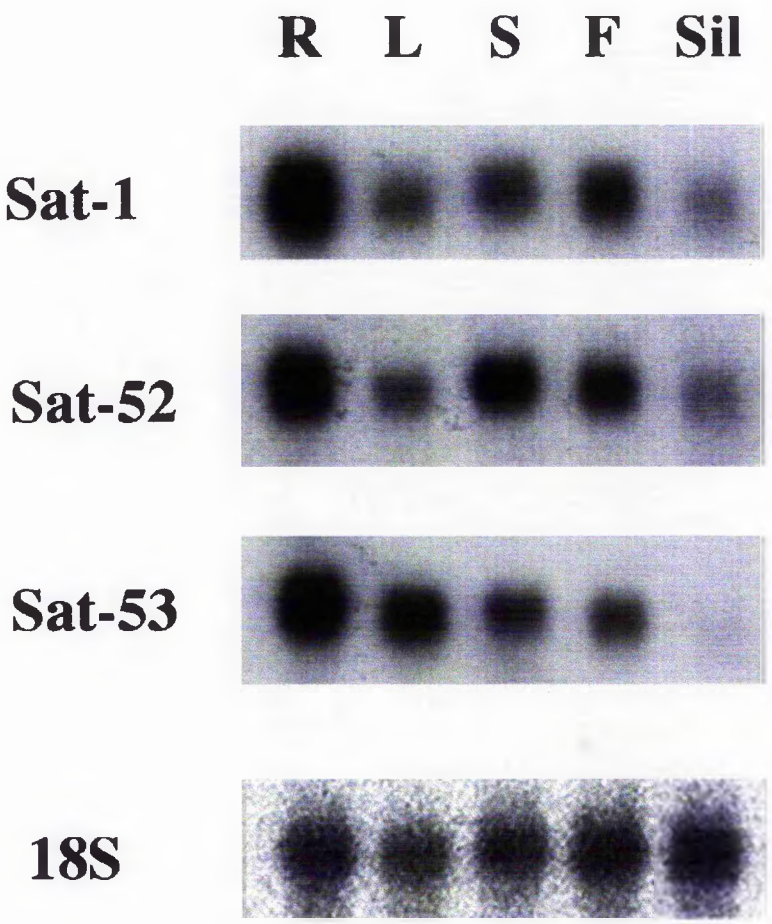
This study compared the abundance of SAT mRNA transcripts in root, rosette leaf, stem, flower and silique tissue of *A. thaliana* (see Figure 2.1 for *A. thaliana* anatomy). Figure 4.1 shows the relative abundance of the SAT mRNA transcripts in each organ. The 18S rDNA probe indicates that the lanes on the filter contain equal amounts of RNA.

Sat-1 transcript levels are shown to be highest in root tissue and approximately a quarter of this level in flowers. Transcript abundance in leaf and stem tissues is approximately equal, the hybridisation bands to RNA from these organs being approximately a tenth as intense as in the root. The *Sat-1* transcript is shown to be least abundant in silique tissue, the intensity of the hybridisation band appearing to be about half as intense as those in leaf and stem tissues.

Sat-52 mRNA transcript is most abundant in stem and root tissues showing approximately equally intense hybridising bands to RNA extracted from these organs. The next highest level of *Sat-52* transcript is found in flower tissues followed by levels in leaf tissue. The *Sat-52* hybridisation signal to RNA extracted from leaf tissue was approximately a fifth as intense as observed in the root. Again, a weak hybridisation of the Sat-52 specific probe to RNA from siliques was observed.

Sat-53 transcript was also found to be most abundant in root tissues. Hybridisation to leaf RNA indicated *Sat-53* transcript abundance approximately half of that in observed in root tissue. Abundance in stem and flower tissues was approximately equal and appeared to be half the level found in leaves. Hybridisation of the Sat-53 probe to silique RNA was barely detectable.

Figure 4.1 Organ-specific expression of the *Sat-1*, *Sat-52* and *Sat-53* mRNAs. Total RNA was extracted from root (R), rosette leaf (L), stem (S), flower (F) and silique (Sil) tissue of compost grown *A. thaliana* plants and subjected to northern blotting. Hybridisations were performed using Sat-1, Sat-52 and Sat-53 gene-specific probes. An 18S rDNA constitutive probe was used to demonstrate equal loading of RNA from each tissue on the filter.


R L S F Sil
Sat-1
Sat-52
Sat-53
18S

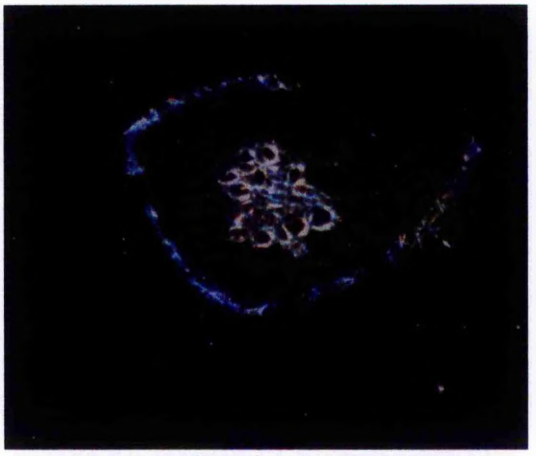
4.2.2 In situ hybridisation of Sat-52 and Sat-53 in A. thaliana

Northern hybridisation indicated different levels of SAT mRNA transcript in the various organs of *A. thaliana* plants (section 4.2.1). The transcription of SAT mRNAs to different extents in the different organs of the plant may be related to the role of the isoform they encode in cysteine biosynthesis in those tissues. Each of the organs from which RNA was extracted for the northern hybridisation studies is made up of many cell-types with a variety of structural and functional roles. *In situ* hybridisation techniques were employed to determine the spatial distribution of SAT transcript at a cellular level within root, leaf and stem tissues grown as outlined in section 2.2.2.

The SAT gene-specific probes used in the northern hybridisation studies were also used in the preparation of riboprobes for *in situ* hybridisation. The gene-specific probes were amplified by PCR and cloned into the pGEM-T (Promega UK Ltd) or pBluescript (Stratagene Ltd, UK) vectors using primers outlined in Table 3.3. The pGEM and pBluescript vectors are designed for riboprobe preparation, possessing M13 oligonucleotide binding sites for insert amplification, and T7, T3 and/or SP6 RNA polymerase transcription initiation sites for sense or antisense transcription of the insert. Antisense riboprobes, complementary to the SAT mRNAs, and sense probes, which will not hybridise to any mRNAs and therefore can be used as controls to determine levels of non-specific background hybridisation signal, were transcribed for probing mounted tissue sections. Results observed following hybridisation with the sense probe (Figures 4.2a-c) indicate the level of non-specific background signal which can be expected in the antisense probe hybridisations. The level of background signal observed on the control slides is taken into account when describing the results in this section. A probe specific to the *O*ASTL gene *Atcys-3A* (Barroso et al. 1995) was used as a positive control. This cDNA,

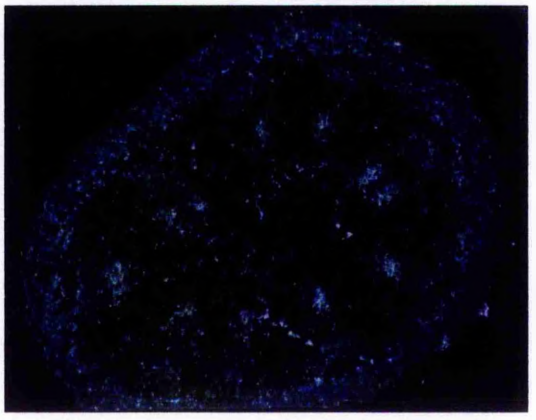
Figure 4.2 Control *in situ* hybridisations. a), b) and c) show hybridisation of a sense riboprobe to leaf (including a leaf trichome), root and stem tissue cross-sections respectively. As the control sense probe does not hybridise to any mRNA, only non-specific background signal is observed. See Figures 4.2 and 4.3 for light-field illumination of similar tissues. d) shows an *in situ* hybridisation against a leaf tissue cross-section using the *A. thaliana* OASTL *Atcys-3A* probe, under light-field (left) and dark-field (right) illumination. Hybridisation signal is represented by silver grains which appear as bright white dots under dark-field illumination.The strong hybidisation signal in the trichome cell reproduces the results of Gotor et al. (1997). Figure labels: l, leaf blade; t, trichome; v, vascular tissue. Solid scale-bar represents approximately 1 mm.

a)

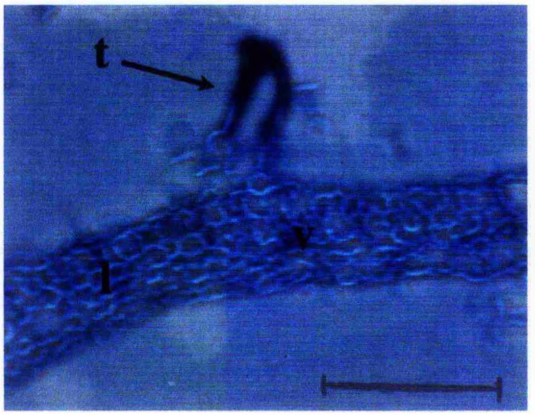


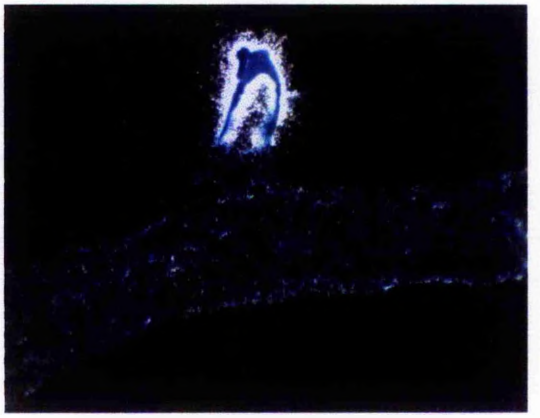
b)

c)



d)





which encodes a putatively cytoplasmic form of *O*ASTL, has recently been the subject of an *in situ* hybridisation study. Extremely high levels of *Atcys-3A* mRNA expression were detected in leaf and stem trichomes (Gotor et al. 1997). This was also found when the tissue sections prepared for this study were hybridised with the *Atcys-3A* riboprobe (Figure 4.2d).

4.2.2.1 Sat-1 in situ hybridisations were unsuccessful

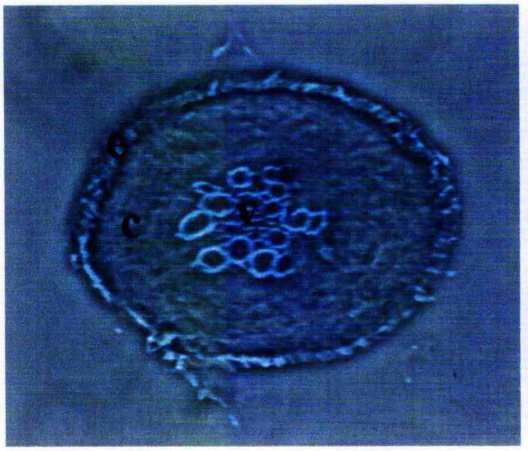
Experiments with the *Sat-1*-specific riboprobe were unsuccessful with no hybridisation observed on any slides. Several errors in the experimental procedure may have caused the failure of the Sat-1 probe to produce a hybridisation signal. Ribonuclease contamination may have resulted in probe-denaturation at some point during preparation. Ribonuclease contamination of the mounted tissue sections may, similarly, have denatured the mRNA within the plant cells. Alternatively, poor coverage of the slide by the hybridisation solution may have prevented the radiolabelled riboprobe accessing the plant tissue sections. The lack of non-specific background signal on Sat-1 probed slides suggests that errors in riboprobe preparation are most likely to have caused the failure of the these hybridisations.

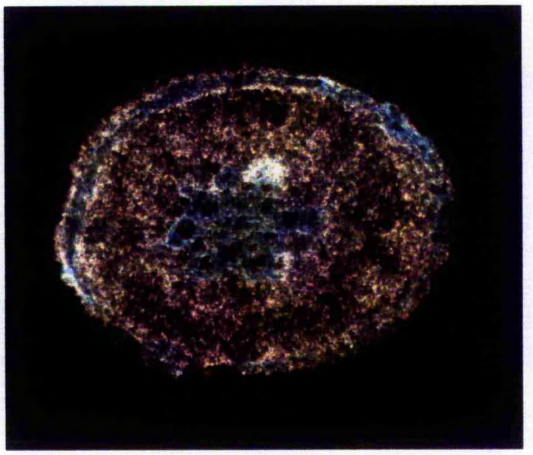
4.2.2.2 In situ hybridisation using the Sat-52-specific probe

In situ hybridisation of the *Sat-52*-specific probe against leaf tissue revealed signal above background levels in all cell types indicating transcription of *Sat-52* throughout the leaf (Figures 4.3b and 4.3c). As had previously been reported for *Atcys-3A*, the *Sat-52* transcript was expressed at extremely high levels in the leaf trichomes (Figure 4.3b). The silver grains deposited on these structures, during photographic development of the hybridised tissue, were visible even during light-field microscopic inspection

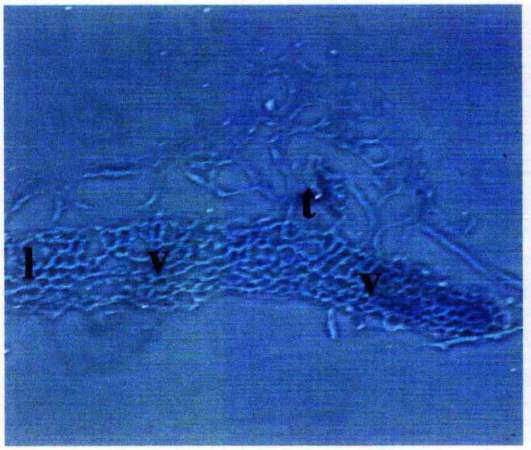
Figure 4.3 *In situ* hybridisation showing distribution of *Sat-52* mRNA in cross-sections of a) the root, b) the leaf blade and trichome, c) the leaf midrib and d) the stem of *A. thaliana*. Each section is shown under light- (left) and dark- (right) field illumination allowing visualisation of the tissue structure and hybridisation respectively. Hybridisation signal is represented by silver grains which appear as bright white dots under dark-field illumination. Figure labels: l, leaf blade; t, trichome; v, vascular tissue; e, epidermis; c, cortex. Solid scale-bars represent approximately 1 mm.

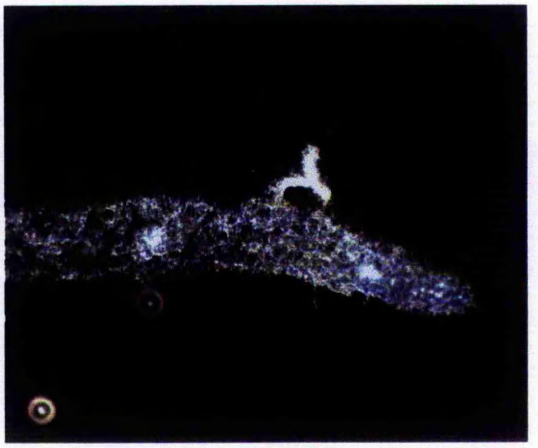
a)



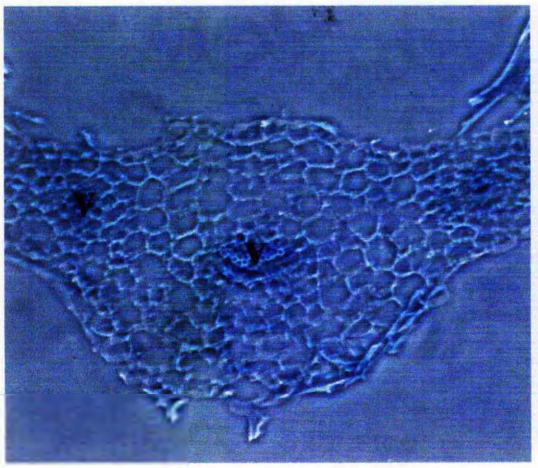


b)

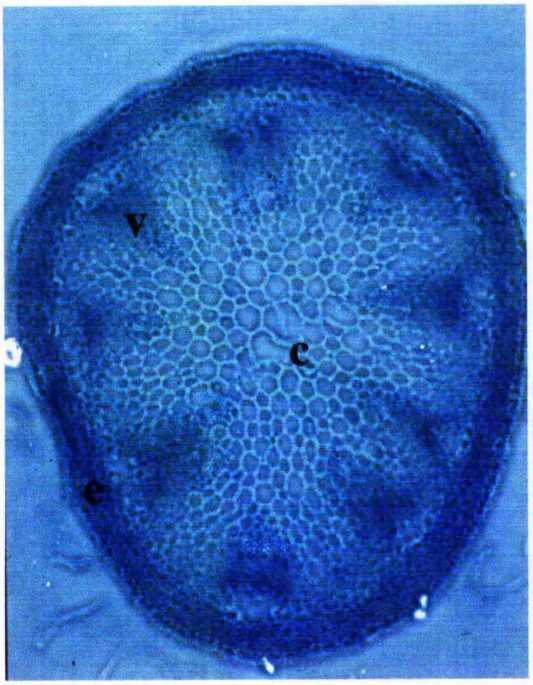


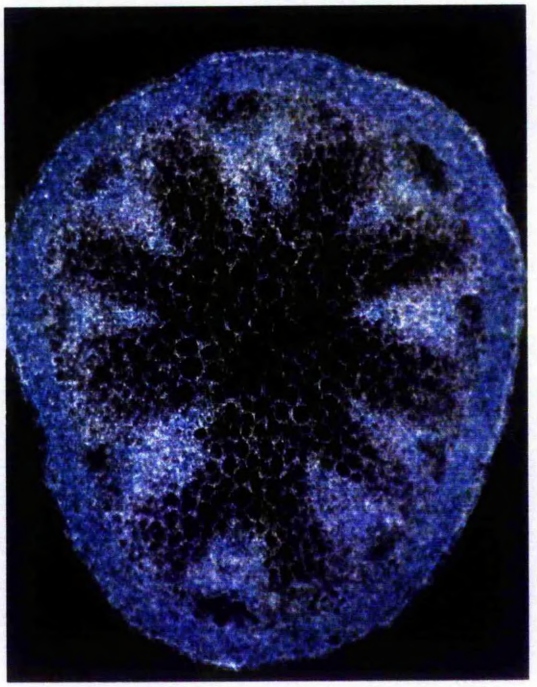


c)



d)





of the slides. High levels of *Sat-52* mRNA were also detected in the cells of the leaf vascular tissues as shown in Figures 4.3b and 4.3c.

Root sections hybridised with the *Sat-52*-specific riboprobe revealed an even distribution of *Sat-52* transcript throughout the epidermal and cortical cells. In comparison, certain cells of the vascular bundle of the root tissue contained higher levels of *Sat-52* transcript (Figure 4.3a).

Stem tissues hybridised with the *Sat-52*-specific riboprobe showed low levels of transcript abundance throughout the cortex. Higher levels of *Sat-52* transcript were detected in the epidermis and also in the cells of the vascular bundles (Figure 4.3d), as was observed in root and leaf.

4.2.2.3 In situ hybridisation using the Sat-53-specific probe

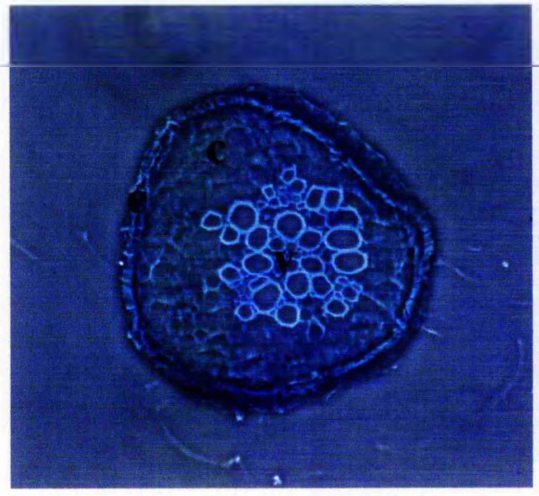
The *Sat-53*-specific probe detected extremely high transcript abundance in the leaf trichomes (Figure 4.4b), as was the case with *Sat-52* (Figure 4.3b) and *Atcys-3A* (Gotor et al. 1997 and Figure 4.2d). No signal above background was detected in the rest of the leaf indicating that the *Sat-53* gene may be exclusively expressed in trichomes.

