Investigation into the formation of a protein-protein complex between ATP sulfurylase and APS reductase in onion (*Allium cepa* L.).

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This thesis has attempted to obtain evidence, both *in vitro* and *in vivo,* of complex formation between chloroplastidic isoforms of APS-reductase (APSR; EC 1.8.4.9) and ATP-sulfurylase (ATPS; EC 2.7.7.4) of *Allium cepa* (onion).

Genes encoding APSR and ATPS from onion were expressed in *E. coli*, and the recombinant proteins were expressed and purified with glutathione Sepharose 4B chromatography and ion exchange chromatography. SDS-PAGE separation and Coomassie blue staining revealed an ATPS recombinant protein with a molecular mass of 50 kDa, and full length and a truncated form of APSR, with molecular masses of 55 kDa and 45 kDa, respectively.

Three different approaches were used to investigate complex formation *in vitro.* Using an ELISA-based technique, an association of recombinant ATPS with recombinant APSR was shown at pH 7.4, with a proposed 1:1 stoichiometry. However, when these ELISA experiments were conducted at pH 9.4, no evidence for complex formation was observed, suggesting that the complex is dependent on the pH of the buffered solution used.

The second method was the ligand binding assay where recombinant protein APSR was immobilised onto PVDF membrane and then incubated at 25 °C with a solution containing ATPS protein. The detection of bound ATPS was achieved using anti-ATPS IgG, and it was possible to detect a putative ATPS-APSR complex at pH 7.4, although the complex was unable to be detected at pH 7.8 or pH 9.4.

The third method used was the immunoprecipitation assay, where anti-ATPS IgG that was conjugated onto Sepharose resin was used to precipitate recombinant ATPS (and any proteins bound to ATPS). The proteins precipitated were identified firstly by their molecular mass and subsequent western analysis with either biotinylated ATPS IgG or APSR IgG antibodies. Using this technique, recombinant APSR was able to be precipitated from solution using recombinant ATPS, at pH 7.4.

To investigate the role of glutathione as a possible co-factor in the mediation of the protein complex, the tripeptide was added to the buffer used (final concentration between 0-10 mM) in the ELISA experiments. These experiments showed that glutathione had no effect on the formation of the complex. However investigation of the role of glutathione using the immunoprecipitation assay (added at 5.0 mM) demonstrated that glutathione did result in an increase in the amount of the ATPS-APSR complex.

The immunoprecipitation technique was also used in attempt to isolate the complex *in vivo* from onion chloroplast extracts. However the ATPS-APSR complex was unable to be detected using this technique. In this thesis, the term *in vivo* is used to refer to experiments with chloroplast extracts and so will include ATPS, APSR and other proteins that may also contribute to any ATPS-APSR complex. The term *in vitro* refers to the direct interaction between recombinant ATPS and APSR only.

To determine if the ATPS-APSR complex had any effect on the control of ATPS activity, the specific activity of ATPS was measured *in vitro* using recombinant ATPS and APSR. Results from these experiments demonstrated that the complex formation did not alter the activity of ATPS.

A second technique was used to detect the ATPS-APSR complex *in vivo*. An ATPS affinity column, made with anti-ATPS IgG antibodies conjugated to Sepharose, was firstly incubated with recombinant ATPS, and the chloroplast extract from onion was then passed through the column and any bound proteins were eluted with solutions with high or low pH. Using this technique, APSR was unable to be identified as a protein that associated with ATPS, but a number of other proteins, with molecular masses of *ca.* 48 kDa, 45 kDa, 40 kDa and 28 kDa, were identified as being putative protein partners to ATPS.

Another approach to signal whether ATPS complex formation with other protein partners (including APSR) has occurred is to examine changes in the kinetic properties of the enzyme. To do this, the K_m values of ATPS for inorganic phosphate were determined in two cultivars of onions, grown hydroponically with varying sulfur supply. Measurements that were taken prior to and during bulbing showed that for at least one cultivar (Texas Grano) with sufficient sulfur, an increase in the K_m value from 3.8 nM (prior to bulbing), to 0.15 μM (during bulbing) was observed. It is possible that this change in K_m value is due to the formation of a protein-protein complex.

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I couldn't have done this without you, and am glad that I have the rest of my life to make it up to you.
I love you so much.

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List of Abbreviations

Ab$_{280/340/405/590/595\ nm}$	Absorbance at 280, 340, 405, 590 or 595 nm
ACC	1-aminocyclopropane-1-carboxcylic acid
ACO	ACC oxidase
ACSO	sulfur-alk (en) yl cysteine sulfoxide
Alliin	3-(2-propenylsulfinyl)-L-alanine
Aliinase	allin alkyl-sulfenate-lyase
AMP	5' adenosine monophosphate
Amp^{100}	ampicillin (100mg/ml)
AP	alkaline phosphatase
APS	5' adenosine phoshosulfate
AP^{35}S	5' adenosine phosho ^{35}sulfate
APSK	5' adenosine phosphosulfate kinase
APSR	5' adenosine phosphosulfate reductase
ATP	5' adenosine triphosphate
ATPS	5' adenosine triphosphate sulfurylase
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
°C	degrees Celsius
ca.	circa.
cDNA	complementary DNA
cm	centimetre
cpm	counts per minute
Cys	cysteine
DEA	diethanolamine
DEAE	diethylamonioethyl
DNA	deoxyribonucleic acid
DTE	dithioerythreitol
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECSO	sulfur-ethyl cysteine sulfoxide
EDTA	ethylenediamine tetra-acetic acid

ELISA	enzyme linked immunosorbent assay
ELSA	enzyme linked sorbent assay
Fd	ferredoxin
FW	fresh weight
FPLC	fast protein liquid chromatography
g	gram
g	acceleration due to gravity
Grx	glutaredoxin
GS4B	glutathione Sepharose 4B
GSH	glutathione (reduced)
GSSH	glutathione (oxidised)
GST	glutathione S-transferase
HPLC	high pressure liquid chromatography
hr	hour
Hsp	heat shock protein
IgG	immunoglobulin G
IPTG	isopropyl-β-$_D$-thiogalactopyranoside
kDa	kilodalton
K_{eq}	equilibrium constant
K_m	substrate concentration at half maximum reaction rate
kPa	kilopascal
L	litre
LB	Luria-Bertani
M	Molar, moles per litre
mAmp	milliampere
MCSO	sulfur-methyl-L-cysteine sulfoxide
MD-ACO2	ACO-2 from apple (*Malus sp.*)
Meq	milliequivalent
Milli-Q	water that has been purified by Milli-Q ion exchange chromatography
min	minute
ml	millilitre
μg	microgram
μl	microtitre

μm	micrometer
μmol	micromole
mol	moles
M_r	relative molecular mass (g mol^{-1})
mRNA	messenger RNA
NAD$^+$/ NADH	nicotinamide adenine dinucleotide reduced/ oxidised
NADPH	dihydrotriphosphopyridine nucleotide
NBT	*p*-nitro blue tetrazolium chloride
ng	nanogram
nmol	nanomole
NR	nitrate reductase
*O*AS	*O*-acetylserine
*O*AS-TL	*O*-acetylserine thiol lyase
ox	oxidised
1-PECSO	*trans*- sulfur- (1-propenyl)- L-cysteine sulfoxide
2-PECSO	*trans*- sulfur- (2-propenyl)- L-cysteine sulfoxide
PA	1, 10-phenanthroline
PAGE	polyacrylimide gel electrophoresis
PAPS	adenosine 3'-phosphate 5'-phosphosulfate
PBS	Phosphate buffered saline
PCSO	sulfur-propyl- L-cysteine sulfoxide
pers. comm.	personal communication
pH	- log [H$^+$]
PP$_i$	inorganic phosphate
ppm	part per million
psi	pounds per square inch
PVDF	polyvinylidene diflouride
red	reduced
RO	reverse osmosis
rpm	revolutions per second
RUBISCO	ribulose bisphosphate carboxylase/oxygenase
s	second
SAT	serine acetyltransferase
SAT-p	plastid-localised serine acetyltransferase

SAT-m	mitochondrial-localised serine acetyltransferase
SBTI	soy bean trypson inhibitor
SDS	sodium dodecyl sulfate
SiR	sulfite reductase
TBS	tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
Thr	threonine
Tris	tris (hydroxymethyl)-aminomethane
$Trx_{red/ox}$	thioredoxin reduced/oxidised
Tween-20	poly (oxyethylene) sorbitane-monolaurate
UV	ultraviolet light
V_{max}	maximum rate of reaction
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

1.0   Introduction

1.1   Overview

Members of the genus *Allium*, which include Chinese chive (*A. tuberosum*), garlic (*A. sativum*) and onion (*A. cepa*), have unique intense flavours, often described as pungent. These flavours are popular in medicine, and in cooking, where they are used for their distinctive flavour and the ability to enhance the flavour of other foods. The distinct flavours of *Alliums* are due largely to a class of sulfur-containing compounds, termed sulfur-alk (en) yl cysteine sulfoxides (ACSO) that occur in high concentrations. ACSO compounds themselves are not volatile, but require the hydrolysis by an allin alkyl-sulfenate-lyase, commonly referred to as alliinase (EC 4.4.1.4), which is activated in response to wounding. The activity of alliinase on ACSO as a substrate produces the distinctive flavours (Randle and Lancaster, 2001).

Onion (*Allium cepa*), a common vegetable crop grown in New Zealand, contains five major ACSO compounds: (+)-S-methyl-L-cysteine sulfoxide (MCSO), (+)-S-ethyl cysteine sulfoxide (ECSO), (+)-S-propyl- L-cysteine sulfoxide (PCSO), *trans*- (+)-S- (1-propenyl)- L-cysteine sulfoxide (1-PECSO) and *trans*- (+)-S- (2-propenyl)- L-cysteine sulfoxide (2-PECSO), and it is the 1-PECSO that contributes the most to the typical onion flavour (Bernard, 1970).

Pungency is a heritable trait, and this allows cultivars to be selected on the basis of flavour, as intensity varies between cultivars. For example, 'Canterbury Longkeeper', a more pungent cultivar, would generally contain more of the non-volatile precursor ACSO compounds. In contrast, a less pungent cultivar, e.g. 'Houston Grano', has less detectable ACSO compounds, but more free sulfate (McCallum et al., 2002).

Differences in pungency between cultivars has recently been shown to be due to differences in the regulation of the sulfur assimilation pathway (Randle et al., 1995; McCallum et al., 2002), as the biosynthesis of ACSO compounds is dependent on reduced sulfate in the form of cysteine. In *Allium* species, cysteine is converted to various ACSO compounds (Lancaster and Shaw, 1989). However, very little is known about the

regulation of the sulfur-assimilation pathway in onion, or if this regulation differs between onion cultivars with different levels of ACSO compounds.

As part of this overall investigation of the regulation of the sulfur assimilation pathway in onion, this project proposes to investigate the significance of protein-protein interactions, principally between ATPS and APSR as a candidate for a major control point in the pathway. Further, cultivar differences may arise from differences in the efficiency of sulfur assimilation that is regulated by the formation of these complexes. However, a distinct comparison of these cultivars is not the immediate aim of this project. As a starting point, it is therefore necessary to introduce the reductive sulfur assimilation pathway in higher plants.
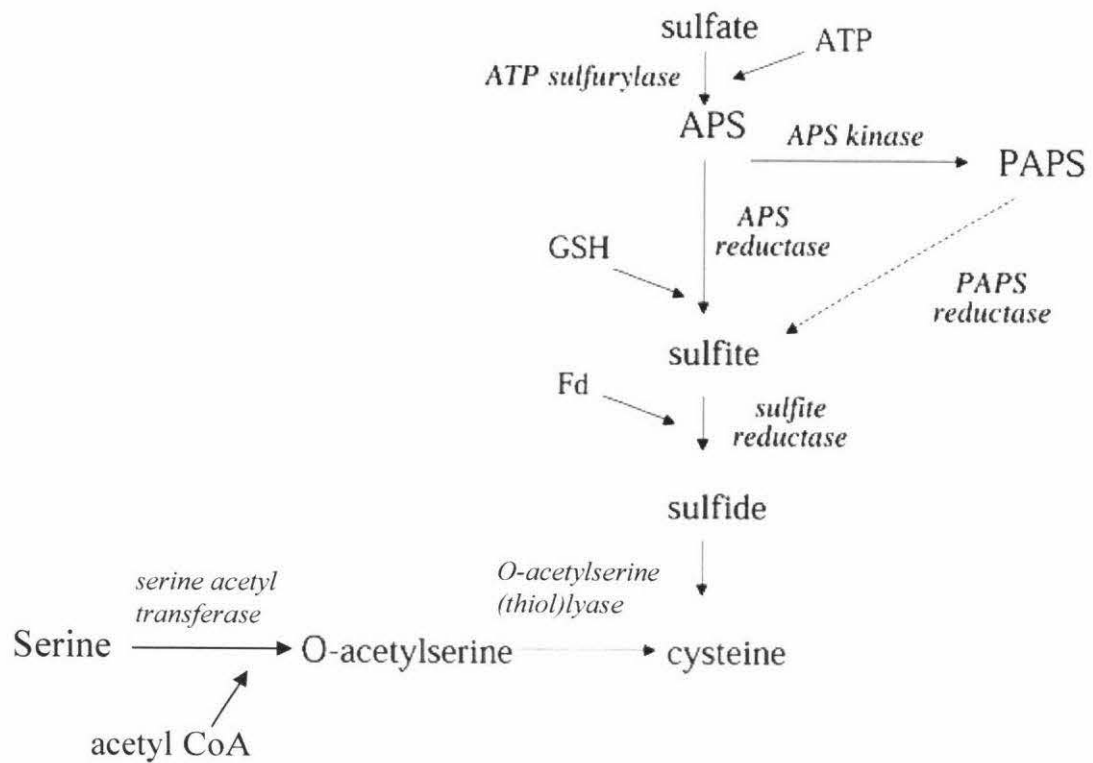
Figure 1.1: Sulfur assimilation pathway in higher plants. Derived from Kopriva and Koprivova (2004). Abreviations; *ATP*, adenosine triphosphate, *APS*, adenosine-5'-phosphosulfate, *PAPS*, adenosine 3'-phosphate 5'-phosphosulfate, *GSH*, glutatione (reduced), *Fd*, ferredoxin (reduced), *CoA, coenzyme A.*

1.2   Cysteine biosynthesis.

Sulfate is the major source of uptaken sulfur for higher plants and is usually obtained from the rhizosphere by the roots. Cysteine is the major product of the sulfur reduction pathway, which is regulated by a number of factors (Leustek et al., 2000). Transport of sulfate into cells is largely due to plasma membrane-localised H^+/SO_4^{2-} co-transporters that are dependent on the electrochemical gradient produced by a proton pump. In *Arabidopsis*, 14 genes have been identified that code for sulfur transport proteins (Takahashi et al., 1997; Takahashi et al., 1999; Takahashi et al., 1999; Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). These are involved with the loading of sulfate into the root, long distance transport of sulfate through the plant, and for the uptake of sulfate into the root plastids or leaf chloroplasts. The site of sulfur assimilation occurs largely within the chloroplasts of leaves, although evidence suggests that limited sulfur assimilation also occurs in the cytoplasm, and in the plastids of roots (Lunn et al., 1990; Rotte and Leustek, 2000).