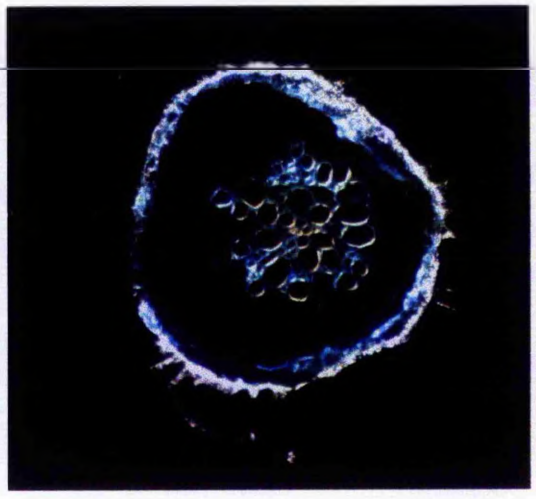
The *Sat-53*-specific riboprobe detected *Sat-53* transcript largely in the root epidermis (Figure 4.4a). However, the epidermis and vascular bundle also appear highly illuminated due to refraction of light by these structures, rather than due to the hybridisation signal. Some refraction of light can also be seen from the epidermis and vascular cells of the control, sense probed, root section in Figure 4.2a. The specific hybridisation signal, caused by deposited silver grains, appears as bright white dots under dark-field illumination. Any other illuminated areas caused by light refraction must be ignored during analysis.

The *Sat-53* transcript in stem tissue cannot be detected above background levels as shown in Figure 4.4c. Refraction of light by the

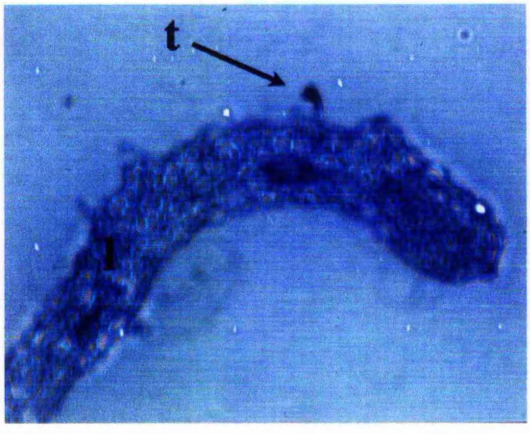
Figure 4.4 *In situ* hybridisation showing distribution of *Sat-53* mRNA in cross-sections of a) the root, b) the leaf blade and trichome and c) the stem of *A. thaliana*. Each section is shown under light- (left) and dark- (right) field illumination allowing visualisation of the tissue structure and hybridisation respectively. Hybridisation signal is represented by silver grains which appear as bright white dots under dark-field illumination. Figure labels: l, leaf blade; t, trichome; v, vascular tissue; e, epidermis; c, cortex. Solid scale-bars represent approximately 1 mm.

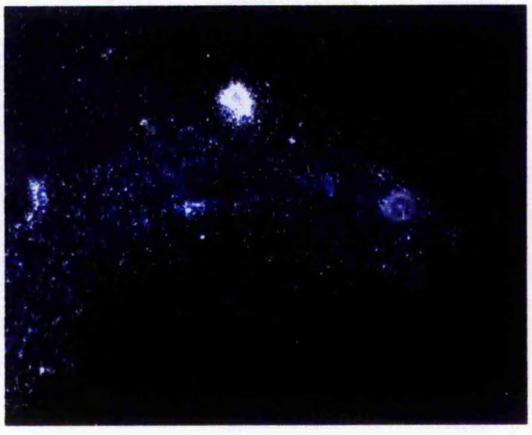
a)



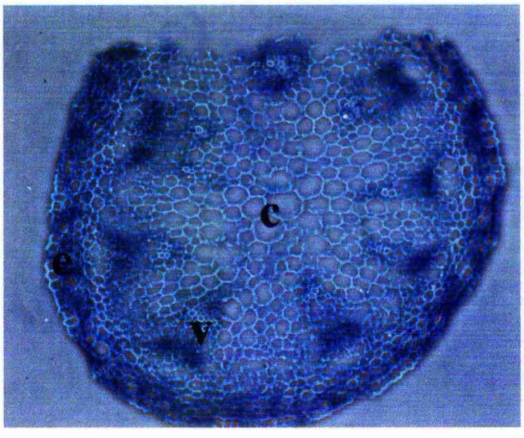


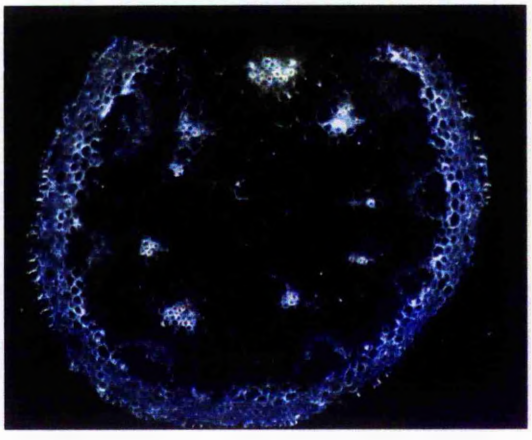
b)





c)





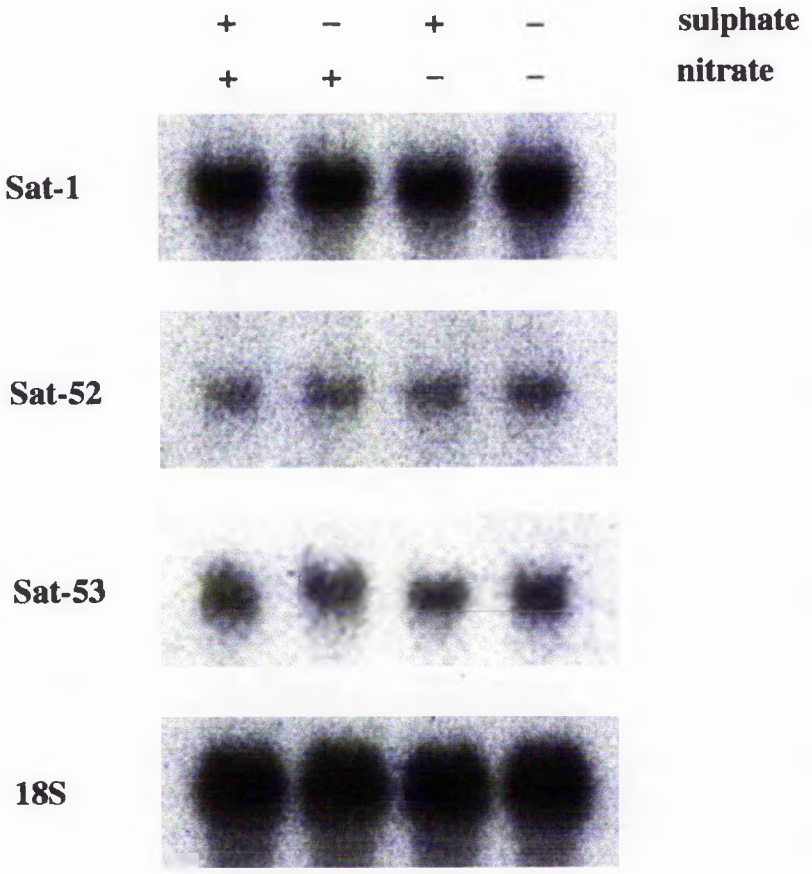
epidermal and some vascular cells under dark-field illumination gives the initial impression of hybridisation. Close examination clearly shows no hybridisation signal is present in these regions. *Sat-53* transcript was detected in stem tissue by the northern hybridisation described in section 4.3.1. Therefore, a longer exposure time may have been required to detect the *Sat-53* transcript in stem cells by *in situ* hybridisation

4.2.3 Expression of SAT gene-family under sulphate and nitrate deficiency

As previously mentioned in section 1.3.1, SAT and *O*ASTL form a complex which combines sulphide and *L*-serine forming *L*-cysteine in higher plants. This effectively represents the point of convergence between sulphate and nitrate assimilation. *O*AS, the product of SAT activity, also has a role in the regulation of reductive sulphate assimilation and cysteine biosynthesis in plants (Neuenschwander et al. 1991; Saito et al. 1994a), ensuring a balanced supply of sulphur-containing amino acids is available for protein synthesis. It was of interest to determine whether sulphate or nitrogen nutrition had any regulatory effect on the expression of the SAT gene-family.

A. thaliana plants were grown in sterile Petridishes containing MS medium with 5% sucrose, solidified with 1% agar. Three weeks after germination plants were transferred to four different media, 1) MS -sulphate (S) + nitrate (N), 2) MS +S -N, 3) MS -S -N and 4) MS +S +N as a control. Sulphate salts in the MS -S, and nitrate salts in the MS -N media, were replaced with chlorides. Agar used in all cultures had been washed, as outlined in section 2.4.2, to remove contaminating salts. Four days after transfer to these media, RNA was extracted from approximately 80 whole plants and subjected to northern blotting. The blotted membrane was sequentially hybridised with Sat-1, Sat-52 and Sat-53 gene-specific probes and an 18S ribosomal DNA constitutive probe as shown in Figure 4.5. No

Figure 4.5 Transcript abundance of the SAT gene-family from *A. thaliana* in response to sulphate and nitrate nutrition. Total RNA was extracted from whole, *in vitro* grown plants for northern blotting/hybridisation. Plants were grown on complete MS medium for three weeks before being subject to 4 days of sulphate and/or nitrate starvation on modified MS media. + Sulphate + Nitrate plants were transferred to complete MS medium for the same four day period. In modified media, sulphate and/or nitrate salts were replaced with chlorides as required. See section 2.4.1 for MS medium composition. An 18S rDNA constitutive probe was used to demonstrate equal loading of RNA in each lane on the filter.


+ − + − sulphate
+ + − − nitrate
Sat-1
Sat-52
Sat-53
18S

change in abundance, compared to control plants, was observed in any of the SAT transcripts in response to 4 days of sulphate and/or nitrate starvation.

4.2.4 The effect of salt stress on Sat-53 expression

The high levels of *Sat-52*, *Sat-53* and *Atcys-3A* transcript detected in *A. thaliana* trichomes by *in situ* hybridisation (section 4.3.2) suggests a specialist requirement for cysteine biosynthesis in these structures. As trichome involvement in stress response and stress tolerance has been widely reported (Salt et al. 1995; Foley & Singh 1994; Parra et al. 1996; Kononowicz et al. 1992; Yamagushi-Shinozaki & Shinozaki 1993) work was carried out to study the expression of *Atcys-3A* transcript in response to a variety of stress conditions (Gotor C, pers. comm., University of Seville). Salt (sodium chloride) and osmotic (conferred by mannitol) stress were found to increase *Atcys-3A* transcript abundance by up to 300% in stem and leaf tissue with low levels of increase also observed in root (Figure 4.6b). ABA, commonly identified as the signalling compound involved in plant responses to environmental stress (Giraudat et al. 1994), increased *Atcys-3A* transcript abundance to a similar magnitude as salt stress, possibly implicating it as a regulatory factor for the *Atcys-3A* gene. Collaboration with the workers who carried out the *Atcys-3A* studies led to experiments to determine the effect of salt, mannitol and ABA on *Sat-53* transcript. *Sat-53* and *Atcys-3A* encode putatively cytoplasmic isoforms of SAT and *O*ASTL respectively.

Gotor and co-workers found that salt stress induced higher levels of *Atcys-3A* transcript than mannitol (Figure 4.7b). Salt stress was therefore analysed in greater detail. *Atcys-3A* expression was studied in root, leaf and stem tissue over a five day period. Equivalent experiments were performed with respect to *Sat-53* expression as part of this PhD and are described

below. The effects of mannitol and ABA on *Sat-53* transcript abundance are described in section 4.2.5.

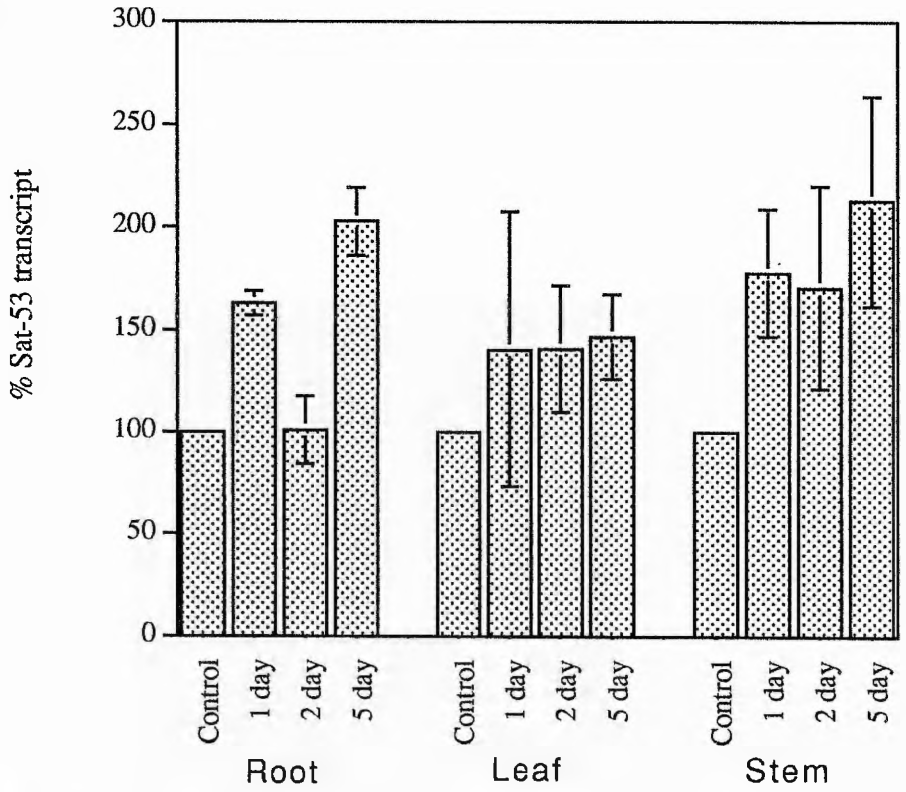
A. thaliana plants were grown in vermiculite supplemented with half strength Hoagland's solution (as outlined in section 2.2.2) for 40 days by which time they had reached the early stages of flowering. Control plants continued to be grown in half-strength Hoagland's solution whilst samples of approximately 20 plants treated with half-strength Hoagland's solution containing 0.17 M NaCl. Root, leaf and stem tissue was harvested from NaCl treated plants after 1, 2 and 5 days. RNA extracted from harvested samples was subjected to northern blotting and hybridisation with the *Sat-53* gene-specific probe designed in section 3.2.5. Transcript abundance was quantified using a bioimaging analyser.

Figure 4.6a shows the mean results of 3 replicate experiments, comparing *Sat-53* transcript abundance in NaCl stressed tissues to abundance in unstressed control tissues. Whilst large standard deviations were often recorded, as indicated by error bars on the histogram in Figure 4.6a, a general increase in *Sat-53* transcript abundance was observed in all tissues in response to NaCl stress. The highest increases in abundance were observed in root and stem tissue with up to 200% of control levels being recorded. *Sat-53* transcript was observed to decrease in abundance between 1 and 2 days in all the root replicates, before increasing again in tissue which had been subject to 5 days NaCl treatment. The second peak in transcript abundance after 5 days may be due to a secondary induction, perhaps as a consequence of tissue senescence. It was noted that after 5 days in 0.17 M NaCl, the plants were beginning to wilt and leaves were yellowing. *Sat-53* transcript abundance in leaf tissue, being subject to quite large variation between replicates (Figure 4.6a), increases to approximately 140% of control levels after 1 day NaCl stress remaining at this level throughout the 5 day experiment. *Sat-53* transcript abundance in stem tissue followed a similar

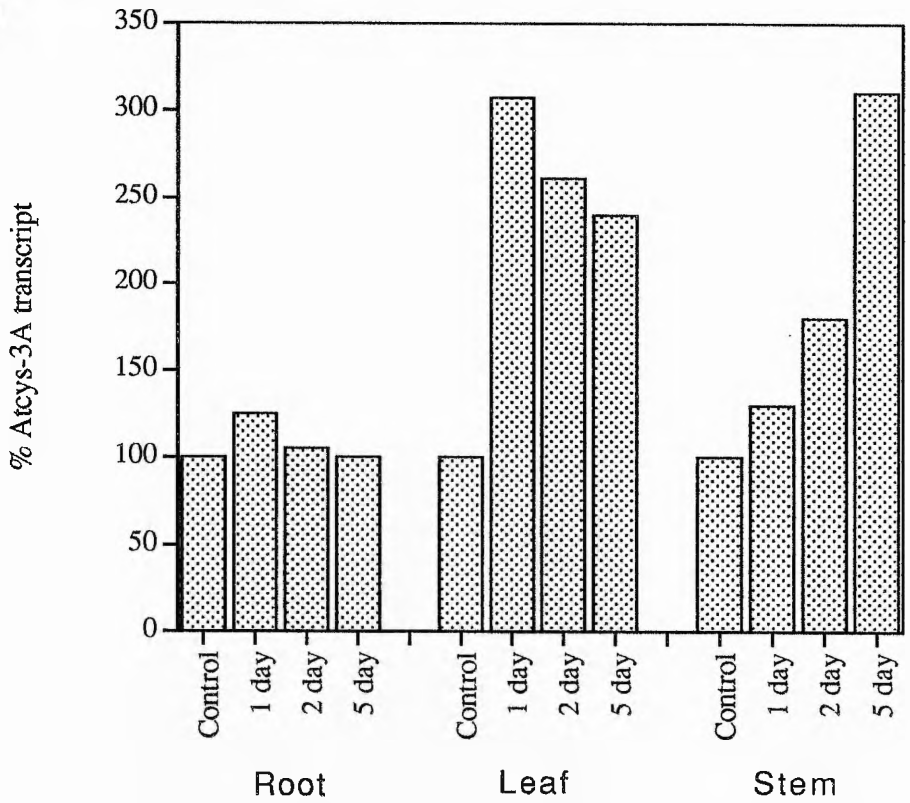
Figure 4.6 a) Effect of salt stress on *Sat-53* expression in *A. thaliana*. 40 day old plants were subject to salt stress by the addition of 0.17 M NaCl to the growth medium. Total RNA was extracted from root, leaf and stem tissue after 1, 2 and 5 days and subject to northern blotting followed by hybridisation with the Sat-53 gene-specific probe. Hybridising-band intensity was quantified on a bioimaging analyser and transcript levels calculated as a percentage of levels detected in control, unstressed tissues. Levels of *Sat-53* transcript were normalised to the level of 18S rRNA for each extraction. Error bars represent the standard deviation from the mean of three experiments.

b) Effect of salt stress on *Atcys-3A* expression in *A. thaliana* root, leaf and stem tissue after 1, 2 and 5 days. Results of one experiment are shown. This experiment was carried out by C. Gotor and co-workers at the University of Seville, Spain, (C. Gotor, pers. comm.) and is presented for comparison with the *Sat-53* results shown in a).

a)


% Sat-53 transcript
300
250
200
150
100
50
0
Control
1 day
2 day
5 day
Root
Leaf
Stem

b)


% Atcys-3A transcript
350
300
250
200
150
100
50
0
Control
1 day
2 day
5 day
Root
Leaf
Stem

pattern of induction to that observed in leaves, increasing to approximately 175% of control levels within 1 day of NaCl stress and not significantly increasing or decreasing from this level over the 5 day experimental period.

4.2.5 The effect of mannitol and ABA on Sat-53 expression

Whilst a more detailed study was carried out to determine the effect of NaCl stress on *Sat-53* transcript levels (section 4.2.4), northern hybridisation to RNA extracted from mannitol- and ABA- treated plants was also performed. Mannitol was dissolved in half strength Hoagland's solution to subject the plants to hyper-osmotic, 'drought-like' conditions.

Sat-53 transcript induction under saline conditions may be due either to a secondary response, as a consequence of the ionic imbalance, or a direct up-regulation by a salt-induced signalling factor. ABA, a naturally occurring plant hormone, is widely recognised as the chemical mediator involved in many signalling processes related to environmental stress (Giraudat et al. 1994). Applications of ABA were carried out to induce the plant ABA-response without subjecting them to ionic or osmotic imbalance.

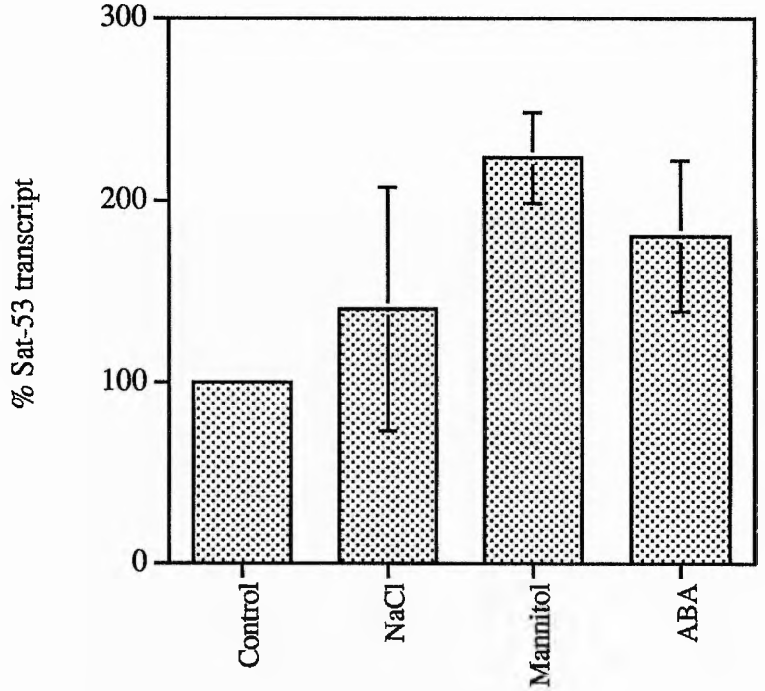
Plants were grown as outlined in section 4.2.4 with 0.3 M mannitol or 100 μM ABA being added to the half strength Hoagland's solution to induce the stress response in 40 day old plants. After 24 hours, RNA was extracted from leaf tissue of approximately 20 plants per treatment. The experiment was repeated three times.

Figure 4.7a shows the *Sat-53* transcript levels in the leaves of mannitol and ABA treated plants when compared to unstressed controls. 224% and 181% of control *Sat-53* transcript levels were detected in mannitol- and ABA- treated tissue respectively.

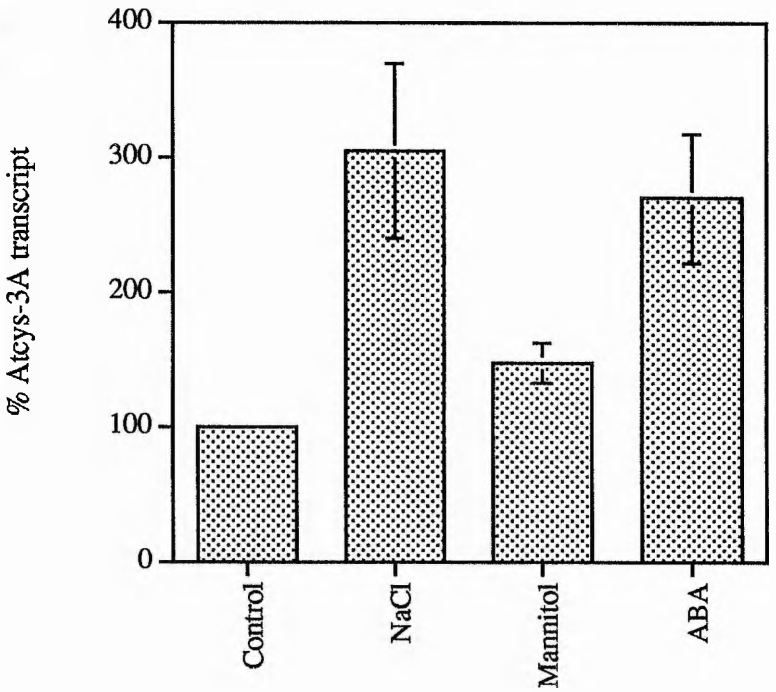
Figure 4.7 a) Effects of salt, mannitol and abscisic acid (ABA) on *Sat-53* expression in the leaves of *A. thaliana*. 40 day old plants were exposed to 0.17 M NaCl, 100 µM ABA or 0.3 M mannitol by addition of these compounds to the growth medium. Total RNA was extracted after 1 day and subject to northern blotting/hybridisation with the Sat-53 gene-specific probe. Hybridising-band intensity was quantified on a bioimaging analyser and transcript levels calculated as a percentage of levels detected in control, untreated tissues. Levels of *Sat-53* transcript were normalised to the level of 18S rRNA for each extraction. Error bars represent the standard deviation from the mean of three replicate experiments.

b) The effects of 1 day salt, mannitol or ABA treatment on *Atcys-3A* expression in *A. thaliana* leaves. Standard deviations from the mean of three experiments are shown. Experiments were performed by C. Gotor and co-workers at the University of Seville, Spain (C. Gotor, pers. comm.) as described in a) and are presented for comparison with the *Sat-53* results.

a)


% Sat-53 transcript
300
200
100
0
Control
NaCl
Mannitol
ABA

b)


% Atcys-3A transcript
400
300
200
100
0
Control
NaCl
Mannitol
ABA

4.3 Discussion

4.3.1 SAT mRNA distribution in A. thaliana tissues

The first expression study in this chapter shows the transcript distribution of *Sat-1*, *Sat-52* and *Sat-53* in the various organs of *A. thaliana*. No evidence of organ-specific expression of the gene-family was observed, each of the SAT isogenes being expressed in all tissues. Expression of each gene was also found to be highest in root tissue. The high SAT expression in roots combined with high root expression of the known *O*ASTL isoforms (Hell et al. 1994; Gotor et al. 1997) suggests that the root is an important site for cysteine biosynthesis in *A. thaliana*, although enzyme assays would be required to confirm this. It should be noted that the *Sat-1* transcript, which encodes a putatively chloroplastic SAT isoform, was also highly expressed in roots suggesting that the root proplastids may be significantly involved in plant cysteine biosynthesis. A previous report that AtOAS7.4 transcript (which encodes a putatively chloroplastic isoform of *O*ASTL (Hell et al. 1994)) is highly expressed in *A. thaliana* roots was attributed to the development of photosynthetic capacity in root cells under *in vitro* culture conditions (Hell et al. 1994). ATP sulphurylase, APS reductase, sulphite reductase and APS kinase are also highly expressed in the roots of *in vitro* grown plants (Takahashi et al 1997). As RNA used in the SAT transcript distribution experiments in this chapter was extracted from compost grown plants, the findings here are free from artifacts of *in vitro* culture. All three available APS reductase mRNA transcripts, which encode putatively chloroplastic isoforms, have also been shown to be highly expressed in compost-grown root tissue (Gutierrez-Marcos et al. 1996). This molecular evidence of *Sat-1* and APS reductase gene expression in root tissue suggests that the root plastids are capable of both reductive sulphate assimilation and

cysteine biosynthesis. This would contradict some early hypotheses that these processes occur almost exclusively in leaves (*e.g.* Evans 1975).

It is also noted that very little hybridisation was observed between any of the SAT gene-specific probes and RNA extracted from silique tissue. The low SAT expression in siliques may indicate that reduced sulphur compounds, produced in other tissues, are imported into the developing seed capsules rather than being produced *in situ*. Enzyme assays and/or radioactive-metabolite tracing would be able to test this hypothesis on the cysteine biosynthetic capacity of *A. thaliana* siliques.

Whilst northern hybridisations to RNA extracted from the various organs of *A. thaliana* show the transcript distribution of each SAT mRNA individually, it would also be of interest to compare the abundance of the SAT mRNAs within each organ. This would determine whether certain isoforms are predominantly expressed. Such an experiment, which could be performed using ribonuclease protection techniques (Gilman 1993), may indicate which isoforms make the greatest contribution to total cysteine biosynthesis. *Sat-1* may be expected to be the predominantly transcribed SAT gene in *A. thaliana* tissues. 88 of the 110 SAT cDNAs isolated by functional complementation were found to be of the Sat-1 type (see section 3.2.6). Assuming that no selection against any mRNAs occurs during cDNA library preparation or functional complementation, *Sat-1* may be expected to account for the majority of SAT transcripts in *A. thaliana* green tissue, from which the original cDNA library was prepared.

4.3.2 Cellular localisation of Sat-52 and Sat-53 as detected by in situ hybridisation

Following studies on the distribution of SAT mRNA transcript within the various organs of *A. thaliana*, the transcript distribution between different cell types within tissues was investigated by *in situ* hybridisation. The failure of hybridisation of the Sat-1 riboprobe to tissue sections was discussed in section 4.3.2 and the Sat-106 cDNA was isolated after the completion of the *in situ* experiments detailed here. Hybridisations using these probes have still to be carried out.

The Sat-52 probe hybridised specifically to the vascular tissues of *A. thaliana* root, leaf and stem. This may indicate a role for SAT52, specific to the vascular system. Rennenberg et al. (1979) detected cysteine, methionine and glutathione in the phloem sap of *Nicotiana tabacum*. The SAT52 protein may be involved in cysteine biosynthesis in vascular transfer cells, which transfer solutes into and out of the xylem and phloem elements. Cysteine, and its derivatives methionine and glutathione, may be produced in the transfer cells for loading directly into the vascular system.

A second hypothesis for the high expression of *Sat-52* in vascular tissue relates to the putative mitochondrial location of the SAT52 protein (see section 3.2.13). Transfer cells surrounding the xylem elements of the vascular system contain numerous mitochondria due to the high energy and metabolic rate requirement of solute movement into and from the xylem. Mitochondria can account for up to 20% of the volume of transfer cells (Gunning & Steer 1996; Newcomb 1997). If expression of the gene encoding mitochondrial SAT was related to the mitochondrial content of the cell, high expression may be expected in the transfer cells of the vascular system.

A high level of *Sat-52* transcript was also detected in the leaf trichomes, as was *Sat-53* and *Atcys-3A* which encodes a putatively

cytoplasmic isoform of *O*ASTL (Gotor et al. 1997 and Figure 4.2d). These findings suggest that the *A thaliana* trichome has a specialised requirement for cysteine biosynthesis. *A thaliana* trichomes are branched, single cell structures expanded out of the plane of epidermal cells (Larkin et al. 1996). With respect to cysteine biosynthesis, literature searches reveal several metabolic processes which may explain the high expression of certain SAT and *O*ASTL genes in trichomes.

A function of the leaf trichomes of many species is the accumulation of heavy metals, detoxifying the plant by acting as a sink for harmful metal ions. Manganese (Blamey et al. 1986), lead (Martell 1974) and cadmium (Salt et al. 1995) have been shown to be accumulated in the trichomes of a variety of plants. The plant compounds responsible for chelating heavy metal ions are cysteine rich peptides such as metallothioneins (MT) and phytochelatins (PC). The toxic metal ions complex with four proximal cysteine residues of both MT and PC peptides (Salt et al. 1995; Zenk 1996). Five MT genes have been cloned from *A. thaliana*, all possessing cysteine-rich chelating domains (Zhou & Goldsbrough 1995). In *Vicia faba*, an MT-like gene with two cysteine rich domains has been shown to be extremely highly expressed in foliar trichomes using *in situ* hybridisation techniques (Foley & Singh 1994).

The PCs have the consensus sequence [glutamate-cysteine]$_n$-glycine, and are produced from glutathione in response to metal stress rather than being genetically encoded. Production of PCs has been found to cause rapid depletions in the glutathione pool in response to metal stress (Zenk 1996).

The ability of *A. thaliana* trichomes to accumulate heavy metals has not yet been studied, but cadmium was shown to be accumulated in the trichomes of *Brassica juncea*, a close relation of *A. thaliana* (Salt et al. 1995). If *A. thaliana* is found to accumulate heavy metals in its trichomes, the high expression of *Sat-52*, *Sat-53* and *Atcys-3A* genes in these structures may be to provide the cysteine for production of MTs or for the replenishment of the

glutathione pool in response to PC production. Although it has been reported that one MT gene (MT2a) is strongly induced by Cu^{2+} in *A. thaliana*, another (MT2b) was expressed at a relatively high basal level, increasing only slightly in response to Cu^{2+} stress (Zhou & Goldberg 1995). Certain cell types, expressing high basal levels of MTs, may require high levels of cysteine biosynthesis even when not subject to metal stress. This may explain the high *Sat-52*, *Sat-53* and *Atcys-3A* transcript abundance in the trichomes of unstressed *A. thaliana*, as observed in Figures 4.3a and 4.4a.

It will be of interest to further investigate any correlation between heavy metal tolerance and SAT and *O*ASTL expression. Initially a northern blot/hybridisation study could be carried out to assess transcript levels in plants subject to a variety of heavy metal stresses. Tracing radioactively-labelled metal ions in *A. thaliana* may also confirm the role of trichomes in heavy metal detoxification.

4.3.3 Expression of SAT genes does not appear to be regulated by sulphate or nitrate nutrition

No response in *Sat-1*, *Sat-52* or *Sat-53* transcript abundance was observed in whole, *in vitro* cultured *A. thaliana* plants after 4 days of growth on sulphate and/or nitrate deficient medium. These results suggest that the SAT gene-family is constitutively expressed under varying nitrate and sulphate nutrition. A recently published report also showed no effect of 2 days sulphate starvation on *Sat-52* or *Sat-53* transcript levels in *A. thaliana*, but found a 2 to 3-fold increase in *Sat-1* transcript during this period (Takahashi et al. 1997). Although no increase in *Sat-1* transcript abundance in response to sulphate starvation was observed in this study, it is possible that transcript abundance increases temporarily, returning to control levels by 4 days. Performing the same experiment, but sampling plants at intervals over a

several day time period, would allow the response of SAT expression to sulphate starvation to be studied in more detail. Temporal expression responses of the other SAT transcripts, *Sat-52*, *Sat-53* and *Sat-106*, may also be detected by such an experiment.

*O*AS, the product of SAT activity, has been shown by several studies to have a key role regulating the reductive assimilation of sulphate in plants to ensure balanced supplies of sulphide are provided for cysteine biosynthesis (see section 1.3.4 and references therein). *O*AS concentrations have also been reported to increase up to 7-fold under sulphur deficiency (Kim et al. 1997). It appears, from the northern hybridisations in this chapter, that increases in *O*AS concentrations under sulphate starvation are not caused by increased SAT expression at the level of transcription. *O*AS levels are more likely to be increased by a reduction in sulphide availability for cysteine biosynthesis. Expression of the *Sat-106* transcript under nitrate and sulphate starvation has not yet been tested.

4.3.4 Sat-53 expression is regulated by salt, mannitol and ABA

Significant increases were observed in *Sat-53* transcript levels in response to salt (NaCl) and osmotic (conferred by mannitol) stress. Similar responses could be induced by exogenous applications of ABA (Figures 4.6a and 4.7a). Increases have also been observed in *Atcys-3A* transcript levels in response to salt, mannitol and ABA (Figures 4.6b and 4.7b; C. Gotor, pers. comm.). As the response of *Sat-53* expression to salt and osmotic stress was mimicked by the application of endogenous ABA, it appears that ABA may be responsible for upregulation of the *Sat-53* gene or another gene whose expression is closely linked to that of *Sat-53*. The presence of several G-box-like transcription factor recognition sequences, involved in ABA regulation of gene expression (Giraudat et al 1994), within the promoter region of the

Sat-53 gene (see Figure 3.11) suggests that *Sat-53* expression is directly regulated by ABA. Alternatively, expression of the *Sat-53* gene under salt and osmotic stress may be induced by the ATMYB2 protein which would bind to the MYB transcription factor recognition sequence present in the *Sat-53* promoter region (Urao et al 1993; Figure 3.11). The *Atmyb2* gene itself is however induced by ABA, possibly indicating an indirect link between ABA-signalling and *Sat-53* expression under salt and osmotic stress.

A study of the role of ABA in *Sat-53* expression under environmental stress conditions, using *A. thaliana* ABA mutants, was started during this PhD but remains unfinished. The *A. thaliana* mutants involved were *aba-1* (Koornneef et al. 1982), which is sensitive to ABA but is defective in its biosynthesis, and mutants *abi1-1* and *abi1-2* (Koornneef et al. 1984), which are deficient in ABA signal recognition. If the observed responses in *Sat-53* transcript abundance to salt and osmotic-stress conditions are mediated by ABA directly, no response would be expected in *aba-1*, *abi1-1* or *abi1-2* plants under the same conditions. However, the increase in *Sat-53* transcript would be expected to be inducible in the *aba-1* mutant, by exogenous application of ABA, but not in the *abi1-1* or *abi1-2* mutants, due to their ABA-insensitivity.

The major reason for increased SAT expression under salt and mannitol stress treatments is likely to be an increased requirement of cysteine biosynthesis for adaptation to saline and drought conditions. One compound important in plant adaptation to saline and drought environments is the tertiary sulphonium compound β-dimethylsulphoniopropionate (DMSP), a derivative of methionine. In salt or drought stressed plants DMSP is produced as a non-toxic osmolyte protecting cells from disrupted osmotic balance (Rhodes & Hanson 1993). Availability of sulphur is a limiting factor for DMSP metabolism, and production is inhibited under sulphate-starvation (Storey et al. 1993). Therefore it would not be unexpected that plants

optimise their capacity for sulphate reduction, cysteine biosynthesis, and consequently DMSP metabolism, under saline or drought conditions by upregulating expression of genes involved in these processes.

A second hypothesis for the upregulation of cysteine biosynthetic genes in response to salt and drought stress would be to increase the production of glutathione in stressed cells. The generation of active oxygen species (*e.g.* O_2^-), which are extremely toxic to living organisms, in response to a variety of environmental stresses is well documented (Smirnoff 1993). Reduced glutathione has a key role in the recycling of ascorbate (via the ascorbate-glutathione cycle) which is responsible for scavenging active oxygen (Aono et al. 1997 and references therein). The increases in *Sat-53* and *Atcys-3A* transcript observed in response to salt and drought stress may therefore act to increase the plant's resistance to oxidative stress by increasing availability of cysteine for glutathione metabolism.

As shown in Figure 4.6a, whilst significant increases in *Sat-53* transcript are observed in root, leaf and stem tissues subject to salt stress, the size of some of the standard error bars obscure the magnitude of transcript increase and the temporal pattern of expression. Work is currently underway (at both the Universities of St. Andrews and Seville) to repeat this work, involving additional replicate experiments, larger sample sizes and probing the same northern filters with *Sat-53*- and *Atcys-3A*-specific probes. This will allow more accurate comparisons between the expression of cytoplasmic SAT and *O*ASTL genes in response to salt stress and determine whether their expression is controlled by the same regulatory mechanism under saline conditions.

Chapter 5: Transgenic Manipulation of SAT Expression and Antibody Production for the Analysis of Transformants

5.1 Introduction

5.1.1 Genetic manipulation of plants

The availability of four cDNAs encoding SAT from *A. thaliana* will allow the role of the gene-family in cysteine biosynthesis to be studied using transgenic technology. Over the last twenty years the development of *Agrobacterium tumefaciens*-mediated gene-transfer systems has made perhaps the most important contribution to plant molecular biology and physiology. The ability to express or inactivate plant genes via the stable introduction of specific transgenes has become a powerful tool in the study of physiological, biochemical and molecular systems (reviewed in: Hooykaas & Schilperoot 1992; Birch 1997; Azpiroz-Leehan & Feldmann 1997; Koorneef et al. 1997).