Assimilation of uptaken sulfate is initiated by the adenylation of sulfate by the magnesium-dependent enzyme, ATP-sulfurylase (ATPS; EC 2.7.7.4) which generates adenosine-5'-phosphosulfate (APS) and pyrophosphate (Renosto et al., 1993) (Reaction 1.1).

<div align="center">
ATP-sulfurylase

$$SO_4^{2-} + MgATP \leftrightarrow MgPPi + 5\text{'-adenylylsulfate (APS)}$$
</div>

<div align="right">Reaction 1.1</div>

Once this first activation step occurs, the reduction of activated sulfate (APS) takes place as two steps. The first reaction indirectly transfers two electrons from glutathione (GSH) to APS producing sulfite (Bick et al., 1998). This reaction is catalysed by APS-reductase (APSR; EC 1.8.4.9) (Reaction 1.2).

<div align="center">
APS-reductase

$$APS + 2\ GSH \rightarrow SO_3^{2-} + 2\ H_2O + GSSG + AMP$$
</div>
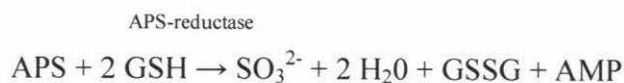
<div align="right">Reaction 1.2</div>

Sulfite is then reduced further by sulfite reductase (SiR; EC 1.8.7.1), which catalyses the reduction of sulfite to sulfide through a 6-electron transfer from ferredoxin (Fd) (Reaction 1.3) (Nakayama et al., 2000).

Sulfite reductase

$$SO_3^{2-} + 6 \text{ ferredoxin}_{red} \rightarrow S^{2-} + 6 \text{ ferredoxin}_{ox}$$
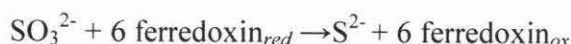
Reaction 1.3

Once sulfate is reduced to sulfide, it is then assimilated into the amino acid cysteine. Sulfide reacts with O-acetylserine (OAS) to form cysteine in a reaction that is catalysed by OAS thiol-lyase (OAS-TL; EC 4.2.99.8) (reaction 1.5). The formation of OAS occurs by the addition of acetyl CoA to serine and is catalysed by the enzyme serine-actyltransferase (SAT; EC 2.3.1.30) (Reaction 1.4).

serine-acetyl-transferase

$$\text{serine} + \text{acetyl-CoA} \rightarrow OAS + \text{CoA}$$

Reaction 1.4

OAS thiol-lyase

$$O\text{-acetylserine } (OAS) + S^{2-} \rightarrow \text{L-cysteine} + \text{acetate}$$
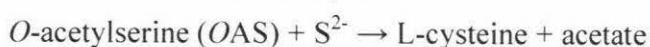
Reaction 1.5

The reduction of sulfate involves an 8-electron transfer, which reduces sulfate to sulfide and also requires one ATP (for the ATP-sulfurylase reaction). The sulfide is largely incorporated into cysteine. From there, cysteine is incorporated into proteins and other sulfur-containing compounds including the ACSO compounds produced by members of the *Allium* genus (Lancaster and Shaw, 1989; Jones et al., 2004). Reaction 1.6 shows the summary of the reduction of sulfate.

$$SO_4^{2-} + ATP + 8 \text{ e}^- + 8 \text{ H}^+ \rightarrow S^{2-} + 4 \text{ H}_2O + AMP + PPi$$
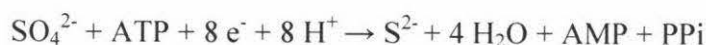
Reaction 1.6

Prior to 1996, before the activity of APS reductase was identified (Gutierrez-Marcos et al., 1996; Setya et al., 1996) it was thought that the sulfur assimilation pathway in higher plants was homologous to the pathway that operates in fungi and bacteria. In this pathway the activation of the sulfate is achieved in two steps rather that one. First, the sulfate is bound to an adenosine group in a reaction that is similar to that which occurs in higher plants (Reaction 1.2). Once APS is formed, an APS kinase (EC 2.7.1.25) adds an

additional phosphate group yielding adenosine 3'-phosphate 5'-phosphosulfate (PAPS) (Leyh, 1993) (Reaction 1.7).

<div align="center">APS-kinase</div>

$$ATP + APS \leftrightarrow PAPS + ADP$$
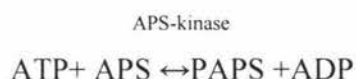
<div align="right">Reaction 1.7</div>

PAPS is then reduced by a thioredoxin-dependent reaction catalysed by PAPS reductase (EC 1.8.4.8) to form sulfite (Reaction 1.8). This pathway is referred to as sulfation.

$$PAPS + Trx_{red} \rightarrow SO_3^{2-} + Trx_{ox} + ADP$$

<div align="right">Reaction 1.8</div>

Two independent groups then determined that higher plants contain a APS-reductase (Gutierrez-Marcos et al., 1996; Setya et al., 1996), suggesting a pathway that by-passes the requirement for PAPS reduction. It is now generally agreed that this pathway (APS reduction) is the major route for reductive sulfate assimilation in higher plants (Bick and Leustek, 1998; Leustek et al., 2000). However, reduction of PAPS should not be excluded in plants since some evidence suggests that this pathway may be present. In the moss, *Physcomitrella patens,* mutants that had the APSR gene knocked out could grow on sulfate as the sole sulfur source. However, the authors did suggest that *P. patens* contained both pathways, and that higher plants have lost the sulfation pathway (Koprivova et al., 2002).

In *Arabidopsis,* at least three genes have been found to encode for an APS-kinase (Reaction 1.7), and at least one of these is located in the chloroplast (Lee and Leustek, 1998; Schiffmann and Schween, 1998), confirming that PAPS is formed in higher plants. However, it is unconfirmed as to whether higher plants contain PAPS reductase, as only one report exists in which PAPS reductase has been isolated from spinach leaves (Schween, 1989). A survey of the *A. thaliana* and rice (*Oryza sativa*) genomes do not contain any genes homologous to the *E.coli* PAPS reductase, other than domains within APSR. However it is possible that plant PAPS reductases have a different sequence and structure to *E.coli* APSR (Kopriva and Koprivova, 2004). In higher plants, PAPS can act as a sulfuryl donator, where a sulfate is added to oxygen by sulfotransferases thus

forming a sulfur ester bond. An example of this reaction is seen in the biosynthesis of the plant hormone group, the brassinosteriods (Rouleau et al., 1997).

In terms of the important enzymes in the pathway in onion, McCallum *et al.* (2002) identified ATP sulfurylase as a possible candidate for the observed differential regulation of the sulfur assimilation pathway between onions of high and low ACSO content. Also it is well known that the subsequent enzyme, APS reductase, has been identified as a key regulatory enzyme of the pathway. A number of factors have been described that effect gene expression or activity, including the levels of sulfur, carbon and nitrogen (Brunold et al., 1987; Kopriva et al., 2002), light (Kopriva et al., 1999), and oxidative stress (Bick et al., 2001). Thus APSR is another likely candidate responsible for cultivar-specific regulation of sulfur assimilation in onion. Therefore, these two enzymes will be introduced in more detail.

1.3   ATP-sulfurylase.

The first step of sulfur assimilation is the activation of the sulfate as a prelude to subsequent reduction. ATP sulfurylase (ATPS) catalyses the reaction which breaks the bond between the β- and the γ-phosphate of ATP and the sulfate is then subsequently bound to the γ-phosphate (Hiltz and Lipmann, 1955), resulting in the formation of APS and PPi (Reaction 1.1). The energy stored between the β- and the γ-phosphate bond is essential for the successful reduction of the inert sulfate.