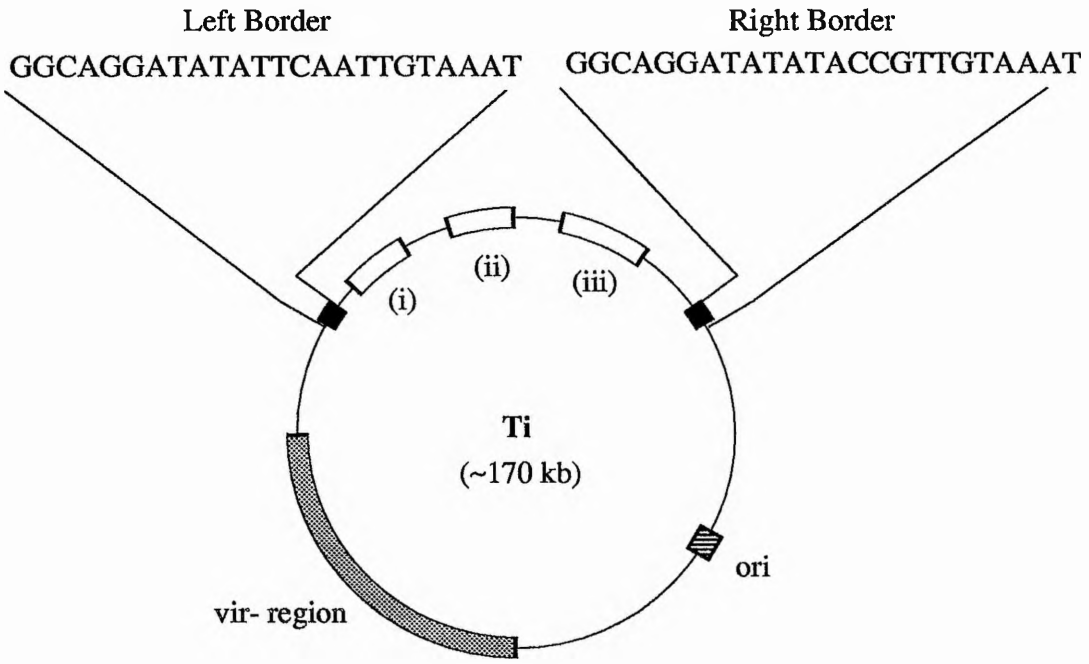
The first step towards modern transgenic techniques came with the discovery that a large extrachromosomal plasmid of virulent strains of the 'Crown Gall' bacterium *A. tumefaciens,* possessed all the genes necessary for gall-tumour induction (Zaenen et al. 1974). The plasmid, named the Ti plasmid due to its Tumour-inducing role, is shown in diagrammatic form in Figure 5.1a. The ability of gall cells to proliferate *in vitro* without exogenous application of phytohormones led to genetic investigations of gall cultures. In one study, analysis of genomic DNA from aseptically grown gall cells detected 20 copies of a section of the Ti plasmid from *A. tumefaciens* (Chilton et al. 1977). The DNA section which had transferred to the plant genome, referred to as the T-DNA or Transfer-DNA, was found to encode genes for the biosynthesis of opines which are amino acid derivatives not usually associated with plant metabolism (Tempé & Goldmann 1982).

T-DNA transfer takes place as follows. Proteins encoded by a 40 kb operon of the Ti plasmid located outside the T-DNA, known as the *vir-* or virulence region, synthesise a single stranded template of the T-DNA. Initially,

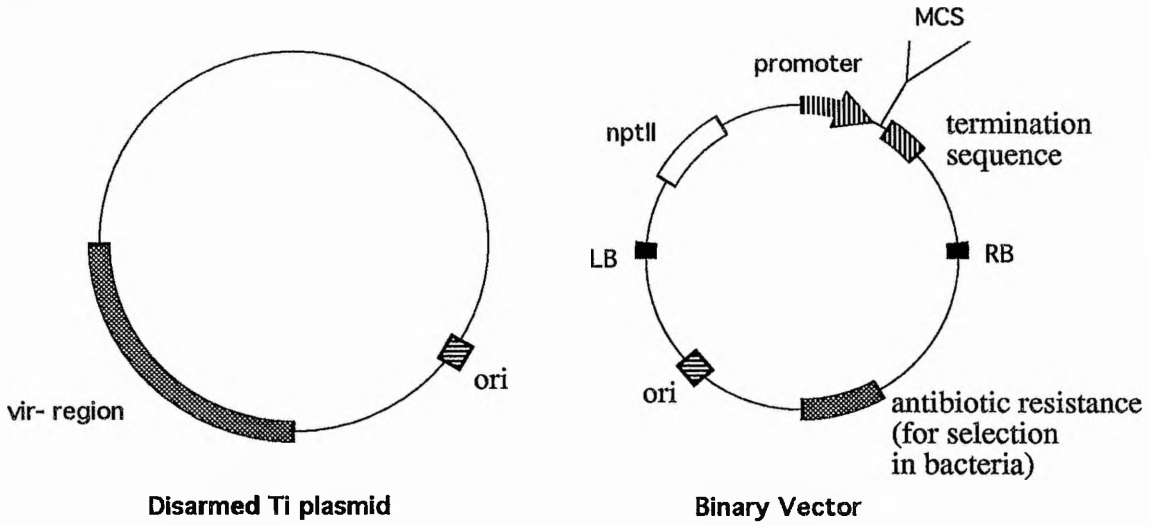
Figure 5.1 a) Diagram of the Ti-plasmid from *Agrobacterium tumefaciens*. 24 bp imperfect repeat sequences of the left and right T-DNA borders are shown. T-DNA regions i, ii and iii encode opine, auxin and cytokinin biosynthesis genes. **ori** is the origin of replication.

b) Diagrammatic representation of a binary transformation vector system. The disarmed Ti plasmid possesses the genes required for virulence but has had the T-DNA region removed. The binary vector possesses a multicloning site (MCS), into which the transgene is inserted, flanked by promoter and polyadenylation sequences. T-DNA borders and antibiotic resistance genes which allow selection of the plasmid in both plants and bacteria are also included. **ori** is the origin of replication.

a)



b)



Disarmed Ti plasmid **Binary Vector**

'nicks' are formed in 24 bp imperfect-repeat sequences at the left (LB) and right (RB) borders of the T-DNA (Figure 5.1a). A single-stranded T-DNA template is then synthesised from the sequence between the nicks (Stachel et al. 1986). Although it is not known whether the T-DNA is transferred to the host plant cell in a single-stranded (ss) or double-stranded (ds) form, plant cells are known to rapidly convert artificially introduced ssT-DNA molecules into dsDNA (Rodenburg et al. 1989). The T-DNA is accompanied into the host cell by a *vir* operon-encoded protein, VirD2, which contains sequences for nuclear targeting in plant cells and which also protects the T-DNA from nuclease denaturation prior to incorporation into the plant genome (Herrera-Estrella et al. 1990). Once inside the plant nucleus, insertion of the T-DNA into the genome appears to be at random. Segregation analysis of large numbers of transgenic *A. thaliana* lines suggests an average of 1.5 independent T-DNA insertions per diploid genome (Feldmann 1991).

It was realised that the transfer of DNA between the imperfect-repeat borders of the T-DNA by *A. tumefaciens* could be exploited for specific genetic engineering purposes. As none of the genes within the T-DNA region are essential for virulence or DNA transfer, and neither is a physical linkage between the T-DNA and the rest of the Ti plasmid (Hoekema et al. 1983), a number of strategies were devised to integrate a gene or DNA sequence of choice stably into a plant genome. The most commonly used of these systems is the binary vector (or BIN vector) system which is employed in this chapter. Binary refers to two separate plasmids which are introduced into *A. tumefaciens* to allow transfer of a chosen gene (see Figure 5.1b). The first plasmid is a modified version of the naturally occurring Ti plasmid, which has been 'disarmed' by the removal of the T-DNA region including the 24 bp border repeat sequences. This prevents the transfer of tumour-inducing genes to the host plant and allows transformants to grow as wild-type, with the exception of the transgene's influence. The *vir* region is fully represented and functional on the disarmed Ti plasmid (Hoekema et al.

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1983). The second plasmid of a binary vector system, often referred to as the binary vector, is smaller, easier to manipulate in the laboratory, and possesses artificial T-DNA borders between which is a multicloning site (MCS). The MCS is often flanked by promoter and transcription termination sequences which induce transcription of the transgene in the host plant and act as a polyadenylation signal respectively. The 35S cauliflower mosaic virus (CaMV) promoter, which generally induces high level expression in most plant tissues, and terminating sequences of octopine synthetase or nopaline synthetase are commonly used. A gene conferring antibiotic resistance is also included between the T-DNA borders to allow selection of positively transformed plants on growth media containing the relevant antibiotic. The neomycin phosphotransferase (*nptII*) gene, which originates from the bacterial transposon Tn5, confers resistance to kanamycin and is most commonly used in binary vector systems (Bevan et al. 1983). Any gene can be cloned into the MCS of the T-DNA region and will be expressed in a host plant once incorporated into its genome. However, the level of transgene expression can be unpredictable due to 'position' effects. The number of copies of the T-DNA, and the positions at which they are incorporated into the host genome, result in a wide variation of expression levels between transformants. For this reason, the levels of transgene expression must be characterised for each transgenic line before experiments can be performed. Southern and northern hybridisations, using probes specific to the transgene, can be used to analyse the number of T-DNA insertions and the levels of mRNA transcript expression respectively, when comparing transgenic lines to untransformed wild-type plants.

Whilst a transgene can be overexpressed in an *A. tumefaciens*-transformed plant, another application of the plant transformation system is the down-regulation of an endogenous 'target' gene by antisense mRNA expression. Using a Ti-based system, a transgene is expressed in reverse (antisense) orientation, resulting in 3' to 5' transcription. Following transformation, expression of the antisense gene leads to a down-regulation of the enzyme encoded by the

complementary endogenous gene (Weintraub et al. 1985). Whilst debate continues as to the mechanism of antisense technology, it is believed that the antisense mRNA binds to the complementary endogenous mRNA inhibiting translation of the target protein (Kumria et al. 1998).

Several methods exist for the transformation of plants by *A. tumefaciens*-mediated gene transfer. *In vitro* transformation involves the co-cultivation of plant tissue and the required *A. tumefaciens* strain, followed by proliferation of antibiotic resistant tissues in axenic culture (An et al. 1986; Valvekens et al. 1988). Alternatively, co-cultivation of plant seed with an *A. tumefaciens* culture (Feldmann & Marks 1987), or a series of *A. tumefaciens* inoculations to severed apical shoots (Chang et al. 1994; Katavic et al. 1994) can be performed to avoid *in vitro* proliferation. Such methods are known as *in planta* transformation. *In planta* transformation methods tend to be preferred as they allow the introduction of a transgene whilst avoiding the effects of somaclonal genomic mutations. Non-specific mutations are frequently associated with *in vitro* regeneration of plants from vegetative tissue using phytohormones, and can obscure the effects of the transgene.

The transformation method used in this chapter has been developed from Bechtold et al. (1993), and is known as *in planta* vacuum infiltration. This is the most efficient, reproducible, and now the most widely used method for the transformation of *A. thaliana*. The protocol for vacuum infiltration is outlined in section 2.17. Whole *A. thaliana* plants, which have had recently emerged infloresences severed at the base, are immersed in an *A. tumefaciens* culture and subjected to a vacuum. This allows the *A. tumefaciens* to infiltrate the premature flower buds which develop after the severing of previous inflorescences. Transfer of the T-DNA to one of the gametophytic tissues of the developing flower will result in transformants which can be selected from the T_1 seed (Chang et al. 1994). Prior to biochemical or physiological analysis of transformants, homozygous transgenic lines, with respect to the T-DNA insertion, are obtained

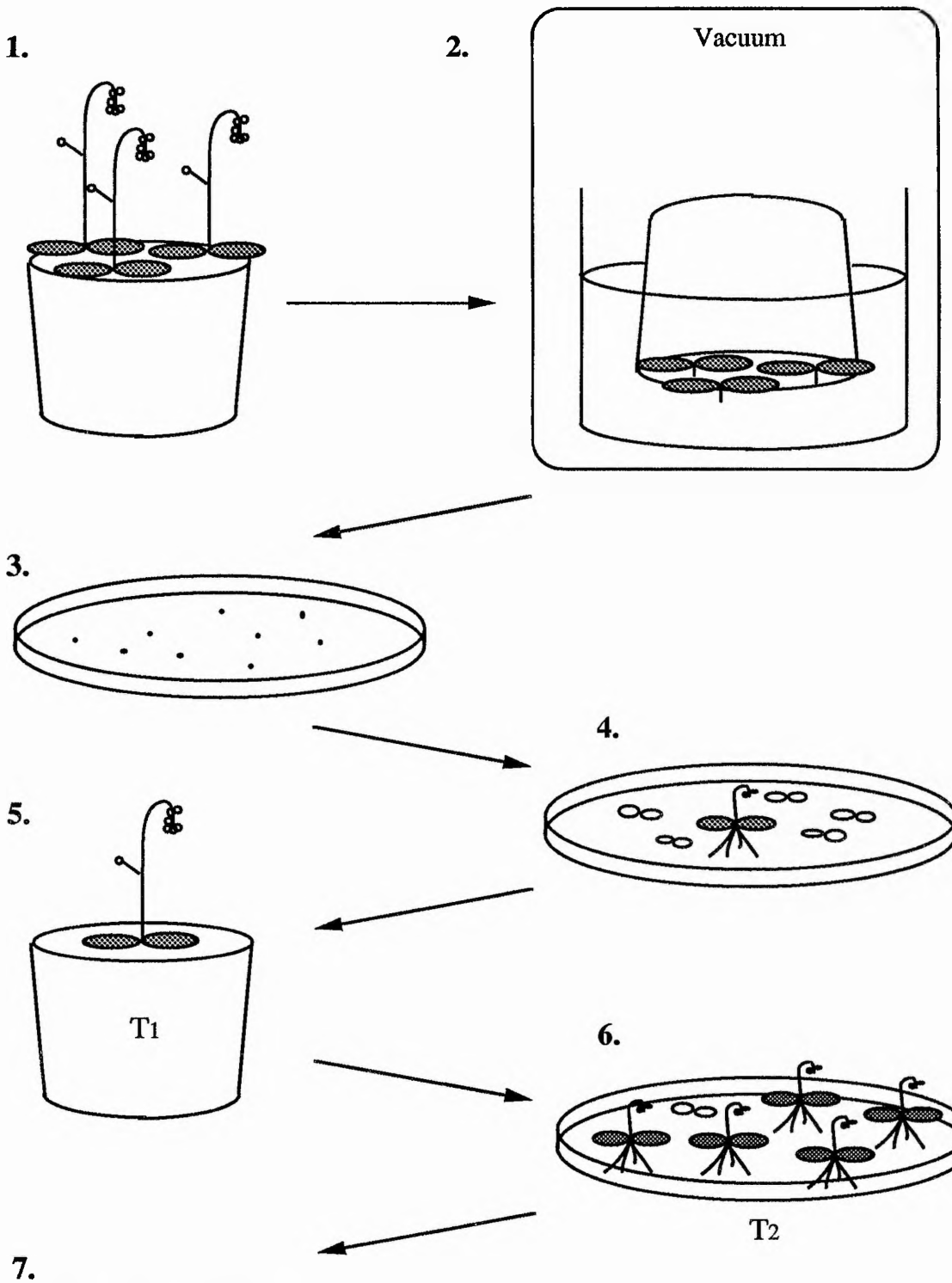
by plating subsequent generations (*i.e.* T_2, T_3, T_4,....) on suitable antibiotic-containing selection media. Seed lines which result in 100% antibiotic-resistant progeny and which exhibit stable levels of transgene expression are chosen for experimentation. The stages of plant transformation by vacuum infiltration and selection of transformants are shown in Figure 5.2.

5.1.2 Approaches to transgenic manipulation of SAT expression

Transgenic techniques have already been used in the analysis of reductive sulphate assimilation and cysteine biosynthesis in higher plants. In the first of these studies, tobacco plants transformed with an *O*-acetylserine (thiol) lyase (*O*ASTL) gene from wheat were used to determine the role of the enzyme in H_2S detoxification (Youssefian et al. 1993). Plants overexpressing *O*ASTL were resistant to fumigation by toxic levels of H_2S gas. Transformation of tobacco was also used to assign subcellular locations to the CysA, CysB and CysC *O*ASTL proteins from spinach, by fusing cDNA fragments encoding putative targeting peptides to the β-glucuronidase (GUS) reporter gene (Takahashi & Saito 1996). Recently, an *A. thaliana* ATP sulphurylase (ATPS) has been overexpressed in tobacco cells to determine whether the enzyme has a limiting role in reductive sulphate assimilation in plants. Eight-fold increases in ATPS activity were not observed to affect sulphate uptake by the transgenic cells, indicating that the enzyme is unlikely to limit the rate of reductive sulphate assimilation (Hatzfeld et al. 1998). Finally, a study by Saito et al. (1994a), using transgenic tobacco plants overexpressing a spinach *O*ASTL, indicated that supply of *O*AS was limiting for the biosynthesis of cysteine. This finding made the study of plants genetically modified with respect to SAT activity of great interest, SAT being the enzyme which produces *O*AS.

This chapter describes the first advances towards the study of SAT in higher plants using transgenic technology. By down-regulating the SAT gene-

Figure 5.2 Schematic diagram showing the stages involved in *in planta* vacuum infiltration, and the subsequent selection of transformants. **1.** Wild-type *A. thaliana* plants are grown until inflorescences are approximately 5 cm high. **2.** Inflorescences are severed and 4 days later the plants are immersed in 200 ml of an *A. tumefaciens* culture and subjected to 650 mmHg vacuum for 5 to 10 minutes. **3.** Seed from inflorescences which grow following vacuum infiltration are plated on selection medium containing the appropriate antibiotic. **4.** Positive transformants (T_1 plants) grow healthily on the selection medium. Non-transformed plants grow poorly and die. **5.** T_1 plants are removed from culture and grown to maturity in compost. Tissue can be tested by PCR analysis to verify the presence of the transgene at this stage. **6.** Seed from the T_1 plant (the T_2 generation) are plated on selection medium. Several positive transgenic lines from the T_2 generation are grown to maturity for co-segregation tests. **7.** Subsequent generations of seed are plated on selection media in co-segregation tests to isolate homozygous lines. Homozygous transgenic lines are those which produce 100% antibiotic resistant seed.


1.
2.
Vacuum
3.
4.
5.
T1
6.
T2
7.
Co-segregation tests to
isolate homozygous lines
with respect to the T-DNA
insertion.

family using an antisense gene with high homology to all the family-members and individually overexpressing each SAT isogene in sense vectors, it is hoped to determine the role the gene-family, and each individual isoform, has in cysteine biosynthesis and its regulation. Studies of the effects of manipulated SAT expression on the transcription of genes involved in cysteine biosynthesis and reductive sulphate assimilation will be possible due to the availability of cDNA clones encoding all the steps of the APS reductase-dependent sulphate assimilation pathway. Cysteine, glutathione and *O*AS levels in transgenic lines can also be measured by established HPLC methods (Rauser et al. 1991; Kim et al. 1997) in order to determine biochemical effect of manipulating SAT activity. As Saito et al. (1994a) showed that supply of *O*AS is limiting for the biosynthesis of cysteine in higher plants, the transgenic SAT experiments outlined in this chapter may determine whether production of *O*AS is limited by SAT expression. If so, one or more isoforms of SAT may be candidates for overexpression in plants subject to adverse environments, such as low sulphate, saline or drought soils (see section 4.3.4), which require increased biosynthesis of reduced sulphur-containing compounds. Alternatively, acetyl CoA and serine, the substrates of SAT, may be found to limit the biosynthesis of cysteine.

This chapter describes the work to date on the transgenic study of SAT in *A. thaliana*. So far this includes the construction of an antisense vector designed to confer a general down-regulation of SAT activity. The gene-fragment expressed in antisense orientation was derived from a 532 bp region of the Sat-52 cDNA which shares a high nucleotide homology with the Sat-1, Sat-53 and Sat-106 sequences. The production of 7 transgenic *A. thaliana* lines transformed with this SAT antisense construct is also presented. The design and construction of a sense vector for the overexpression of Sat-53 is also described.