The genome of *Arabidopsis* contains four genes encoding ATP sulfurylase, designated *AtAPS1, AtAPS2, AtAPS3* and *AtAPS4* (Leustek et al., 1994; Klonus et al., 1995; Murillo and Leustek, 1995). Each of these genes encodes proteins that contain a chloroplast transit peptide, suggesting that each enzyme is localised in the chloroplasts. In spinach (*Spinacia oleracea*), an ATPS isoform has been shown to be localised in the cytosol (Lunn et al., 1990), but thus far in *Arabidopsis*, despite detectable ATPS activity in the cytosol (Rotte and Leustek, 2000), a gene coding for a putative cytosolic isoform is yet to be identified. Hatzfeld *et al.* (2000), however, suggest that *AtAPS-2* may play a cytosolic

role, since an alternative translation start point is proposed to result in an active *AtAPS-2* without the transit peptide (Hatzfeld et al., 2000).

Previous reports of ATPS kinetics in plants have reported K_m values using purified ATPS from the chloroplast of 0.046 mM for MgATPS and of 0.25 mM for SO_4^{-2} and for the reverse reaction K_m values of 64×10^{-85} nM for APS and of 10 μM for PP_i have been reported (Renosto et al., 1993).

In thermodynamic terms, the ATPS enzyme in all species carries out an unfavorable reaction, with a $K_{eq} \sim 10^{-8}$ M (Farooqui, 1980; Murillo and Leustek, 1995). To move the reaction in the forward direction, the products APS and PP*i*, must be efficiently removed (Seubert et al., 1985). Since APS or PP_i cannot accumulate for sulfate assimilation to continue, an inorganic pyrophosphatase, ATP reductase and/or APS kinase must be readily available to accommodate this removal of substrate. To overcome this problem, other species have alternative methods for efficient APS synthesis. For example an ATPS isolated from *E.coli* was shown to contain two subunits, one that catalyses the APS synthesis reaction, while the other subunit is responsible for the hydrolysis of GTP. The hydrolysis of GTP is linked to APS synthesis and the transfer of energy is required for the reaction to occur (Leyh and Suo, 1992). So far, the GTPase subunit has not been identified in eukaryote ATPS proteins.

In several animal species, ATPS is conjugated with the subsequent enzyme, APS-kinase (Li et al., 1995; Jullien et al., 1997; Venkatachalam et al., 1998) but in plants and fungi these enzymes exist separately (Jain and Leustek, 1994; Murillo and Leustek, 1995). An alternative mechanism to encourage APS synthesis exists, in which APS channeling occurs between the ATPS and APS-kinase (Lyle et al., 1994).

In plants, the C-terminal domain of ATPS has high sequence homology and is structurally very similar with APS-kinase in yeast and *Penicillium chrysogenum* suggesting that ATPS may have evolved from APS-kinase. Despite the similarities ATPS does not act as a kinase, although in *P. chrysogenum* it does bind to PAPS, the product of APS-kinase, thus allowing allosteric control over ATPS (Martin et al., 1989; Renosto et al., 1990; MacRae et al., 2001). From an evolutionary perspective, plant ATPS enzymes, seem to be more closely related to animal and fungal ATPS enzymes than bacterial ATPS enzymes (Murillo and Leustek, 1995; Leustek, 1996).

The regulation of ATPS has been noted in a number of plant studies, but as yet no mechanisms have been described. In canola (*Brassica napus*), maize (*Zea mays*) and *Arabidopsis*, ATPS mRNA levels decline and an excess of reduced sulfur compounds (e.g. glutathione, cysteine) will also induce the down-regulation of ATPS mRNA after prolonged exposure to sulfur-deprivation (Lappartient and Touraine, 1996; Bolchi et al., 1999; Vauclare et al., 2002). However, the exposure of *Brassica juncea* to cadmium, which chelates glutathione and decreases the cellular concentration of glutathione, induces expression of ATPS mRNA (Heiss et al., 1999). Low nitrogen levels will also reduce the mRNA levels of not only ATPS, but also of APSR (Koprivova et al., 2000), in a coordinated manner.

In a detailed study, Rotte and Leustek (2000) reported that plastid-associated enzyme activity of ATPS in *Arabidopsis* is down regulated as the plant matures, but the activity of the cystolic localised isoform of ATPS increases, suggesting a different function for the cystolic form of ATPS.

1.4   APS-reductase

1.4.1   Overview of APS-reductase

APS reductase (APSR) catalyses the first reduction of activated sulfate (APS), where two electrons are transferred from a glutathione to the sulfate, producing AMP and sulfite (reaction 2). This step became universally accepted as a possible sulfate reduction step after the isolation of three isoforms of APSR in 1996 (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Before this, resolution of the APSR catalysing step, it was thought that an APS sulfotransferase activity existed, whereby the reduced sulfate is transferred to a thiol acceptor producing a thiosulphate, such as glutathione (Schmidt, 1975; Varin et al., 1997). This hypothesis was refuted when it was shown that the cloned APS sulfotransferase from *Lemna minor* (Suter et al., 2000) displayed very high identity to an APSR cloned from *A. thaliana* (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Later, Suter et al. (2000) showed that APSR exclusively used APS to produce sulfite. Despite the identification of the APS reduction pathway, the presence of a PAPS pathway should not be excluded in plants, because of the identification of both APS kinase and PAPS

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reductase enzyme activities (Schween, 1989; Lee and Leustek, 1998; Koprivova et al., 2002).

Three genes encoding APSR have been identified in the genome of *Arabidopsis*, which are designated *AtAPR1, AtAPR2* and *AtAPR3*. All contain a chloroplast transit peptide, a reductase domain and a thioredoxin-like domain (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Suter et al., 2000).

1.4.2   Localisation of APSR

Observation of the full length cDNA of all plant APSR enzymes reveals a chloroplast localisation sequence, suggesting that APSR is found in the chloroplast (Suter et al., 2000). By western blot analysis, APSR protein, before the entry into the chloroplast, is determined to be approximately 58 kDa, but APSR detected in the chloroplast is processed to give a 45 kDa product (Prior et al., 1999). Using immunogold electron microscopy, APSR was detected in the chloroplasts of three *Flaveria* species, with 70 % of detectable APSR localised near or associating with the thylakoid membrane (Koprivova et al., 2001).

1.4.3   The reductase domain

The reductase domain has high similarity with that of bacterial PAPS reductases, although one important difference between APSR and bacterial PAPS reductase exits. This is the presence of two additional cysteine pairs in the N-terminal domain of the plant APSR enzyme, which are essential for the binding of the cofactor [4Fe-4S], which confers specificity towards APS binding over PAPS (Kopriva et al., 2001; Kopriva et al., 2002). It is thought that this domain is responsible for the reduction of APS, although the reductase domain is unable to be catalytically active without the C-terminal domain or the addition of reduced thiols (Weber et al., 2000).

1.4.4   The thioredoxin-like domain

The C-terminal domain of APSR has sequence identity with thioredoxin (EC 1.8.4.10), a class of thiol:disulphide oxidoreductases (Gutierrez-Marcos et al., 1996; Setya et al., 1996). In *E.coli*, APSR activity with the C-terminal removed was low, but this was restored after the addition of thioredoxin (Bick and Leustek, 1998). In plants, despite the C-domain containing high sequence identity with thioredoxin, it is now accepted that the C- terminus domain acts as a glutaredoxin (which uses reduced glutathione) and not as a thioredoxin reductase (EC 1.8.1.9) (as the thioredoxin domain does). This conclusion was reached after a series of studies that showed firstly that glutathione was more effective as a hydrogen donor (Bick et al., 1998; Prior et al., 1999), that the C-terminal domain of the enzyme can catalyse glutathione-dependent reactions (Bick et al., 1998; Prior et al., 1999) and that it can also complement *E.coli* mutants lacking glutaredoxin (Bick et al., 1998). Taken together, these studies confirm the notion that the C-terminus acts as a glutaredoxin and not a thioredoxin.

1.4.5   The reaction mechanism of APSR

The catalytic mechanism of APSR can be divided into three steps (figure 1.3). For the first step, the sulfur from APS covalently binds to a conserved Cys_{248} in *Arabidopsis* within the reductase region. Mutagenesis of the Cys_{248} to Ser_{248} resulted in the loss of activity due to the formation of a stable $APSR-SO_3^-$ intermediate which does not require the interaction of the C-terminal (Weber et al., 2000). The second step releases the sulfite from the enzyme. This requires the reduced C-terminal domain which oxidises conserved cysteine residues forming a disulfide bound within the C-terminus and release of free sulfite. Sulfite is unable to be released if the C-terminal is absent, or if reduced thiols are not present, resulting in a stable intermediate (Weber et al., 2000). In order for active enzyme to be regenerated, two molecules of reduced glutathione are required, which reduce the di-sulfide bond between the cysteines of the C-terminal and N-terminal domains, restoring the APSR enzyme to an active state.

Consequently, APSR activity is increased by oxidative stress, *via* oxidation of the pair of cysteines required for function. This is caused by an abundant supply of oxidised

glutathione, a consequence of reactive oxygen species, and thus demonstrates a post translational mechanism for control of APSR (Bick et al., 2001).

1.4.6   Kinetic properties of APSR

The measured activity for APSR varies substantially between species with rates ranging from 0.4 to 0.8 nmol min^{-1} mg^{-1} protein in poplar (*Populus tremula* × *P. alba*) (Hartmann et al., 2004), 0.77 ± 0.09 nmol min^{-1} mg^{-1} protein in *Arabidopsis* (Tsakraklides et al., 2002) to 30-40 µmol min^{-1}mg^{-1} protein in *Lemna minor* (Kopriva et al., 2001). Kinetic properties have been determined for APSR in *L. minor*, where at an APS concentration of 40 µM, a K_m value of 16.8 ± 5.3 mM has been calculated for GSH and at 40 mM glutathione K_m value of 12.2 ± 4.6 µM has been calculated for APS (Suter et al., 2000).

| TP | Reductase domain | trx/grx domain |

Figure 1.2: **Structural domains of APSR**. The N-terminus includes the chloroplast transit peptide (TP), which is removed from the peptide as part of the mechanism of chloroplast entry. The reductase domain, homologous to the PAPS reductase domain in *E. coli*, contains a conserved cysteine that allows SO_4^{2-} to bind (Weber et al., 2000). The C-terminus contains a domain homologous to thioredoxin, yet acts like a glutaredoxin, oxidising glutathione (Bick et al., 1998). Reproduced from Bick and Leustek (1998)
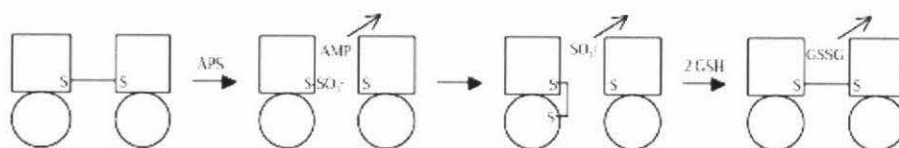
Figure 1.3: **Proposed reaction mechanism for the catalytic cycle of APSR** (see text for details). The circles and square represents the N-terminal and C-terminal domain of APSR, respectively. S represents the thiol group of catalytically significant cysteine residues. Adapted from Kopriva and Koprivova, (2004)

1.5　　　　Regulation of the sulfur-assimilation pathway

Regulation of the sulfur assimilation pathway is important. The ability to sense environmental signals results in the regulation of metabolic processes in plants accordingly and thus in the efficient use of the nutrients. The regulation of sulfur assimilation is part of an intricate web of control which is dependent on the supply of sulfur, nitrogen, carbohydrates and the occurrence of oxidative stress (Prosser et al., 1997; Koprivova et al., 2000; Kopriva et al., 2002; Hesse et al., 2003; Nikiforova et al., 2003).

1.5.1　　　　Developmental regulation of ATPS and APSR

At the gene level, the gene expression of ATPS and APSR are both regulated during plant development. In general, the highest expression of both genes occurs in the actively growing regions of the plant. The expression of ATPS and APSR mRNA declined in parallel with one another as the plant aged, suggesting similar regulatory cues. For ATPS, Rotte and Leustek (2000) determined that the ATPS protein can be localised in either the cytosol or the chloroplast. The cytosolic and chloroplastic ATPS have different temporal patterns of abundance, where the chloroplastic, the most abundant isoform, is most active in the young growing leaves, and decreases as the plant develops (3-fold decrease). In contrast, the cystolic isoform shows highest activity in the mature leaves (a 5-fold increase). Rotte and Leustek (2000) suggested that the cystolic isoform functions to provide APS for sulfate biosynthesis in the cytosol.

1.5.2　　　　Regulation by sulfur status and products of the sulfur assimilation pathway.

The external sulfur supply plays a major role in controlling the rate of sulfur assimilated. Under limited external sulfur supply, sulfate uptake, ATPS and APSR enzyme activities, an increase the transcript level of sulfate transporters, ATPS, APSR, and SAT are observed (Reuveny et al., 1980; Smith, 1980; Lappartient and Touraine, 1996; Takahashi et al., 1997; Yamaguchi et al., 1999). Under sulfur stress, the extracted protein levels,

activity and transcript levels of ATPS from roots of *A. thaliana* increase significantly after 3 days. Further, supplying sulfate to sulfur starved plants reduced the ATPS activity back to a basal level (Lappartient and Touraine, 1996; Lappartient et al., 1997). In another study, *Arabidopsis thaliana,* grown in sulfur deprived media for 2 days before northern blot analysis, showed an increase in transcription of a high affinity sulfate transporter and APSR in both the shoots and the roots (Takahashi et al., 1997). The mRNA levels of APSR in poplar (*Populus tremula* x *P.alba*) increased after 3 days of sulfur deprivation, but unlike herbaceous species, only occurred in the roots (and not in shoot tissues). Also, unlike *A. thaliana,* an increase of ATPS mRNA was observed only after prolonged sulfur deficiency (four weeks). It is suggested that poplar may have a larger store of sulfur which is a possible reason as to why an immediate down regulation of ATPS, observed in herbaceous species, is not observed (Kopriva et al., 2004). These observations suggest that APSR is more susceptible to control from sulfur stress, when compared with ATPS.