5.1.3 Production of antibodies for analysis of transgenic plants

Northern blotting and hybridisation are used to analyse the level of transgene expression in transformed plants compared to that of the endogenous gene in wild-type plants. The effect of the transgene expression on protein levels can be analysed by western blotting using antibodies raised against the analogous protein. This chapter describes the production of antibodies to recombinant SAT52 protein, for the analysis of transformants. Whilst it would be ideal to have antibodies specific to each SAT isoform, it is accepted that a polyclonal antiserum raised to one SAT may cross-react with other SAT proteins due to the high amino acid homology between them (see section 3.2.12). The cross-reactivity of the SAT52 antiserum is also examined in this chapter.

Recombinant SAT52 protein was expressed and purified using the GST (glutathione S-transferase)-fusion protein purification kit (Pharmacia Biotech, UK) using the pGEX expression vector (Figure 5.7) to produce a GST-fusion protein. Problems encountered whilst purifying SAT1 and SAT53 by GST-fusion led to the use of a different system for the purification of these proteins. The second system used was the IMPACT™ I: One step purification system (New England Biolabs Ltd, UK). The cDNA is cloned into a pCYB expression vector (Figure 5.10) in-frame with the 5' end of intein protein cleavage and chitin binding domains. The two protein purification systems used in this chapter are shown diagrammatically in Figures 5.7 and 5.10.

5.2 Transgenic Manipulation of SAT Expression

5.2.1 Design and construction of a SAT antisense vector

The strategy used for the construction of an antisense vector to downregulate all SAT isoforms, utilised the pSLJ series of vectors designed by, and publicly

available from, Jones et al. (1992) (currently at The Sainsbury Laboratory, John Innes Centre, Norwich, UK). The vector construction required two subcloning steps, the first to insert the required cDNA fragment between promoter and termination sequences, and the second to insert this transgene into the binary vector (Figure 5.3). A *Bgl*II/*Cla*I fragment of the Sat-52 cDNA (nucleotides 414 to 945 in Figure 3.3) was chosen to be expressed in antisense orientation as it represented the region of highest homology between the four known SAT cDNAs. The pSLJ4KI vector had *Cla*I and *Bam*HI restriction sites which allowed excision of the GUS gene and replacement with the SAT antisense fragment by directional subcloning as described below (*Bam*HI and *Bgl*II digestions result in compatible 'ends' for ligation). All plasmid digestions involved in subcloning steps were separated by electrophoresis on agarose gels and the resulting bands sized in relation to λHindIII DNA markers as outlined in section 2.9.1. DNA fragments required for subcloning were eluted from agarose gels using Qiagen QIAEX DNA Gel Extraction columns. Between each subcloning step, sequencing using vector-derived primers and plasmid digestion analysis were used to verify successful ligation of insert and vector.

As the MCS of pSLJ vectors are positioned within a *lacZ* gene, ligations in subcloning steps could be selected, when transformed into a suitable bacterial host (*e.g.* DH5α, XL1-Blue), by α-complementation, also known as blue/white bacterial colony screening (Ullmann et al. 1967). Positive ligation of an insert produces a blue colouration in cells exposed to the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Self-ligation of the vector (negative ligation) results in white bacterial colonies. This useful tool facilitates subcloning into vectors compatible with the *lacZ* system.

The SAT antisense vector was constructed as follows. Subcloning Step 1: Plasmid pSLJ4K1 (Jones et al. 1992), consisting of a GUS coding region flanked by CaMV 35S promoter and *nos* termination sequences cloned into the *Eco*RI and *Hin*dIII restriction sites of pUC118, was digested with *Cla*I and *Bam*HI. This

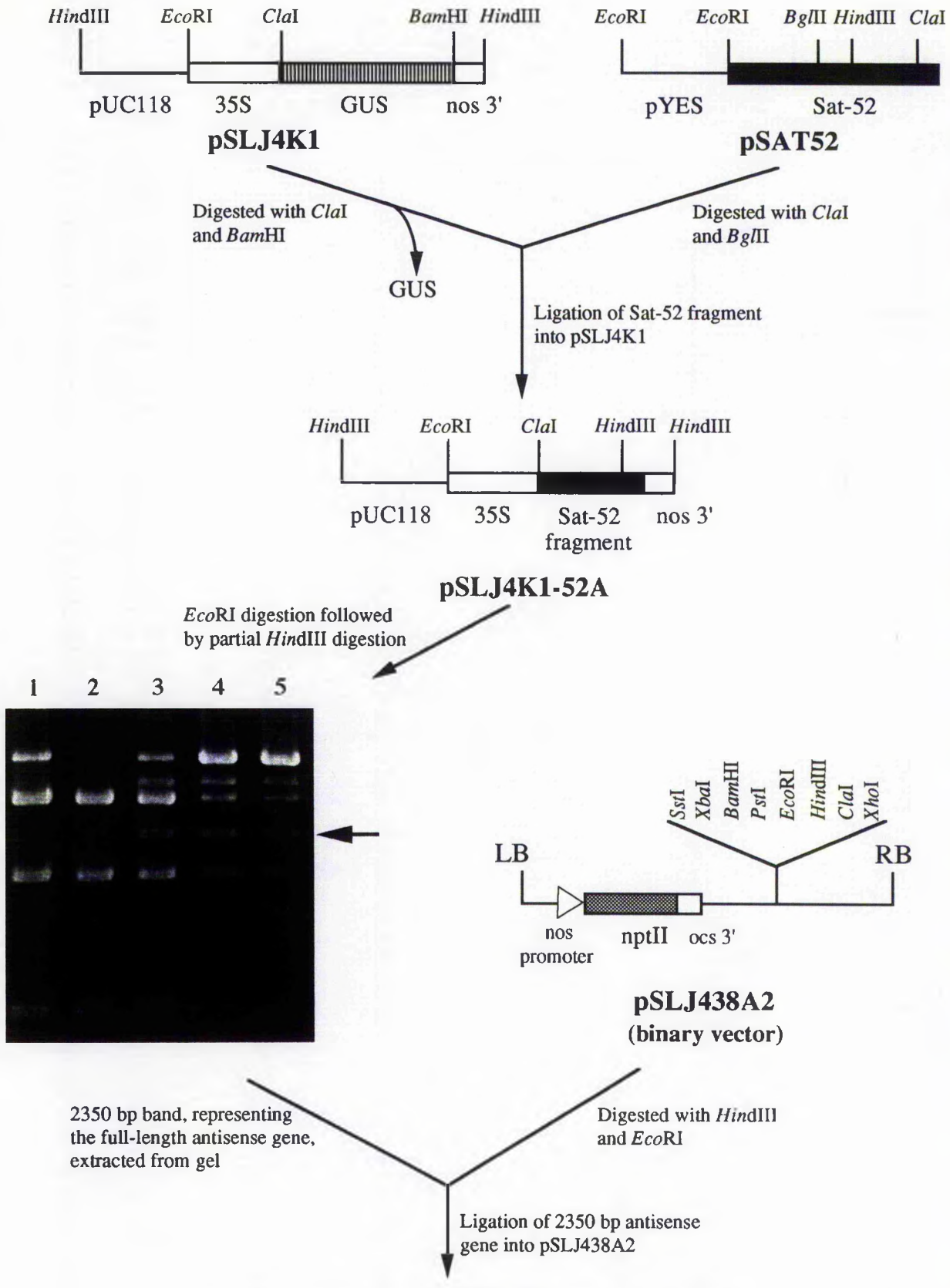
digestion excised the GUS coding region, linearising pSLJ4KI. The 532 bp Sat-52 fragment was excised from pSAT52 with *Cla*I and *Bgl*II and directionally subcloned in antisense orientation into the *Cla*I and *Bam*HI site of pSLJ4K1 in place of the original GUS gene. The resulting plasmid, containing the SAT antisense construct (Sat-52 antisense fragment flanked by CaMV 35S and *nos* termination sequences), was named pSLJ4K1-52A.

Subcloning Step 2: A second subcloning step was used to insert the 2350 bp SAT antisense construct between the T-DNA borders of the binary vector pSLJ438A2 (Jones et al. 1992). pSLJ438A2 is a 25.7 kb pRK290 (Ditta et al. 1980)-based binary vector which allows the insertion of a wide range of transgene 'cassettes' for the production of kanamycin resistant transgenic plants (Jones et al. 1992). The antisense construct was excised from pSLJ4K1-52A by a partial digestion with *Eco*RI and *Hin*dIII. As there is an internal *Hin*dIII restriction site 118 bp from the 5' end of the Sat-52 antisense fragment, a partial digestion of pSLJ4K1-52A had to be performed as outlined in section 2.8.1. Restriction fragments from the partial digestion were separated by electrophoresis and the band of 2350 bp, which contained the complete antisense gene uncut at the internal *Hin*dIII site, was extracted for subcloning (see Figure 5.3a). pSLJ438A2 was fully digested with *Eco*RI and *Hin*dIII to allow directional subcloning of the Sat-52 antisense gene. The resulting 28.1 kb SAT antisense binary vector was named pSLJ-52A (Figure 5.3b), and consisted of the 532 bp *Bgl*II/*Cla*I fragment of the Sat-52 cDNA (nucleotides 414 to 945 in Figure 3.3) in reverse orientation between a CaMV 35S promoter and nopaline synthase (nos) termination signal. An *nptII* kanamycin-resistance gene between the LB and RB of the T-DNA and a tetracycline resistance gene outside the T-DNA region allowed selection of plant transformants and bacterial hosts respectively.

Figure 5.3 Schematic representation of SAT antisense vector construction. a) The steps involved in subcloning the Sat-52 fragment (which shares high nucleotide homology with all SATs from *A. thaliana*), into the multicloning site of pSLJ4K1, and subsequent subcloning of the transgene into the binary vector pSLJ438A2. Lanes 1 to 5 on the gel photograph inset contain *Eco*RI cut pSLJ4K1-52A which has been digested with a serial dilution of *Hin*dIII as described in section 2.8.1. The bands indicated by the arrow are the 2350 bp antisense gene-fragment required for subcloning. DNA in these bands was eluted from the gel and ligated into the binary vector pSLJ438A2.

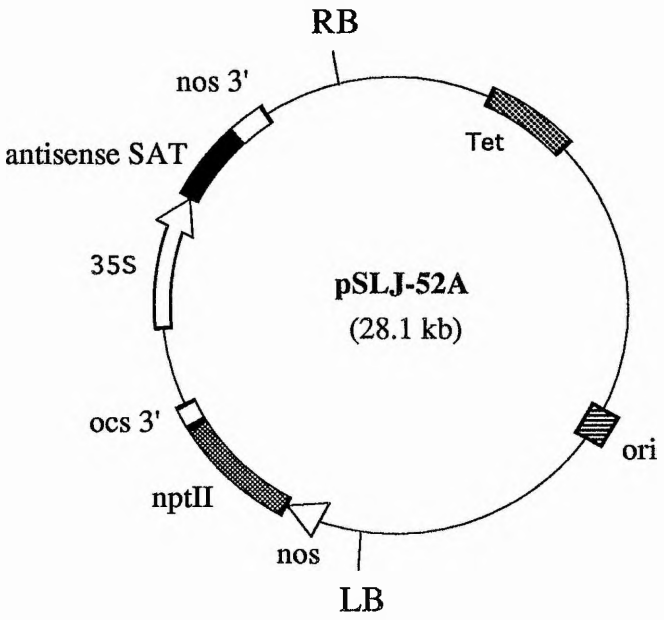
b) Plasmid map of the 28.1 kb SAT antisense vector pSLJ-52A which was used to transform *A. thaliana*. **ori** represents the origin of replication.

a)



Sat-52 antisense vector **pSLJ-52A** (See Figure 5.3b)

b)


RB
nos 3'
antisense SAT
Tet
35S
pSLJ-52A
(28.1 kb)
ocs 3'
ori
nptII
nos
LB

5.2.2 Choice of medium for selection of pSLJ-52A-transformed plants

As pSLJ-52A transformants may express SAT at extremely low levels it is possible that toxic levels of sulphide could build up due to low *O*AS production. It was decided that the selection medium used for plating the T_1 generation seed should consist of MS supplemented with a reduced sulphur source, and all sulphate salts replaced by chlorides. Transformants could then grow in the absence of SAT activity due to the presence of reduced sulphur (in the form of cystine) as the sole sulphur source. To determine the concentration of cystine required for the selection medium, growth of wild-type *A. thaliana* plants *in vitro* on MS deficient in sulphate but supplemented with a range of cystine concentrations was compared to growth of plants on complete MS medium. The cystine concentration which resulted in growth phenotype similar to plants on complete MS would be used for selection.

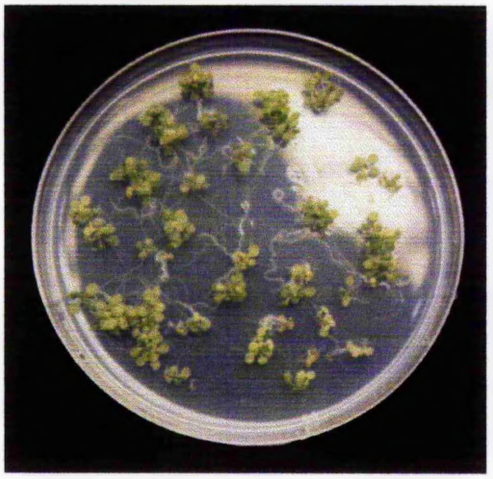
Plants growing with 0.01 and 0.05 mM cystine as the sole sulphur source had small yellow leaves, a feature of plants exposed to sulphur-starvation, and a shorter root system than the plants on complete MS. Plants growing with 1 mM cystine or higher as the sole sulphur source had small leaves and had a very poorly developed root system. 0.5 mM cystine was found to induce growth most similar to that of plants on complete MS, so was chosen for selection of the SAT antisense lines (Figure 5.4).

5.2.3 Vacuum infiltration and selection of transgenic lines

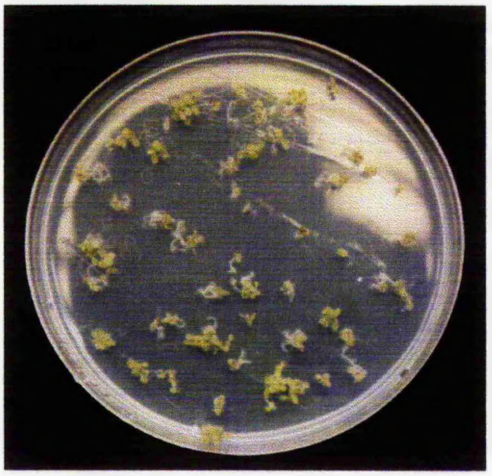
The SAT antisense vector, pSLJ-52A, was transformed into *A. tumefaciens* strain GV3101 by triparental mating as described in section 2.17.2. Strain GV3101

Figure 5.4 Choice of medium for selection of *A. thaliana* plants transformed with antisense vector pSLJ-52A. Transgenic plants exhibiting extemely low SAT activity may be difficult to select on complete MS medium due to a build up of toxic sulphide which cannot be incorporated into cysteine. Reduced-sulphur containing compounds when provided as the sole sulphur source should allow 'normal' growth in the absence of SAT activity. Wild-type growth on media containing various concentrations of reduced-sulphur (in form of cystine) as a sole sulphur source was compared to growth on complete MS to determine the concentration most suitable for the selection medium. a) Wild-type *A. thaliana* plants growing on complete MS medium (control). Wild-type *A. thaliana* plants growing on b) 0.01 mM, c) 0.05 mM, d) 0.5 mM and e) 1 mM cystine as the sole sulphur source are shown for phenotype comparison to the control plants.

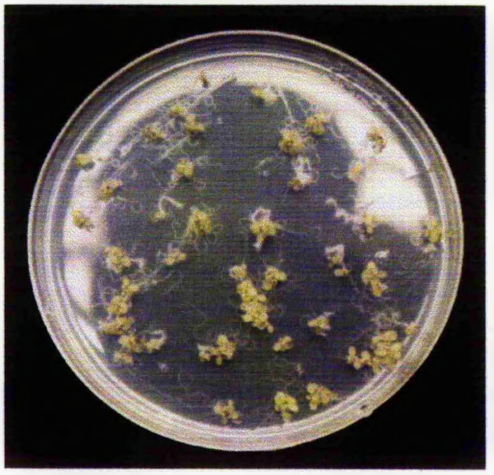
a) control (complete MS)



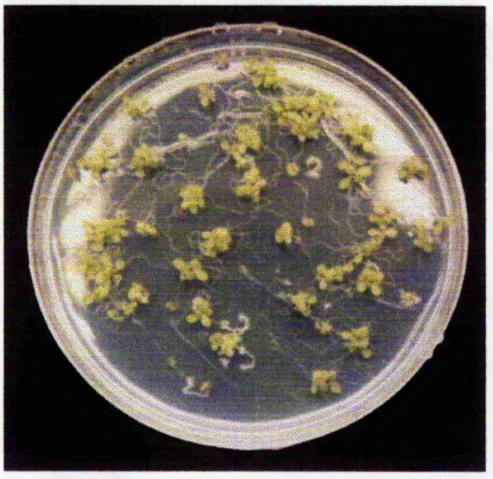
b) 0.01 mM cystine



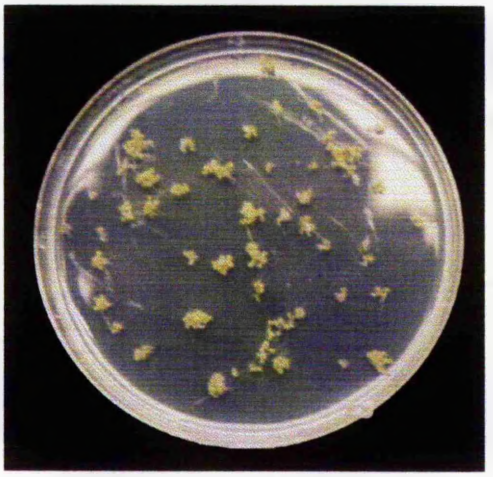
c) 0.05 mM cystine



d) 0.5 mM cystine



e) 1 mM cystine

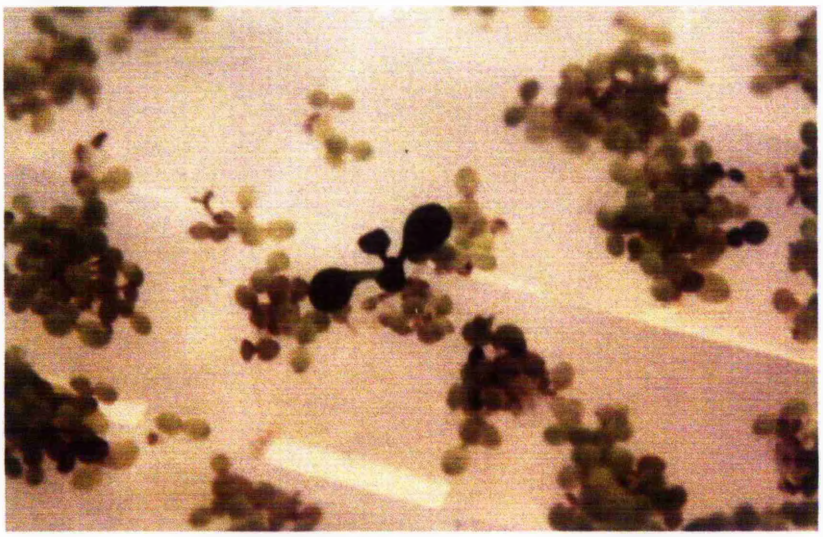


possesses a chromosomally-located rifampicin resistance gene for selection purposes and contains the disarmed Ti plasmid C58pTi, which is stably maintained at temperatures below 29°C. *A. tumefaciens* cells harbouring the pSLJ-52A vector after triparental mating were selected on LB agar plates containing tetracycline (to which pSLJ-52A confers resistance) and rifampicin. *A. thaliana* plants were infiltrated with *A. tumefaciens* containing the antisense vector under a 650 mmHg vacuum for 5 to 10 minutes. Seed from infiltrated plants (the T_1 generation) were sterilised and sown on modified MS medium containing 0.5 mM cystine as a sole sulphur source (see section 5.2.2) and 30 mg/l kanamycin to select for transformants.