More recently, the role of reduced sulfur compounds such as glutathione, cysteine, *O*-acetyl-L-serine and H_2S have becoming increasingly apparent as important in sensing the sulfur levels, either as signals of sulfur stress or by directly regulating the sulfur assimilation pathway. (Neuenschwander et al., 1991; Lappartient and Touraine, 1997; Bolchi et al., 1999; Clarkson et al., 1999; Westerman et al., 2001; Vauclare et al., 2002) Glutathione (a product of the sulfur reduction pathway), which is commonly associated with scavenging of radicals produced by oxidative stress, is also linked with the storage and transport of reduced sulfur and as a modulator of enzyme activity (May et al., 1998; Noctor and Foyer, 1998). Glutathione is produced by the conjugation of cysteine to glutamate and glycine by γ-glutamyl-cysteine synthase (EC 6.3.2.2) and glutathione synthase, respectively (EC 6.3.2.3) (Hell and Bergmann, 1988, 1990). Glutathione is a likely candidate as a signaling molecule for sulfur stress. Here nutritional status can be determined in the roots and glutathione acts as a signal to the shoots, as it is easily translocated within the phloem (67–70% of reduced sulfur in the phloem is glutathione) (Rennenberg et al., 1979). Glutathione content also reflects the sulfur status of the plant, as the tripeptide requires reduced sulfur for synthesis (Meyer and Fricker, 2002). This 'demand-driven' model was developed by Lappaertient and Touraine (1996) who demonstrated that glutathione transported in the phloem is the signal that controls sulfur

assimilation. Supplied glutathione and cysteine, either externally to the roots or internally through the phloem of canola (*Brassica napus* L. *cv* Drakkar), resulted in reduction of both ATPS transcript levels and ATPS activity and a reduction in sulfate uptake. This repression was inhibited if buthionine sulfoximine, a specific inhibitor of γ-glutamyl-cysteine synthase, which results in a decreased glutathione pool. This inhibitor study suggests that glutathione and not cysteine, is responsible for repression of the expression of ATPS mRNA. (Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Lappartient et al., 1999). However, conflicting results in *Arabidopsis thaliana* have been reported, whereby externally applied oxidised glutathione resulted in no reduction in either ATPS transcript or protein activity levels in root cultures. In contrast to ATPS, APSR mRNA, protein and activity reduced significantly in the presence of 0.2 mM oxidised glutathione, suggesting that APSR is more sensitive to glutathione when compared to ATPS (Vauclare et al., 2002). The regulation of APSR by glutathione has been reinforced in poplars, where exogenously applied glutathione resulted in a strong reduction in APSR mRNA and protein activity, yet no change in mRNA accumulation or protein activity of ATPS, APSR, SiR or OAS-TL was observed. Although this 'demand-driven' control of sulfur metabolism by glutathione is not applicable to all species, other reduced sulfur compounds have been suggested to play a regulatory role.

Bolchi *et al.* (1999) observed that transcript levels of both a sulfate transporter and ATPS increased under sulfur stress in maize (*Zea mays*), then reduced when sulfur supply was restored. Under sulfur stress, a reduction in glutathione pools was also observed. To test if the decrease in glutathione pools induced the increase in transcript levels, buthionine sulfoximine was added to inhibit glutathione synthesis. However, unlike previous reports (Lappartient and Touraine, 1996; Lappartient et al., 1999) reducing the glutathione pool was not sufficient for the up regulation of ATPS or the sulfate transporter. It was determined that L-cysteine, a major product of the sulfur assimilation pathway, successfully down-regulated the sulfate transporter and ATPS mRNA in maize (*Zea mays*) (Bolchi et al., 1999). This provides evidence of a mechanism of control in the absence of glutathione. It maybe that glutathione seems to have a predominate role in regulation in dicot plant species such as canola (*Brassica napus*), poplar (*Populus tremula*), *A. thaliana* but cysteine may be more important in monocots, such as maize.

In addition to ATPS and the sulfate transporter s, the activity of other enzymes in the sulfur assimilation pathway can also be influenced by L-cysteine. Serine acetyltransferase (SAT; EC 2.3.1.30) catalyses the formation of O-acetylserine from serine and acetyl-CoA, which will subsequently form cysteine after a reaction catalyzed by O-acetylserine (thiol) lyase (OAS-TL; EC 4.2.99.8). A number isoforms of SAT have been isolated and characterised from *A. thaliana*. These include SAT-c, a cystolic localised SAT (Ruffet et al., 1995), SAT-p, localised in the plastids and SAT-m, of mitochondrial origin (Roberts and Wray, 1996; Hesse et al., 1999). Interestingly, it is only the SAT-c isoform and not SAT-p or SAT-m, which is sensitive to L-cysteine, allosterically inhibiting the protein activity (Noji et al., 1998). Regulation of cysteine sensitive SAT is due to a region of residues, from Pro276 to Phe285, that are different to the insensitive isoforms, providing evidence for a feedback regulatory mechanism that is specific to the isoform (Inoue et al., 1999).

In addition to L-cysteine, other reduced sulfur compounds, such as H_2S have been observed to have an effect on the sulfur reduction pathway. For example in *Brassica oleracea* L, a reduction in the activity of APSR in the shoots was observed only in the presence of H_2S. The activity of ATPS, SAT and OAS-TL were not effected in this treatment (Westerman et al., 2001).

O-acetylserine (OAS), a product of the reaction catalysed by SAT and is essential for the formation of cysteine, is also proving to play a major role in the regulation of the sulfur assimilation pathway. Unlike glutathione and cysteine, OAS seems to act as a positive regulator, increasing the capacity of the pathway under sulfur deprivation. Under conditions of low sulfur, levels of OAS increase in the plant cell (Kim et al., 1999) and are limiting in high sulfate conditions. OAS acts as a positive regulator of transcription, protein levels and activity of members of the sulfur reduction pathway. In maize (*Z. mays*), the external supply of OAS, with sufficient sulfur supply, leads to an increase in the mRNA of a high affinity sulfate transporter (Smith et al., 1997). Using cell suspensions of *Z. mays* in adequate sulfate nutrition, an increase in sulfate uptake and an increase in ATPS activity was observed after the addition of OAS (Clarkson et al., 1999). The activity of APSR has also been shown to be affected by OAS. The presence of OAS in the media of *Lemna minor* (L.) resulted in an increase in APSR activity but not of ATPS or OAS-TL (Neuenschwander et al., 1991). An increase of internal OAS

concentration obtained by over-expressing *O*-acetyl-serine mRNA, increased levels of cysteine and glutathione, probably due to increasing the substrate levels, as cysteine is known to be the limiting factor in the formation of glutathione. However, it is unknown if the increased levels of cysteine and glutathione observed, are due to an up-regulation of the sulfur assimilation pathway by *O*AS, as has been shown in previous studies (Blaszczyk et al., 1999; Harms et al., 2000; Youssefian et al., 2001).

Recent studies in potato (*Solanum tuberosum* L.) propose that *O*AS is not the signal for the increased gene expression of sulfur-reduction related genes in response to sulfur starvation. It is possible that other proposed signal molecules such as glutathione (Lappartient et al., 1999), cysteine (Bolchi et al., 1999) or sulfide (Westerman et al., 2001), are responsible, as *O*AS levels are thought to be too low to derepress expression at this stage. However, after prolonged sulfur starvation, the *O*AS pool does increase sufficiently, and this may lead to increased expression of sulfur reduction genes (Hopkins et al., 2005).

1.5.3   Regulation by nitrogen

In plants, it is becoming apparent that nitrogen and sulfur levels are tightly linked, whereby nitrogen status can influence the sulfur reduction pathway and vice-versa (Brunold and Suter, 1984; Yamaguchi et al., 1999; Koprivova et al., 2000; Prosser et al., 2001). In *Lemna minor*, the activities of ATPS, APSR and *O*AS-TL decrease under low nitrogen conditions, but are fully restored after the addition of ammonia or nitrate (Reuveny et al., 1980; Brunold and Suter, 1984). Addition of ammonia or nitrate to plants in nitrogen sufficient media resulted in an increase in APSR activity, but no increase in activity was observed for either ATPS or *O*AS-TL.

As described above, under sulfur stress, components of the sulfur assimilation pathway increase, to maximise sulfur uptake and reduction (Prosser et al., 1997; Takahashi et al., 1997). However, under nitrogen deprivation, this response is reduced, suggesting that the sulfur deprivation signal is dependent on the nitrogen supply (Yamaguchi et al., 1999). The regulation of the sulfate assimilation pathway by nitrogen levels is perceived to be possible due to *O*AS as a putative signal molecule. As discussed previously (see section 1.2.2), *O*AS has been seen to positively regulate components of the sulfate reduction

pathway. In *A. thaliana*, a reduction in APSR mRNA, protein levels and protein activity was observed under low nitrogen levels, but the application of *OAS* to these nitrogen deficient plants strongly increased the levels of mRNA of all isoforms of APSR, SiR and also chloroplastic *OAS*-TL. This supports evidence that *OAS* plays an important role as a possible connection between nitrogen and sulfur (Koprivova et al., 2000). Further, in DNA- microarray analysis of 9000 genes of *A. thaliana* isolated after treatment with 1.0 mM *OAS*, more than 100 genes were induced and 548 genes were repressed. Those induced included APSR, *O*-acetyl transferase and a sulfate transporter, although the correlation between the response to *OAS* and sulfur deprivation was not enough to suggest that *OAS* was the signal of sulfur status (Hirai et al., 2003).

1.5.4   Regulation by carbon

Components of the sulfur pathway have been shown to be under regulatory control by light, since the activity of ATPS and APSR and *OAS*-TL was shown to increase in the presence of light (Neuenschwander et al., 1991; Astolfi et al., 2001). A high density oligonucleotide microarray identified genes that exhibited a mRNA cycling which was dependent on a circadian pattern, and included two sulfate transporters, APSR and SAT (Harmer et al., 2000). In maize, APSR is known to be under a diurnal rhythm where maximal enzyme activity and mRNA levels occur during the light period. Interestingly, activity in the dark can be restored by the introduction of sucrose (Kopriva et al., 1999). However the control by light may or may not be attributed to the carbohydrates formed by photosynthesis. Omitting the CO_2 supply in the air resulted in the reduction of APSR mRNA levels and enzyme activity, suggesting transcriptional regulation of APSR. Further, the reduction is relieved if the plants are supplemented with sucrose (Kopriva et al., 2002). In other feeding experiments, 0.5 % (w/v) glucose but not sorbitol or mannitol, resulted in a significant increase in APSR mRNA, enzyme activity and protein accumulation, suggesting that the effect is not dependent on a change in osmotic potential (Hesse et al., 2003).

It is becoming apparent too that *OAS*, discussed earlier as a important molecule in the regulation of the sulfur assimilation pathway is a candidate that links the sulfur, nitrogen

and carbon nutritional status of the plant (Koprivova et al., 2000; Kopriva et al., 2002; Hesse et al., 2003).

1.5.5   Regulation by oxidative stress.

Oxidative stress is caused by active oxygen species which are commonly toxic to the plant cell.   Oxidative stress can result from a number of environmental conditions, including light, chilling, drought, attack from pathogens and nutrient deficiency. One mechanism to reduce the concentration of active oxygen species is by using non-enzymatic antioxidants such as glutathione and ascorbate (May et al., 1998). Not much is known about the effect of oxidative stress on the sulfur assimilation pathway.

In one study, canola (*Brassica napus*) treatment with H_2O_2 to for 8 hrs increased ATPS activity, this effect was found to be independent of the control exerted by reduced glutathione and that a independent regulatory pathway is present (Lappartient and Touraine, 1997).

In contrast the APSR protein has been shown to be regulated by the redox state of glutathione, *Brassica juncea* treated with H_2O_2 resulted in a substantial increase both in APSR protein activity which was due to an increase in the oxidised glutathione: reduced glutathione ratio. However, the addition of reduced glutathione decreases the protein activity demonstrating a oxidative stress regulation dependent on glutathione (Bick et al., 2001). Bick *et al.* (2001) suggested that the regulation of APSR was due to a post-translational modification that may act through a mechanism involving disulfide bonds between conserved cysteine residues.

Alteration of the abundance of sulfur assimilation transcripts by overexpressing SAT and OAS-TL both resulted in an increased tolerance to oxidative stress. In both cases, glutathione and cysteine pools increased, indicating that the sulfur assimilation pathway is important to reactive oxygen resistance, by providing precursors for the antioxidant glutathione (Blaszczyk et al., 1999; Youssefian et al., 2001).

Chilling stress is also known to result in an increase in reactive oxygen and has also been shown to regulate components of the sulfur reduction pathway. Chilling *Z. may* seedlings, by growing at 12 °C, resulted in an increase in mRNA level and protein

activity of APSR, also, under such conditions mRNA is detectable in the mesophyll cells, whereas seedlings grown at 25 °C, mRNA is localised exclusively in the bundle sheath cells, demonstrating a change in the distribution of APSR mRNA (Brunner et al., 1995; Kopriva et al., 2001). Interestingly, this treatment will also eliminate diurnal changes in the activities of APSR (Kocsy et al., 1997).

1.6   Regulation of the sulfur assimilation pathway in onion

1.6.1   Overview of onion production in New Zealand

The onion industry in New Zealand has traditionally been based on volcanic soils (sulfur-rich) in the South Auckland and Waikato region, but is increasingly moving to drier east coast areas on lighter soils under irrigation. As sulfate is highly mobile, the availability of sulfur is easily reduced on such soil, especially as growers have moved to the use of less super phosphate (high in sulfate) and more to the use of urea as fertiliser. It is likely too, that high nitrogen use on such soils can lead to S deficiency, compromising yield and storage as well as creating high nitrate levels in the crop, which is particularly undesirable. Therefore, a better understanding of the physiology of onion and the genetic variation in terms of N/S homeostasis will help develop better crop management systems to provide and optimise fertiliser regimes that can provide desired flavour quality while maximising crop productivity and nitrogen use efficiency.

1.6.2   Impact of sulfur nutrition on the flavour of onions

Agronomic and physiological studies have revealed a significant relationship between genotype and environment interactions, particularly in response to sulfur nutrition which effect the pungency of onions (Randle and Bussard, 1993).
Flavour intensity in onions are commonly measured indirectly by measuring the production of pyruvate produced in a reaction catalysed by alliinase (EC 4.4.1.4) (Randle and Bussard, 1993; Vavrina and Smittle, 1993), or directly, by use of high pressure liquid chromatography (HPLC) (Randle et al., 1995; Yoo and Pike, 1998).

It is known that flavour intensity varies between cultivars and is determined by the level of ACSO compounds.

It has also been suggested that the variability in flavour observed between onion lines is due to differences in sulfur uptake and its metabolism (Randle and Lancaster, 2001) In general, sulfur nutrition plays an important role in the levels of sulfur compounds synthesised. While it is possible to reduce pungency and produce mild onions by reducing the sulfate levels to no greater that 50 ppm (Randle and Lancaster, 2001), this treatment, because of the requirement for sulfate during plant growth and development, will also reduce the bulb yield (Freeman and Mossadeghi, 1970; Hamilton et al., 1997). Onion varieties respond differently to sulfur nutrition although this commonly shows little correlation between total sulfur content and pungency (Randle, 1992; Randle and Bussard, 1993). It is thought that mild varieties accumulate more free sulfate and store it in the vacuole of cells, which excludes it from the incorporation into the flavour pathway (Randle et al., 1999). This is significant as it suggest that a major contributing factor in less pungent, milder lines (with less ACSO compounds) is that these lines can uptake sulfur, but are less efficient at the incorporation of the sulfur into thiols. Therefore, it is critical to define how the pathway of reduced sulfate into thiols and flavour precursors is regulated in mild and pungent varieties, and so test the hypothesis that the genetic variation in flavour is determined at this level.

1.6.3   Regulation of sulfur assimilation at a molecular level in onion

In terms of enzymes in the pathway, ATPS gene expression has been shown to increase with an abundance of sulfur, with notably higher expression in the more pungent cultivars. It is thought therefore that ATPS is an important candidate for the observed divergent sulfur metabolism between phenotypes (McCallum et al., 2002). ATPS could be a possible contender for pathway control because it catalyses the first step in the assimilation of sulfur, but the mechanism of how the differential expression of the ATPS gene regulation is unknown.