Two batches of 100 plants were separately infiltrated and the T_1 seed sown on selection medium. One transformant was selected from the first batch and 6 from the second. The seven transgenic lines were catalogued as At52A1 to At52A7. Transformants were easily identified on the selection plates after 1 to 2 weeks of growth. After this time-period transformants have well-developed dark green leaves and an established root system which penetrates the solid selection medium (Figure 5.5a). Non-transformed plants have poorly-developed yellowing or white leaves and very small roots which do not penetrate the selection medium.

The presence of the transgene was verified in the At52A1 genome by PCR amplification of the *nptII* gene from leaf discs (Figure 5.5b) as described in section 2.17.4. Amplification of part of the endogenous *Sat-53* gene was also carried out as a positive control to make sure the leaf disc PCR amplification method was working. Primers used are outlined in Table 5.1. Leaf disc PCR on At52A2 to At52A7 proved unsuccessful with no amplification of either the *nptII* or endogenous control gene. These PCR amplifications were not repeated to avoid killing the plants by excising more leaf discs. Transgene detection by PCR will be carried out on kanamycin-resistant plants from the next generation of these transgenic lines.

a)



b)

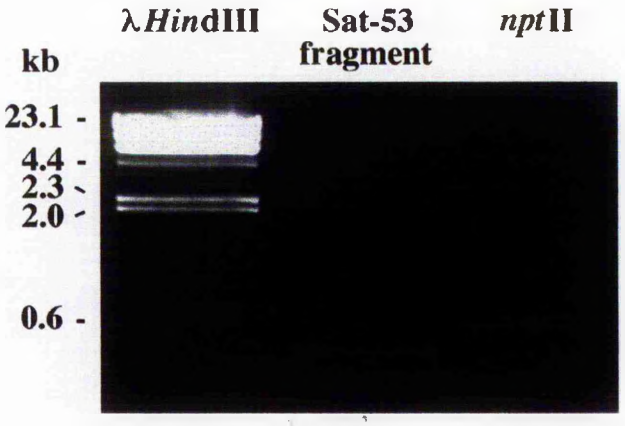


Figure 5.5 a) Appearance of antisense transformant At52A1 growing on selection medium containing 30 mg/l kanamycin and 0.5 mM cystine as the sole sulphur source.

b) Verification of transgene incorporation into the genome of At52A1 by PCR amplification from leaf discs. Lane 1 λ*Hin*dIII base pair markers. Lane 2 control amplification of a fragment of the endogenous *Sat-53* gene. Lane 3 amplification of the *npt*II fragment from leaf discs, indicating the presence of the transgene in the At52A1 genome. Primers used are detailed in Table 5.1.

Table 5.1 Oligonucleotide primers used in transgenic and expression vector construction and transgenic analysis

Primer	Used for:-	Primer sequence (5'→3')
53pBI5'	Amplification of Sat-53 for pBI121 cloning, incorporating 5' *Bam*HI site (underlined)	CGGGATCCATGGCAACATGCATAGAC
53pBI3'	Amplification of Sat-53 for pBI121 cloning, incorporating 5' *Sac*I site (underlined)	CGGAGCTCGTTAAATCACATAATCAG
*npt*II 5'	Amplification of *npt*II gene for verification of T-DNA transfer to plant genome (5')	ATGATTGAACAAGATGGATTG
*npt*II 3'	Amplification of *npt*II gene for verification of T-DNA transfer to plant genome (5')	CTTGAGCCTGGCGAACAGTTCG
53CYB5'	Amplification of endogenous Sat-53 gene fragment as control for leaf disc PCR (5')	GACCATGGCAACATGCATAGACAC
53.gspp	Amplification of endogenous Sat-53 gene fragment as control for leaf disc PCR (3')	CTTTCTTCTAAAACGCTTATG
52GEX5'	Amplification of Sat-52 for pGEX cloning, incorporating a 5' *Eco*RI site (underlined)	AGGAATTCAATATGCCACCGGCCG
52GEX3'	Amplification of Sat-52 for pGEX cloning, incorporating a 5' *Xho*I site (underlined)	AGCTCGAGATAAGCGAATGGTCAG
1CYB5'	Amplification of Sat-1 for pCYB4 cloning, incorporating a 5' *Nco*I site (underlined)	GACCATGGTGCCGGTCACAAGTCGC
1CYB3'	Amplification of Sat-1 for pCYB4 cloning, incorporating a 5' *Xho*I site (underlined)	GACTCGAGAATTACATAATCCGACC
53CYB5'	Amplification of Sat-53 for pCYB4 cloning, incorporating a 5' *Nco*I site (underlined)	GACCATGGCAACATGCATAGACAC
53CYB3'	Amplification of Sat-53 for pCYB4 cloning, incorporating a 5' *Xho*I site (underlined)	GACTCGAGAATCACATAATCAGACCACTCG

This is the stage to which the SAT antisense transformants had been characterised by the end of this study. The future direction of the analysis of these transformants will be referred to in the Discussion.

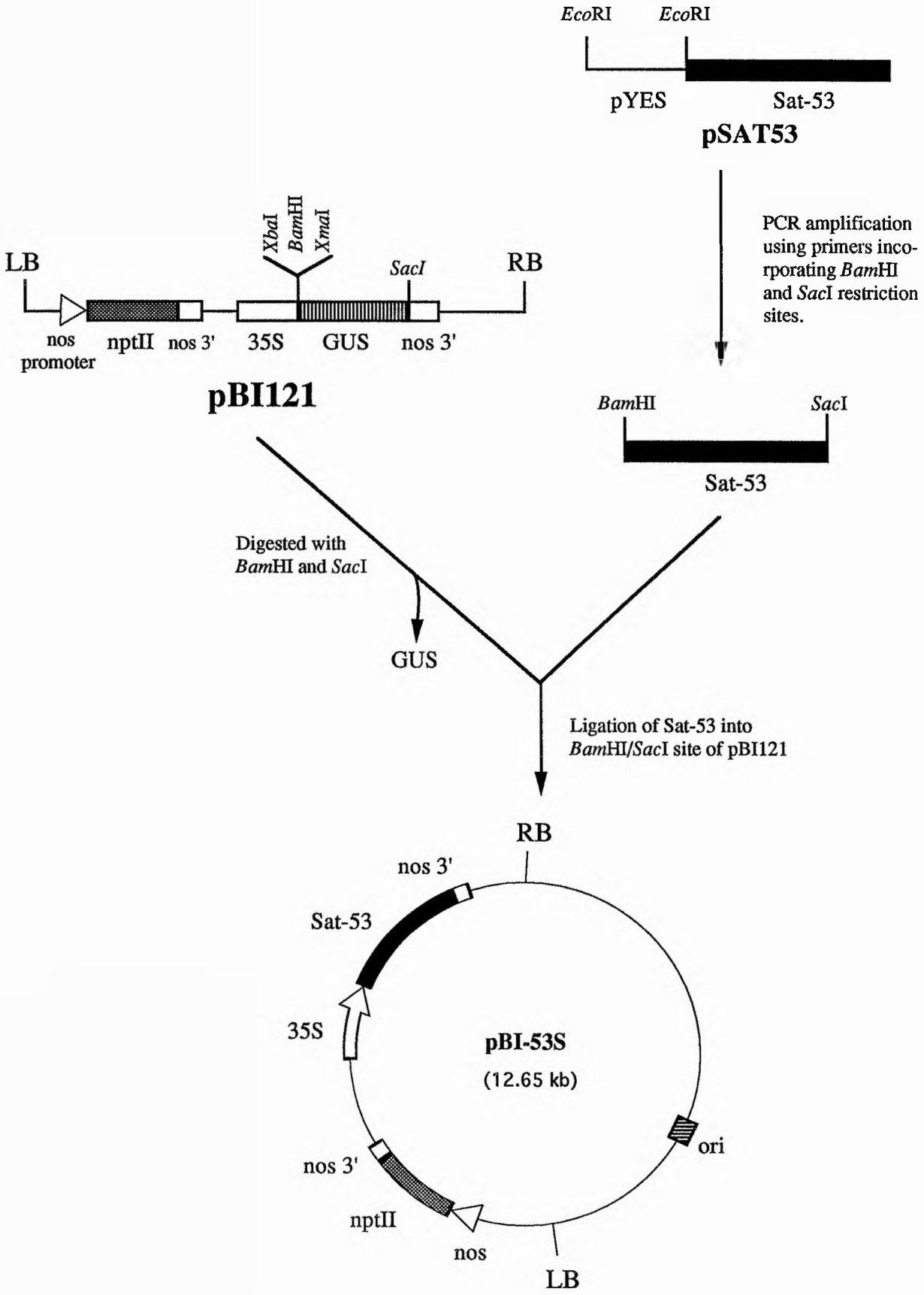
5.2.4 Design and construction of a Sat-53 sense vector

As outlined in the introduction to this chapter, one aim of the transgenic analysis of SAT is to overexpress each isoform individually in *A. thaliana*. A sense vector for the overexpression of Sat-53, which encodes a putatively cytoplasmic isoform of SAT, was designed, constructed and used to infiltrate *A. thaliana* plants. The preparation of the Sat-53 sense construct is outlined below and also shown diagrammatically in Figure 5.6. No transformants had been isolated from T_1 seed by the end of this study, but further infiltrations will be carried out in future.

The two step subcloning procedure required, and difficulties in the manipulation of the pSLJ series of vectors due to their large size (~25.7 kb before transgene insertion), meant that a different binary vector was chosen for the preparation of the Sat-53 sense construct. The vector pBI121, based on pBIN19 (Bevan 1984), has promoter, MCS and termination sequrences included in the binary vector and also has a relatively small size (13 kb). Consequently, transgenic constructs can be assembled more easily in a single subcloning step.

pBI121 possesses CaMV 35S promoter and *nos* termination sequences flanking an MCS, and contains a GUS gene between the promoter and termination sequences. For the production of the Sat-53 sense construct, the GUS gene was excised by digesting pBI121 with *Bam*HI and *Sac*I. The Sat-53 cDNA coding region (nucleotides 50 to 995 on Figure 3.4) was then amplified by PCR using primers incorporating *Bam*HI and *Sac*I restriction sites at the 5' and 3' ends respectively (Table 5.1). High efficiency *Pfu* DNA polymerase was used in PCR amplification reactions to minimise the chance of errors in nucleotide incorporation into the Sat-53 transgene (see section 2.7.2). The PCR product was

Figure 5.6 Schematic representation of Sat-53 sense vector construction. Steps involved in subcloning the Sat-53 cDNA into the multicloning site of pBI121 and the resulting 12.65 kb binary vector pBI-53S are shown.


EcoRI
EcoRI
pYES
Sat-53
pSAT53
PCR amplification using primers incorporating BamHI and SacI restriction sites.
LB
XbaI
BamHI
XmaI
SacI
RB
nos promoter
nptII
nos 3'
35S
GUS
nos 3'
pBI121
BamHI
SacI
Sat-53
Digested with BamHI and SacI
GUS
Ligation of Sat-53 into BamHI/SacI site of pBI121
RB
nos 3'
Sat-53
35S
pBI-53S
(12.65 kb)
ori
nos 3'
nptII
nos
LB

cloned into the vector pGEM-T by T-A overhang ligation (Promega Protocols and Applications Guide), for ease of manipulation. The Sat-53 coding region was then excised by digestion with *Bam*HI and *Sac*I, and directionally subcloned into the *Bam*HI and *Sac*I sites of pBI121. The resulting Sat-53 sense construct was named pBI-53S and was transformed into *A. tumefaciens* strain GV3101 by triparental mating as described in section 2.17.2. Two unsuccessful attempts were made to transform *A. thaliana* plants with pBI-53S by vacuum infiltration. No positive transformants were selected when the T_1 seed were sown onto MS selection medium containing 30 mg/l kanamycin. Infiltrations will be repeated in future.

5.3 Polyclonal Antibody Production

5.3.1 Expression and purification of recombinant SAT52 protein

Phenotype variations resulting from genetic transformation of plants are manifested by altered levels of the enzyme encoded by the transgene. The abundance of the enzyme manipulated in transgenic plants can be compared to levels in wild-type plants by western blotting, if antibodies specific to the protein are available. This section describes the expression and purification of recombinant SAT52 protein to be used in polyclonal antibody production. The SAT52 antiserum can then be used in the analysis of plants with genetically modified SAT activity. Due to the nature of polyclonal antibodies, it cannot be predicted whether serum raised against purified SAT52 will cross-react with other SAT isoforms which share high levels of amino acid homology (see Table 3.6). Cross-reactivity of the SAT52 antiserum will be tested against purified recombinant SAT1, SAT53 and SAT106 proteins (see section 5.3.3). If the SAT52 antiserum is specific to the SAT52 protein, antibodies specific to the other isoforms may similarly be raised for the analysis of transgenic lines. If the SAT52

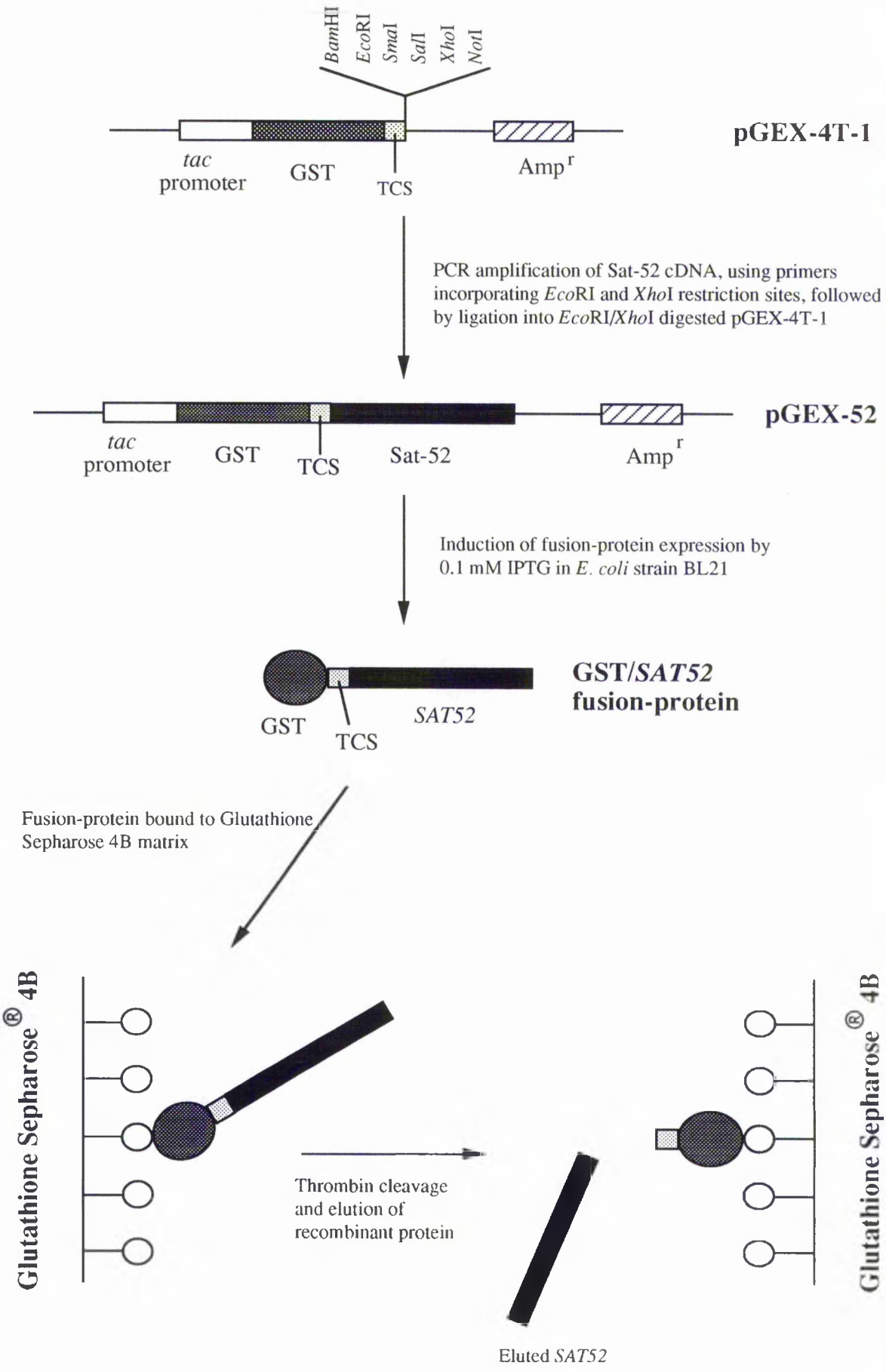
antiserum cross-reacts with all the SAT isoforms it will still be useful in the quantification of total SAT protein in *A. thaliana* extracts.

SAT52 was expressed as a GST-fusion protein as described in section 5.3.1 using the pGEX expression vector system (Figure 5.7). The coding region of the Sat-52 cDNA was amplified by PCR using high-efficiency *Pfu* DNA polymerase and cloned into the vector pGEM-T by T-A overhang ligation (Promega Protocols and Applications Guide). The PCR-amplified insert was then fully sequenced to verify correct nucleotide incorporation. The 5' and 3' primers used for Sat-52 amplification (Table 5.1) incorporated *Eco*RI and *Xho*I restriction sites respectively to allow directional subcloning into the pGEX vector. The primers were also designed so that the expressed SAT52 protein was in-frame with the GST and thrombin cleavage domain of vector pGEX-4T-1. Sat-52 was excised from pGEM-T by *Eco*RI and *Xho*I digestion and ligated into pGEX-4T-1, which had also been digested with *Eco*RI and *Xho*I. The resulting plasmid was named pGEX-52.

For expression of the GST/SAT52 fusion protein, pGEX-52 was transformed into *E. coli* strain BL21 to form strain BL21-52. BL21-52 was screened for fusion protein expression on a small scale prior to large scale purification. A 5 ml culture with an OD_{600} of 0.6 was induced with 0.1 mM IPTG, incubated at 20°C for 120 mins and the crude protein extract was compared to that of a similarly grown uninduced culture. 25 µl of each bacterial culture was boiled for 5 mins and spun briefly in a microfuge. Proteins in the supernatants were then separated by polyacrylamide gel electrophoresis (PAGE). Figure 5.8a shows that the induced culture, compared to the uninduced, expressed high levels of a protein with a molecular weight of approximately 62 kD, the predicted size of a GST/SAT52 fusion protein (SAT52 32.7 kD: GST 29 kD).

The SAT52 recombinant protein was next purified from a large scale culture by the system outlined in Figure 5.7. BL21-52 was grown in a 250 ml culture induced at 20°C over night, and the cells disrupted by sonication. After

Figure 5.7 Schematic representation of SAT52 expression vector construction and subsequent purification of the recombinant SAT52 protein using the GST (glutathione S-transferase)-fusion protein purification system (Pharmacia Biotech, UK). Thrombin Cleavage Site is abbreviated to **TCS.**

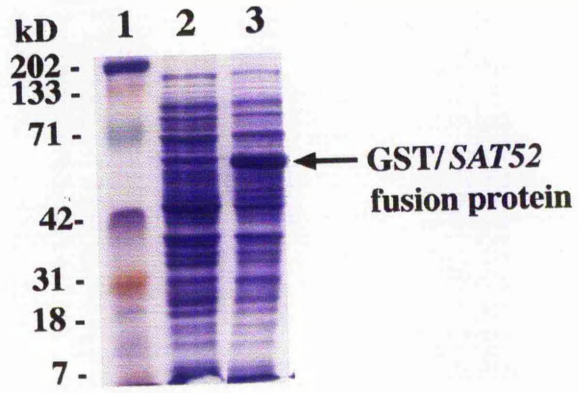

BamHI
EcoRI
SmaI
SalI
XhoI
NotI
pGEX-4T-1
tac promoter
GST
TCS
Amp r
PCR amplification of Sat-52 cDNA, using primers incorporating EcoRI and XhoI restriction sites, followed by ligation into EcoRI/XhoI digested pGEX-4T-1
pGEX-52
tac promoter
GST
TCS
Sat-52
Amp r
Induction of fusion-protein expression by 0.1 mM IPTG in E. coli strain BL21
GST
TCS
SAT52
GST/SAT52 fusion-protein
Fusion-protein bound to Glutathione Sepharose 4B matrix
Glutathione Sepharose® 4B
Thrombin cleavage and elution of recombinant protein
Glutathione Sepharose® 4B
Eluted SAT52

centrifuging the sonicate, the supernatant was applied to a Glutathione Sepharose® 4B column as described in section 2.15.6. Figure 5.8b shows 20 µl protein samples from various stages of the purification procedure separated by PAGE. Molecular weight protein markers are shown in Lane 1. Lanes 2 and 3 contain protein samples from the pellet and sonicate of the induced BL21-52 culture respectively. Lane 4 contains the BL21-52 sonicate flow-through collected after passing through the Glutathione Sepharose® 4B column. Lanes 5 to 8 contain samples of the first four 1 ml GST/SAT52 fusion protein elutions (elution was with 5 mM glutathione in 1x PBS). The first three of these elutions (Lanes 5 to 7) were pooled, as they contained the majority of the fusion protein. To cleave the SAT52 from the GST domain, the eluted fusion protein was mixed with 3 ml of 50% Glutathione Sepharose® 4B for 4 hours at room temperature. After washing with four rinses of 1x PBS, the Sepharose was resuspended in 1 ml of 1x PBS. The SAT52 was then cleaved from the GST domain by incubation over night with 5 U of thrombin and the sepharose-bound GST was removed by centrifugation. Lane 9 contains a 20 µl sample of the supernatant containing the purified, recombinant SAT52 protein.

The protein concentration in the three pooled elutions, used in the thrombin cleavage step, was quantified at 0.41 µg/µl by Bradford's protein quantification assay (section 2.15.2). The concentration of the purified SAT52 sample (Lane 9 on Figure 5.8b) was quantified at 0.66 µg/µl.

It is noted that the GST/SAT52 band in Figure 5.8b (Lanes 5 to 8) appears larger than the expected 62 kD when compared to the molecular markers in Lane 1. However, the Sat-52 insert of pGEX-52 was verified as being in frame with the GST reading-frame by sequence analysis, and the GST/SAT52 band in Figure 5.8a when compared to a different range of molecular markers is measured at approximately 62 kD. This suggests that the markers in Figure 5.8b are not accurately representing their apparent molecular weights.

a)



b)

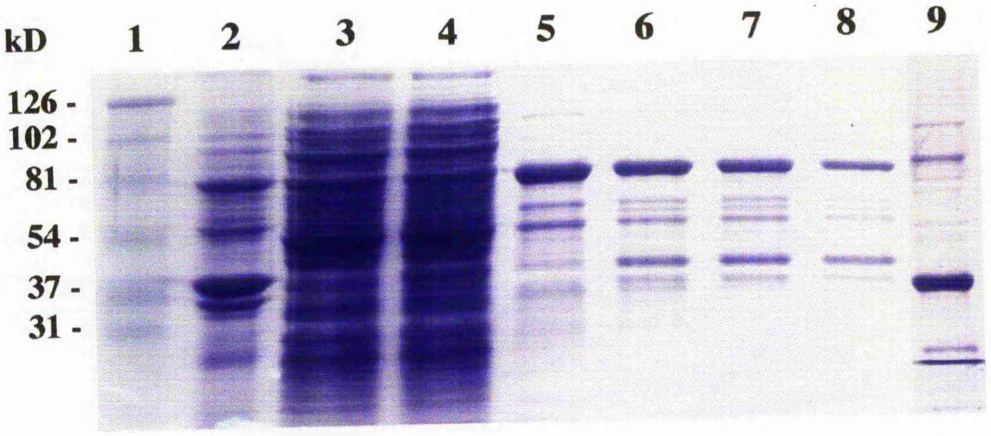


Figure 5.8 a) SDS-PAGE gel showing small-scale induction of GST/SAT52 fusion protein expression. Lane **1** molecular weight markers, **2** bacterial proteins from an uninduced culture and **3** bacterial proteins from a culture induced by 0.1 mM IPTG for 120 mins. 20 µl of protein sample was loaded in each lane. The 62 kD fusion protein band in Lane 3 is indicated by an arrow.

b) Stages of SAT52 purification. Lane **1** molecular weight markers, **2** and **3** protein samples from the pellet and sonicate of the induced BL21-52 culture respectively, **4** sonicate flow-through from the Glutathione Sepharose® 4B column, **5** to **8** samples of the first four 1 ml elutions of the GST/SAT52 fusion protein and **9** purified recombinant SAT52 protein after thrombin cleavage from the GST domain. 20 µl of protein sample was loaded in each lane.

5.3.2 Production of polyclonal antibodies to recombinant SAT52 protein

Polyclonal antibodies were raised to the SAT52 protein in rabbit. A total of 300 µl (198 µg) of the purified protein sample was injected subcutaneously at several sites on the back of the rabbit's neck for the primary inoculation. After 6 days, when antibody titre is expected to be at its peak (Harlow & Lane 1988), a 5 ml test bleed was taken from the rabbit and the polyclonal serum collected as described in section 2.16.3. Four weeks later a secondary 'boost' injection of 100 µl (66 µg) was administered intravenously to induce a secondary immune response, increasing the titre of SAT52 specific antibodies in the serum. A test bleed was again taken 6 days after the second injection. Three more intravenous injections were made at 4 week intervals, a test bleed being taken each time to assess the specificity of the antiserum to SAT52 protein. A 50 ml fatal bleed was taken from the rabbit 6 days after the final injection. Figure 5.9a shows the difference between the antiserum from the first and final fifth bleed when used in western blots against the purified SAT52 sample used for immunisation. Antiserum from the first bleed binds to many bacterial protein bands which were not visible when the protein sample was separated by PAGE (Figure 5.8b). The non-specific bands may be bacterial proteins to which the rabbit had already built up immunity. Antiserum from the final bleed also shows some hybridisation to proteins of different size to that expected for SAT52. However, the majority of hybridisation signal is to the recombinant SAT52 protein of approximately 33 kD. The final antiserum was tested for cross-hybridisation with the SAT53 protein from *A. thaliana* and also against plant protein extracts (see section 5.3.4).

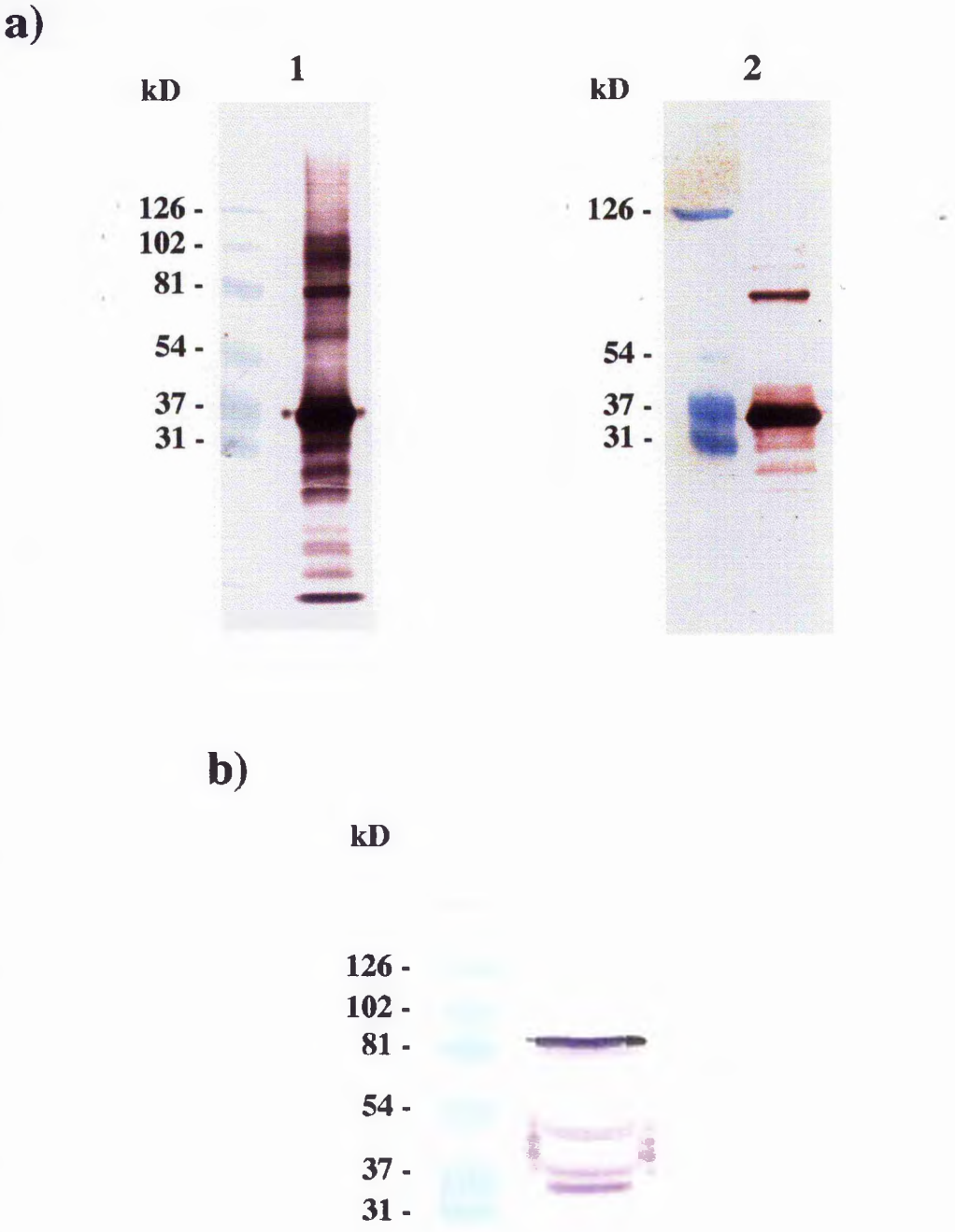


Figure 5.9 a) Western blots of SAT52 polyclonal antiserum from the first (1) and final (2) bleeds, against purified recombinant SAT52 protein.

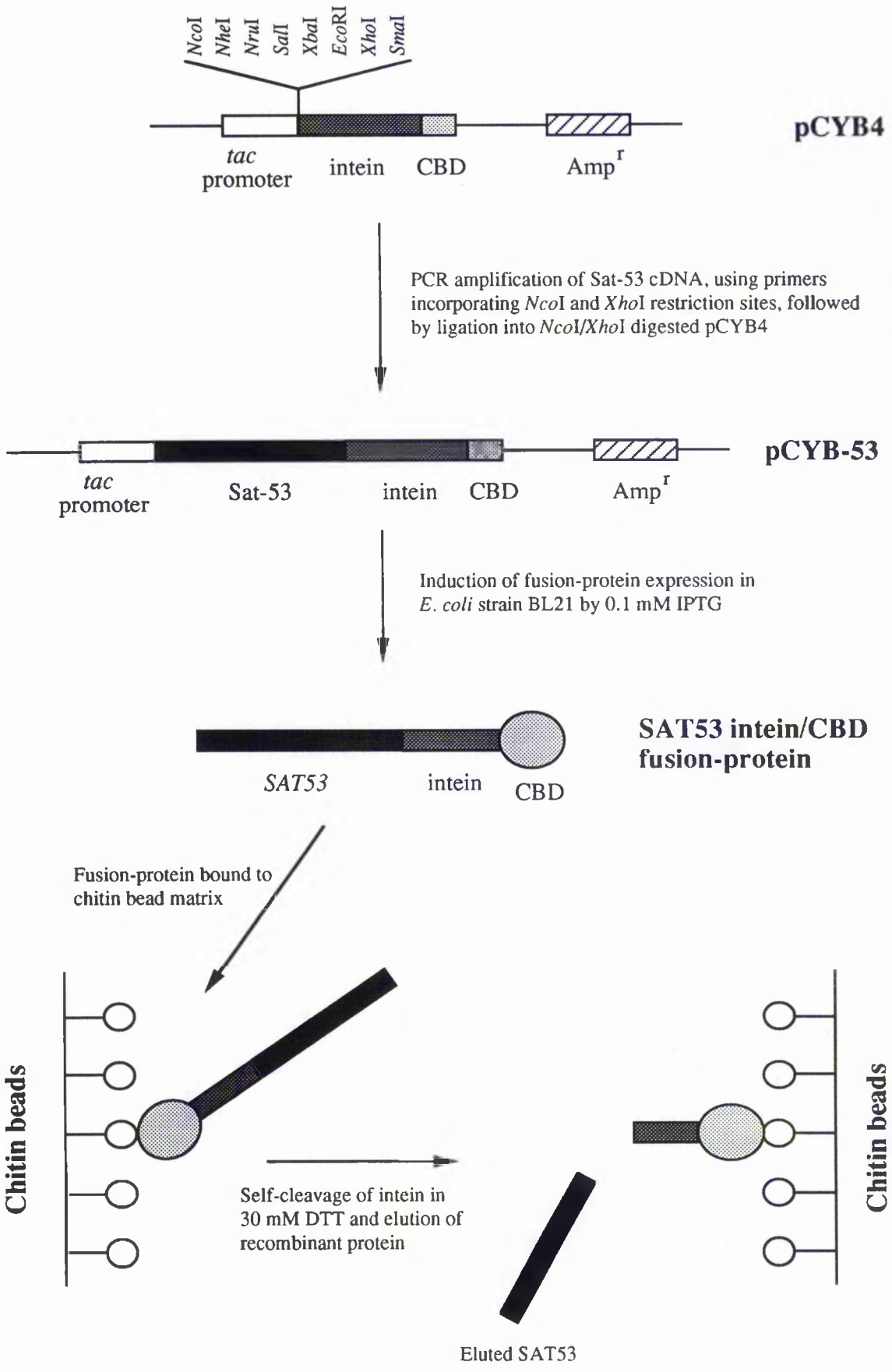
b) Western blot of polyclonal SAT52 antiserum from final bleed, against 25 μg of *A.thaliana* protein.

5.3.3 Cross-reaction of SAT52 antibodies with recombinant SAT53 protein

To analyse western blots using the polyclonal antiserum raised to SAT52 in section 5.4.2, it was necessary to determine whether it will also cross-hybridise with the other SAT isoforms from *A. thaliana*. Expression and purification of recombinant SAT1 and SAT53 protein was attempted using the pCYB vector of the IMPACT™ I system (Figure 5.10) as described in the introduction to this chapter. The Sat-106 cDNA was isolated after the expression experiments described in this section, but will also be purified and tested for cross-reactivity with the SAT52 polyclonal antiserum in the future.

Subcloning of the Sat-1 and Sat-53 coding regions into pCYB4 was performed essentially as described for subcloning Sat-52 into pGEX-4T-1. However, as the intein/chitin-binding domain is fused to the C-terminus of the protein to be purified, the 3' stop codon of the amplified cDNA is not included. 5' and 3' primers were designed incorporating *Nco*I and *Xho*I restriction sites (Table 5.1) for directional cloning into *Nco*I/*Xho*I digested pCYB4. Primers were also designed to ensure the cDNA reading frames were in-frame with the intein coding domain at the 3' end, and that the ATG of the *Nco*I cloning site (C↓CATGG) encoded the initiating methionine residue on transcription. Using the *Nco*I restriction site in this way ensures that the recombinant protein contains no vector derived amino acid residues at the N-terminus. The enforced guanine (G) nucleotide at position 4 of the coding region, when using this cloning strategy, meant that the second amino acid of the recombinant SAT1 was expressed as a valine instead of a leucine residue. This mutation at position 2 is likely to have a negligible effect on either antigenicity or activity of the recombinant SAT1 protein. As nucleotide 4 of the Sat-53 cDNA coding region is a guanine, no alterations in amino acid sequence are caused by the use of the *Nco*I cloning site of the pCYB4 vector for this cDNA. The resulting plasmids for SAT1 and SAT53 expression were named pCYB-1 and pCYB-53 respectively.

Figure 5.10 Schematic representation of SAT53 expression vector construction and subsequent purification of the recombinant SAT53 protein using the IMPACT™ I: One step purification system (New England Biolabs Ltd, UK). Chitin-binding domain is abbreviated to **CBD**.


NcoI
NheI
NruI
SalI
XbaI
EcoRI
XhoI
SmaI
tac promoter
intein
CBD
Amp^r
pCYB4
PCR amplification of Sat-53 cDNA, using primers incorporating NcoI and XhoI restriction sites, followed by ligation into NcoI/XhoI digested pCYB4
tac promoter
Sat-53
intein
CBD
Amp^r
pCYB-53
Induction of fusion-protein expression in E. coli strain BL21 by 0.1 mM IPTG
SAT53
intein
CBD
SAT53 intein/CBD fusion-protein
Fusion-protein bound to chitin bead matrix
Chitin beads
Self-cleavage of intein in 30 mM DTT and elution of recombinant protein
Chitin beads
Eluted SAT53

pCYB-1 and pCYB-53 were expressed in BL21 and purified on chitin bead columns (see Figure 5.10). Purification of the SAT1 protein proved unsuccessful and must be repeated in future. Figure 5.11a shows the Coomassie Blue stained protein gel from the SAT53 purification procedure. The final purified sample contained 0.33 μg/μl of SAT53 protein. Approximately 10 μg of purified SAT52 and SAT53 protein were blotted for western hybridisation to determine the specificity of the antiserum raised to SAT52. As shown in Figure 5.11b no cross-reaction was observed between the SAT52 antiserum and the SAT53 protein.

5.3.4 Detection of SAT52 in plant protein extracts

The antiserum raised against SAT52 in section 5.3.2 was used to detect SAT protein in *A. thaliana* leaf extracts (Figure 5.9b). Amino acid sequence analysis suggests that the SAT52 protein is targeted to the mitochondrion. Section 3.2.13 describes several features of the N-terminal 45 amino acids of SAT52 that resemble plant mitochondrial targeting peptides. As targeting peptides are cleaved during organelle import, the mature SAT52 protein is expected to have a molecular weight of approximately 29 kD, although a definite cleavage site, and therefore mature protein size, has not been determined. The SAT1 protein, to which the SAT52 antiserum may cross-hybridise, encodes a putative chloroplastic isoform and will also have its targeting peptide cleaved. The mature SAT1 protein may also be expected to have a molecular weight of approximately 29 kD. As the SAT52 antiserum does not appear to cross-hybridise with the SAT53 recombinant protein (section 5.3.3), it is not expected to hybridise to the SAT53 plant protein. However, if the SAT52 antiserum binds to SAT1 or SAT106, several bands may be detected in *A. thaliana* protein extracts between 29 and 35 kD.