1.7   Complex formation and the regulation of sulfur assimilation in onion

Protein complexes do exist as part of the sulfur assimilation pathway in plants. A well characterised example is termed the cysteine synthase complex. This comprises SAT in complex with OAS-TL which catalyses the reaction that yields OAS from serine and acetyl CoA (reaction 1.4). Dissociated OAS-TL then catalyses the formation of L-cysteine from OAS and sulfide (reaction 1.5). Evidence of this complex has come from co-purification experiments with a number of plant species including rape, Chinese chives (*Allium tuberosum*) (Nakamura and Tamura, 1990) watermelon (*Citrullus vulgaris*) (Saito et al., 1995) and spinach (*Spinacia oleracea*) (Droux et al., 1992) and using yeast two-hybrid system in *A. thaliana* (Bogdanova and Hell, 1997).

It was assumed that the formation of the complex would result in a channeling effect, where the OAS formed by the activity of SAT would be converted to cysteine by the association by OAS-TL (Droux et al., 1992; Saito et al., 1995). However, investigation into the kinetic properties of bound and un-bound SAT and OAS-TL revealed that when these components are bound as a complex, the SAT activity increased, while OAS-TL activity reduced. Further, the proposed channeling was challenged when OAS was discovered to be released into the bulk solution. It is known that the formation of the complex is controlled by the level of substrates, whereby sulfide encourages the complex formation and OAS destabilises the association (Droux et al., 1998). These reports suggest a regulatory role for OAS-TL, controlling the catalytic activity of SAT, in addition to the catalytic function.

It is becoming increasingly apparent that more proteins are involved in protein complexes, either as a way of regulating the activity of the protein, or to change the flux of substrates to a desired product (Gontero et al., 2002). Thus in the sulfur assimilation pathway of onion, there may be other complexes that form. A candidate that is investigated in this thesis is the formation of a complex between APSR and ATPS, a major branch point between the reductive (towards cysteine) and oxidative (catalysed by APS kinase and sulfotransferase) sulfur assimilation pathways.

1.7.1   Current knowledge that supports the formation of a protein complex
             between ATPS and APSR.

Substrate channeling has been shown between ATPS and APS kinase catalytic sites if
these exist as the bi-functional enzyme adenosine-5'phosphosulfate kinase (EC 2.7.1.25)
in rat chondrosarcoma (Lyle et al., 1994). Using isotope dilution and enrichment with
labeled APS, it was possible to show that during these reactions, the APS intermediate
was not released into the bulk solution (Lyle et al., 1994). Further investigation in
Sacccharomyces cerevisiae revealed that the C-terminal domain of ATPS is responsible
for channeling by forming a physical connection between the active sites of ATPS and
APSK (Lalor et al., 2003).

In plants, ATPS activity in cabbage (*Brassica capitata* L.), was stimulated 60 % after the
addition of APS kinase (APSK) from *Penicillium chrysogenum*, possibly as an interaction
between the two enzymes that could facilitated substrate channeling (Osslund et al.,
1982). However, the authors concluded that this is not the case because of the different
origins of the proteins (Osslund et al., 1982).

In attempt to provide evidence for a protein complex between ATPS and APSK in plants,
yeast two hybrid screens were performed with APSK from *Arabidopsis* as bait, but no
complex was detected between ATPS and APSK (Lillig et al., 2001).

To date, there has been no evidence that APSR is involved substrate channeling, nor are
there any reports of APSR involved in a protein complex. However, APSR has been
shown to associate with the thylakoid membrane (Koprivova et al., 2001), the site of
photosynthesis. APSR doesn't directly require photosynthetic products, but the formation
of a protein complex between other proteins within the sulfur assimilation pathway, such
as ATPS and SiR, which do require photosynthetic products (ATP and reduced
ferredoxin respectively), would permit an effective supply of substrates. A similar model
has been proposed for enzymes of the Calvin cycle that utilise photosynthetic products
(Suss et al., 1993).

After sulfate is catalysed to APS by ATPS (reaction 1), APS can either be used as a
substrate for APSR (reaction 2) or as substrate for APS kinase (reaction 7) (Burnell and
Anderson, 1973; Lee and Leustek, 1998). Therefore both enzymes will compete for APS
as a substrate. Interestingly, in *A. thaliana* APSK has a V_{max}/K_m ratio for APS of 2.8 x

10^7 L mol^{-1} s^{-1}, making it a more efficient enzyme than APSR by a factor of 10^3-10^4 for catalysing APS. This presumably this would limit the APS available for reduction (Lillig et al., 2001). However, this is not the case. It is unknown as to how the reduction pathway dominates over the sulfation pathway. It is possible that negative regulatory mechanisms control the APSK gene expression or protein activity allowing APS to be reduced by APSR. Regulation of APSK is yet to be described.

In onions, there is a high demand for reduced sulfate, which means that the reduction pathway is required to dominate over the sulfation pathway.

Therefore, a protein complex between ATPS and APSR would allow APS produced by ATPS to be passed to the active site of APSR without being released into the bulk solution, reducing the substrate available for APSK and directing the flux of APS towards a desired product.

One effect of forming a protein complex is to create a local environment where the concentration of an intermediate, shared by two enzymes, is at a higher concentration than that in the surrounding solution. The effect is that enzymes are able to produce more product and this is particularly useful if the protein has a high K_m value (Brooks et al., 1988). It known that ATPS has a very low equilibrium value ($K_{eq} \sim 10^{-8}$) (Farooqui, 1980), which requires the product (APS) to be removed quickly otherwise the reverse reaction will dominate over the forward reaction. A complex between ATPS and APSR would allow swift removal of APS by APSR *via* its reduction to sulfite which would reduce the negative effect of APS on the ATPS forward reaction (Leyh, 1993). The product of the APSR reaction (sulfite) is reactive in cells (as is the sulfide produced by sulfite reductase) and so the reduction of these compounds is also highly desirable to minimise oxidative damage to the cell and invites the possibility that SiR may form a complex with APSR.

1.8　　　Thesis aims

In summary, there is some conceptual evidence for the desirability of an ATPS-APSR complex. This thesis therefore sought experimental evidence for the complex using both *in vitro* and *in vivo* approaches.

To do this, the following objectives were designed:

- Express and isolate sufficient ATPS and APSR enzymes as recombinant proteins.
- Examine the formation of protein complexes *in vitro* using ELISA, the ligand binding assay and the immunoprecipitation assay.
- Provide evidence for protein complex *in vivo* using onion chloroplast extracts, affinity chromatography and kinetic data.

In this thesis, when defining experimental approaches, the term '*in vitro*' refers to the direct use of recombinant proteins in experimental assays. In contrast, the term '*in vivo*' is used to refer to techniques that use whole leaf onion extracts or onion chloroplast extracts and so will include all proteins extracted, including ATPS and APSR. It is understood, however, that the term *in vivo* should strictly refer to the study of interactions *in planta* using non-invasive techniques (e.g. protein complementation assay (PCA), fluorescence-resonance-energy transfer (FRET) or by bimolecular florescence complementation (BiFC)).

2.0   Materials and Methods

2.1   Plant growth conditions

2.1.1   Plant material

- Onion cultivar W202A (a pungent, high sulfur compound storage line sourced from Crop & Food Research, Lincoln)
- Onion cultivar Texas Grano (a mild, low sulfur compound storage line sourced from Crop & Food Research, Lincoln)
- Horticultural grade bark/ peat/ pumice (50: 30: 20) (Dalton Nursery Mix, Tauranga).

2.1.2   Growth of plant material for general experiments