A protein extraction from wild-type *A. thaliana* leaf tissue was subject to western blotting and hybridised with SAT52-raised antiserum as

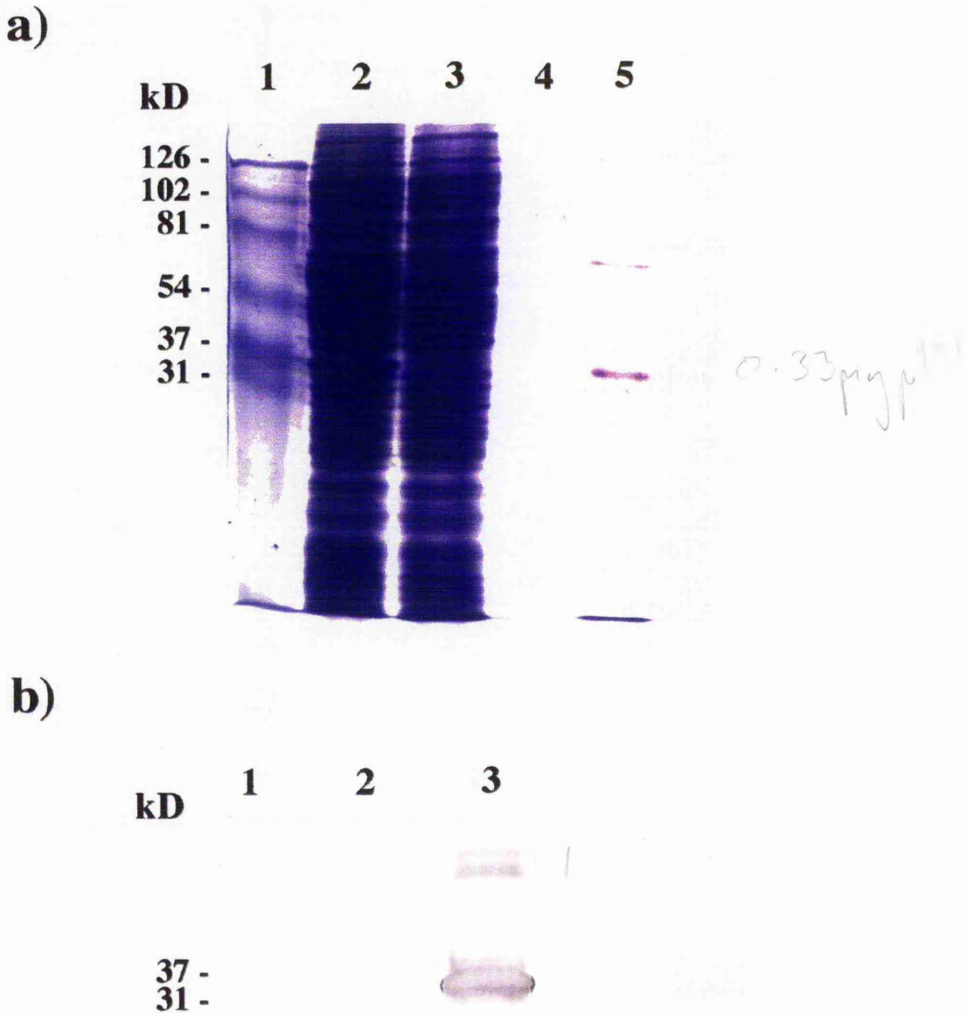


Figure 5.11 a) SDS-PAGE gel showing stages of SAT53 purification. Lane **1** molecular weight markers, **2** induced bacterial sonicate prior to loading on the chitin-bead purification column, **3** sonicate after passing through the purification column, **4** elutant from the column after a 5 minute incubation with cleavage buffer to distribute the DTT throughout the chitin bead matrix and **5** sample of the eluted SAT53 protein after overnight incubation with cleavage buffer. 20 µl of protein sample was loaded in each lane.

b) The SAT52 polyclonal antiserum tested for cross-reactivity with the SAT53 protein purified as shown in a). 10 µg of SAT53 and SAT52 were blotted on to nitrocellulose (Lanes **2** and **3** respectively) and subjected to western blotting with the SAT52 antiserum. Lane **1** contains molecular weight markers.

described in sections 2.15.1, 2.15.4 and 2.15.5. Approximately 25 μg of *A. thaliana* leaf protein was blotted on to nitrocellulose and hybridised with a 1:500 dilution of the SAT52 antiserum. As shown in Figure 5.9b, several plant proteins were found to hybridise with the antiserum. Two bands of approximately the size expected for SAT proteins were detected at approximately 29 and 34 kD. The band of 29 kD may represent the mature SAT52 protein. Whilst two bands of the expected size for SAT proteins were detected in plant protein extracts by the SAT52 antiserum, hybridisation with several larger proteins was also observed. The most intensely hybridising band represents a protein of approximately 81 kD. This is more than 2.5 times the expected molecular weight of the mature SAT52 protein. An attempt will be made to purify antibodies specific to SAT52 from the polyclonal antiserum to eliminate hybridisation of non-specific antibodies.

5.4 Discussion

This chapter describes the initial steps towards the study of SAT in *A. thaliana* using transgenic technology. The pBI121 binary vector proved to be a much easier system for the production of transgenic constructs than the pSLJ system due to the single subcloning step involved an ease of manipulation, and will now be used to prepare the remaining sense constructs for overexpression of Sat-1, Sat-52 and Sat-106. Once introduced into *A. tumefaciens* strain GV3101, vacuum infiltration of *A. thaliana* will be performed as described for the Sat-52 antisense vector. Transformation with the Sat-53 sense vector, already constructed and transformed into GV3101, must also be repeated due to the lack of success from the first attempts.

The seven transgenic lines, At52A1 to At52A7, selected as positive transformants on kanamycin containing growth media can now be characterised. Initially, PCR on genomic DNA will be performed on each line using primers specific to the *nptII* gene to verify the presence of the transgene. The At52A1

genome has already been shown to contain the *nptII* gene using this method (Figure 5.5b), but the other lines remain to be tested. Following verification of transgene insertion by PCR, homozygous lines will be isolated in which the T-DNA insertion is inherited by all progeny of subsequent generations. This will be done by plating several generations of seed from each transgenic line on kanamycin-containing selection media. Segregation during the reproductive phase of the *A. thaliana* life-cycle means that the T_2 generation seed will include a mixture of homozygous positive, heterozygous positive and homozygous negative lines, due to Mendelian inheritance of each transgene insertion. For experimental purposes, homozygous transgenic lines are essential to ensure that 100% of the seed from each generation exhibits uniform level of transgene expression. Transgenic lines producing 100% kanamycin resistant seed at the T_4 generation will be assumed to be homozygous.

Once homozygous transgenic lines have been selected the level of transgene expression, compared to wild type plants, will be assessed by northern hybridisation. Lines will then be chosen which show high levels of transgene expression for analysis of modulated SAT expression on a variety of related genes and metabolic intermediates. Comparisons of *O*AS, cysteine and glutathione concentrations between wild-type and transgenic *A. thaliana* plants can be carried out using established HPLC methods described by Rauser et al. (1991) and Kim et al. (1997). Together with northern analysis of transcript levels of the other genes involved in sulphate reduction and cysteine biosynthesis, data from transgenic experiments should begin to elucidate the contribution of the SAT gene-family members to cysteine biosynthesis. The regulatory role of SAT in sulphate reduction and cysteine biosynthesis will also be further clarified. As mentioned in section 5.2.1, it is not clear what effect the SAT antisense mRNA will have on expression of the individual members of the SAT gene-family. The relatively high nucleotide homology between the Sat-52 antisense fragment and the Sat-1, Sat-53 and Sat-106 sequences may result in down-regulation of these

genes to different extents. Assessment of the mRNA levels of each SAT gene in the At52A lines will indicate the overall effect on SAT expression.

Section 5.3 of this chapter described the preparation of antibodies for use in the analysis of the transgenic plants. The polyclonal antiserum from a rabbit immunised with recombinant SAT52 strongly hybridised with purified SAT52 but not SAT53 protein (Figure 5.11b). This may indicate that the polyclonal serum is specific only to the SAT52 isoform. The hybridisation of SAT52 antiserum to two plant proteins of between 29 and 35 kD may suggest some cross-hybridisation to other SAT isoforms. SAT1 and SAT106 will have to be expressed and tested for cross-hybridisation to verify this.

When the SAT52 antiserum was used in western blots against *A. thaliana* proteins, several bands were detected of molecular sizes much larger than expected for SAT (Figure 5.9b). One of these bands represented a protein of approximately 81 kD and was several times as intense as any of the bands of the expected size for SAT. Non-specific bands may be eliminated by purification of SAT52-specific antibodies from the polyclonal antiserum. Purification of SAT52-specific antibodies from the polyclonal serum can be achieved by adsorption to and elution from their antigen immobilised on a nitrocellulose filter as described in Sambrook et al. (1989). A sample of the recombinant SAT52 protein, to which antibodies were raised, can be loaded along the entire width of an SDS-PAGE gel for electrophoretic separation. After blotting on to nitrocellulose as described in sections 2.15.4 and 2.15.5, the filter can be hybridised with the polyclonal antiserum. A strip of 5mm is then cut from each side of the membrane and these strips subjected to the remaining steps of western analysis and chromogenic visualisation of hybridising bands (section 2.15.5). The stained strips are then realigned with the rest of the filter and a strip of nitrocellulose in the position of the SAT52 band cut out with a scalpel. SAT52-specific antibodies can then be eluted from this strip into a buffer at pH 2.8. The same nitrocellulose strip can be reused for purification up to five times

As the SAT52 antiserum cross reacts with several bands around the expected size of SAT proteins (Figure 5.9b), a strategy to determine which band represents the SAT52 protein is required. Firstly, western blots against *A. thaliana* protein will be carried out using SAT52-specific antibodies purified as outlined above. The ability of the antiserum to cross-hybridise with the other SAT isoforms will then be fully investigated using purified recombinant SAT1, SAT53 and SAT106. If several bands of the approximate size of SAT proteins are still obtained using purified antibodies, determining which band represents SAT1, SAT52, SAT53 or SAT106 should be possible by comparing western hybridisations of protein extracts from wild-type and sense transgenic lines over-expressing the Sat-1, Sat-52, Sat-53 and Sat-106 cDNAs respectively.

Chapter 6: Final Discussion and Future Work

6.1 Discussion and Future Work

The work presented in this thesis represents a detailed molecular study of the serine acetyltransferase gene-family in *Arabidopsis thaliana*, and has initiated research into the regulatory role of the enzyme in the biosynthesis of cysteine. SAT has been shown to be genetically encoded by at least four members of a small gene-family. Three novel cDNA members, Sat-52, Sat-53 and Sat-106, have been isolated and sequenced in addition to the one, Sat-1, cloned previously in this laboratory (Roberts and Wray 1996). Sat-53 is homologous to the *sat5* gene published during this study by Ruffet et al. (1995). Any further members of the gene-family will be detected by the ongoing Arabidopsis Genome Initiative (AGI) sequencing project which promises to be the ultimate genetic resource for *A. thaliana* molecular research in the future.

One major obstacle facing SAT research in *A. thaliana* is the subcellular locations to which the four isoforms are targeted. Experimental determination of subcellular locations of the four SAT isoforms should be carried out as soon as possible. The amino acid sequence analysis in section 3.2.13 (Figure 3.17) gives a good indication that Sat-1 and Sat-52 encode plastidic and mitochondrial isoforms respectively, and that Sat-53 and Sat-106 encode cytoplasmic isoforms. However, evaluation of expression, cellular localisation and regulation experiments will be aided by absolute confirmation of the compartmentation of the SAT isoforms. Takahashi and Saito (1996) determined the subcellular locations of three *O*ASTL isoforms from spinach by fusing 5' *O*ASTL cDNA fragments to the β-glucuronidase (GUS) reporter gene and transgenically expressing the fusion proteins in tobacco. Fractionation of transformed tobacco cells followed by fluorometric determination of GUS activity located CysA, CysB and CysC proteins to the cytoplasm, chloroplast and mitochondrion respectively. This experiment could be performed with respect to SAT from *A. thaliana* using the Sat-1, Sat-52, Sat-53 and Sat-106 cDNAs and the pBI121 binary vector

system used in Chapter 5. pBI121 already contains a GUS gene downstream from its multicloning site (Figure 5.6).

Amino acid sequence analysis of the four SAT isoforms has also provided an indication as to the possible post-translational regulation of the enzyme by cysteine. Section 3.3.2 discussed the conserved methionine residue in the SAT1, SAT52 and SAT53 amino acid sequences which was not present in SAT106. This methionine residue was found to be essential for the inhibition of the *E. coli* SAT protein by cysteine (Denk & Böck 1987), and therefore suggests a differential regulation of the *A. thaliana* SAT isoforms by cysteine feedback. It would be of great interest to test this hypothesis. As described in section 5.1.3, it is the intention to express recombinant proteins of all the SAT isoforms from *A. thaliana* for antiserum cross-hybridisation studies. Recombinant SAT proteins could also be used in SAT assays similar to those performed on crude bacterial extracts in section 3.2.3, but incorporating a range of cysteine concentrations. Assay data should then determine which isoforms are susceptible to feedback inhibition by cysteine. If SAT106 activity was found to be unresponsive to inhibition by cysteine, transgenic plants overexpressing SAT106 may be expected to accumulate higher cellular sulphate, cysteine and glutathione concentrations as a consequence of the lack of feedback inhibition by cysteine. Increased production of *O*AS by the overexpressed SAT106 may be expected to maintain high expression of the sulphate transporter (Smith et al. 1997), ATP sulphurylase and APS reductase genes, which appear to be positively regulated by *O*AS in plants. Plants growing in low sulphate soils or requiring increased levels of reduced sulphur-containing compounds for adaptation to environmental conditions, such as salt, hyperosmotic or heavy metal stresses (see Chapter 3), may benefit from such an overexpression of SAT.

Studies in this thesis have also demonstrated specific tissue and cellular expression patterns of the SAT gene-family in *A. thaliana*. *Sat-1*, *Sat-52* and *Sat-53* have all been shown to be highly expressed in root tissue. High level

expression has also been detected for genes encoding both cytoplasmic and plastidic *O*ASTL in the root of *A. thaliana* (Hell et al. 1994; Barroso et al. 1995). These findings suggest that the root may be an important site of cysteine biosynthesis, contrary to many earlier assumptions. Sulphate had been thought to be transported to the leaves for reduction in the chloroplasts, with flows of reduced sulphur compounds occurring from shoot to root (Cram 1990). High root expression of SAT and *O*ASTL, together with northern data indicating similarly high expression of plastidic APS reductase (Gutierrez-Marcos et al. 1996), suggest that the root may be active in both reductive sulphate assimilation and cysteine biosynthesis. Most characterisations of SAT activity to date exclusively used leaf extracts (Brunold and Suter 1982; Nakamura et al. 1988; Nakamura and Tamura 1990; Ruffet et al. 1994, 1995). Only one report, using radish, has assayed SAT activity in root tissue (Nakamura et al. 1987). No data were provided in that paper for SAT activity in radish leaves but activities comparable to those reported in the leaves of other species were recorded. It would therefore be of interest to determine the relative contribution of the root and shoot to total cysteine biosynthesis in *A. thaliana* using the SAT and *O*ASTL enzyme assays, such as those outlined by Nakamura et al. (1987).

In situ hybridisation studies, in section 4.2.2, revealed high levels of *Sat-52* and *Sat-53* transcript in the trichome cells of leaves. *Sat-1* and *Sat-106* expression has not yet been characterised using *in situ* techniques, but will be carried out in future. It will be of interest to determine whether all the SAT genes are highly expressed in trichomes or whether certain isoforms have a specific role in these structures. As *Atcys-3A*, which encodes the putatively cytoplasmic isoform of *O*ASTL, is similarly expressed (Gotor et al. 1997), it would appear that the trichomes of *A. thaliana* have a specialised requirement for cysteine biosynthesis. In Chapter 4 it was hypothesised that production of cysteine-rich metallothioneins (MTs) and phytochelatins (PCs) in the trichomes, which act as a sink for toxic metal ions in many species, may be the reason for high SAT and

*O*ASTL expression. No evidence has yet been provided for the accumulation of heavy metals in the trichomes of *A. thaliana*, but the relationship between cysteine biosynthesis and heavy metal stress could be characterised in future. Accumulation of heavy metals has been previously established in the trichomes of tobacco (Martell 1974), sunflower (Blamey et al 1986) and Indian mustard (Salt et al. 1995), and an MT gene has been shown to be predominantly expressed in the trichomes of *Vicia faba* (Foley & Singh 1994). *A. thaliana* plants treated with radioactively-labelled heavy metals could be used to monitor heavy metal ion accumulation in trichomes. Severed leaves exposed to photographic film would allow visualisation of radioactive ion accumulation as described in Salt et al. (1995). It would also be of interest to determine, by *in situ* hybridisation, the expression patterns of some of the MT genes cloned from *A. thaliana*. Trichome-specific expression of one or more MT genes would support the hypothesis that trichomes have a high cysteine requirement for the production of MTs. Expression of SAT and *O*ASTL genes in response to a variety of heavy metals may also prove interesting. An induction of expression under heavy metal stress may indicate an increased requirement for cysteine and glutathione for MT and PC biosynthesis (see section 4.3.2).

The upregulation of *Sat-53* in response to salt, mannitol and ABA treatments was discussed in section 4.3.4. The results presented are preliminary, but give an indication of the expected regulation of *Sat-53* under salt and osmotic stress. Further work will be carried out to more accurately determine the timing and level of *Sat-53* transcript increase under these stress conditions. Particular attention will be paid to determining the role ABA plays in the signalling of salt and osmotic stress. The presence of several G-box-like (Giraudat et al. 1994) and MYB (Urao et al. 1993) elements in the promoter region of the *Sat-53* gene (Figure 3.11), suggest that ABA is either directly or indirectly involved in its regulation. Experiments using the *A. thaliana* ABA mutants, as described in 4.3.4, will be completed to assess the effects of salt and mannitol on

Sat-53 expression in plants unable to synthesise or to sense ABA. This should indicate whether ABA is the mediating signal responsible for the upregulation of *Sat-53* observed in sections 4.2.4 and 4.2.5.

Chapter 5 describes the preliminary work towards the study of SAT in *A. thaliana* using transgenic techniques. The ultimate aim of this project is to study the effects of modified SAT expression on sulphate transport and assimilation, and the biosynthesis of cysteine and related reduced-sulphur compounds. This should determine whether SAT has a regulatory role in these processes. Using cDNA probes homologous to genes involved in reductive sulphate assimilation and cysteine biosynthesis, northern hybridisations will be used to monitor mRNA transcript levels compared to levels in wild-type plants. HPLC techniques will also be used to compare cellular concentrations of cysteine, methionine and glutathione. In plants overexpressing SAT, an increase in cellular concentrations of cysteine and increases in transcription of genes encoding sulphate transporters, which are known to be upregulated by *O*AS (Smith et al. 1997), would indicate that SAT has a regulatory influence over sulphate assimilation and cysteine biosynthesis. If transgenic plants overexpressing SAT do not increase the cellular concentrations of cysteine or other reduced sulphur-compounds, the enzyme would not appear to represent a limiting step in cysteine biosynthesis.

Before any analytical evaluation of plants transgenically modified with respect to SAT expression is possible, transgenic lines need to be selected and characterised. As discussed in Chapter 5 further vectors also have to be prepared for use in plant transformations.

6.2 Closing Remarks

The last four years have seen a highly productive period in the research of sulphur metabolism in higher plants. An overview of sulphur metabolism in plants as it was understood at the outset of this PhD, is presented in the detailed review

by Schmidt and Jäger (1992). Many of the 'Open Questions' posed in that review have since been resolved. Research is now extending to new areas which will provide a greater understanding of the physiological and molecular processes involved in the regulation of plant sulphur economy. The current status of research is well documented in several recent reviews (Leustek 1996; Hell 1997; Brunold & Rennenberg 1997; Wray et al. 1998; Bick and Leustek 1998).

Progress in elucidating the mechanisms of sulphate assimilation in plants has been hampered in the past by the low cellular concentrations and instability of many of the enzymes involved, the reactive properties of thiols and a lack of suitable higher plant mutants. However, the advent of molecular cloning stimulated the recent advances in understanding of plant sulphur metabolism. After many years of debate, the biochemical pathway of reductive sulphate assimilation in plants, the elucidation of which was elementary to future research, has been demonstrated (Gutierrez-Marcos et al, 1996; Setya et al. 1996; Wray et al. 1998; Bick & Leustek 1998). cDNA clones are now available which represent each step of sulphate uptake, transport, reduction and the biosynthesis of cysteine, methionine and glutathione (see Chapter 1 and references therein). This molecular information will now provide a basis for all the biochemical investigations required to further understand the processes involved. The molecular evidence has also highlighted the complexity of the plant metabolic system when compared to that of the bacteria initially used to characterise sulphur metabolism. The occurrence of multigene families representing most of the steps involved, indicate that regulation is co-ordinated at the levels of tissues, cells and intracellular compartments in plants. The need for multiple isoforms of some enzymes in the same subcellular compartment is not clear. Isoforms may form separate associations with other enzymes within a subcellular compartment, allowing metabolic channelling into various biochemical pathways. Examination of these hypotheses may provide interesting research areas for the future

The contribution and importance of the various sulphur metabolising centres to the sulphur status of the whole plant, and the regulatory factors controlling them will now provide the focus for research. The prospect of applying recent findings to problems faced by plants in adverse environments should continue to generate exciting and innovative research to plant sulphur metabolism in the coming years.

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