CHAPTER - TWO

REVIEW OF LITERATURE
Sulphur represents the ninth and least abundant essential macronutrient in plants, preceded by carbon, oxygen, hydrogen, nitrogen, potassium, calcium, magnesium, and phosphorus. The content of sulphur in plants is only about one-fifteenth of that of nitrogen. Sulphur plays critical roles in the catalytic or electrochemical functions of the biomolecules in cells. It is found in the amino acids cysteine and methionine, often responsible for protein structure and enzymatic activity; in coenzymes, such as iron-sulphur centres, thiamine, lipoic acid, or Coenzyme A; and in many secondary compounds, e.g. glucosinolates, alliins, etc. Not surprisingly, therefore, sulphur is an essential macronutrient for all living organisms. In most bioorganic metabolites sulphur is found in the reduced form as sulphide (S^{2-}) whereas the majority of sulphur in nature is in the oxidised form of sulphate. The inorganic sulphate thus has to be reduced and incorporated into carbon skeletons. The sulphate assimilation pathway is present in plants, fungi, and many bacteria (Leustek et al., 2000; Kopriva, 2006).

2.1 SULPHUR AS A PLANT NUTRIENT

Sulphur is one of the most versatile elements in life due to its reactivity in different oxidation and reduction states (Hell et al., 2008). Sulphur atom is an essential nutrient for living organisms because it plays a critical role in protein folding, enzyme catalysis and maintenance of redox status of cells. Sulphur is
one of the six macronutrients required by plants. Two important amino acids, cysteine and methionine, and various other metabolites are all derived from sulphur. The sulphur group, a thiol, is strongly nucleophilic (electron-donating), making it ideally suitable for oxidation and reduction reactions (redox reaction). When oxidized two cysteine molecule can form a covalent linkage called a disulphide bond and this is readily broken by reduction to form 2 thiol groups (dithiol). Disulphide ⇌ dithiol interchange is so versatile that nearly all aerobic forms of life, including plants, have evolved to use this reaction as the dominant form of redox control (Leustek and Saito, 1999). Redox control regulates enzymes and protects against oxidative damage. The nucleophilicity of the thiol group, and in particular GSH, plays a role in detoxification of xenobiotics by direct conjugation with sulphhydryl group mediated by GSH S-transferase. Phytochelatins, a polymerized version of GSH, are involved in detoxification of heavy metals by serving as chelating ligands through thiol groups. Sulphur-containing secondary products often have a characteristic smell and are regarded not only as defense compounds against herbivores and pathogenic organisms but also as signaling molecules for fundamental cellular functions (Matsubayashi et al., 2002). Methionine is commonly involved in regulation of enzymatic activity. The sulphur of the methionine is vulnerable to oxidation and can readily form a methionine sulphone. If methionine of a protein is converted to methionine sulphone, the protein is reversibly inactivated (Leustek et al., 2000). Sulphur requirement of plants varies with the plant development stage and with
species. The concentration of sulphur in plants varies between 0.1-1.5% of dry weight (Ahmad et al., 2005a).

2.2 SULPHATE UPTAKE IN PLANT

Plants need to move sulphate across multiple membrane barriers and into several sub-cellular compartments and organelles from root to leaf (Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002; Kataoka et al., 2004a). Sulphur is taken by plants through roots as sulphate, but sulphate reduction and assimilation takes place in leaves. Communication between root and leaf is therefore essential to maintain sulphur homeostasis for a balanced cysteine. In the uptake process of sulphate from roots, there are various transporters, which are involved in sulphate transportation.

Sulphate transporters are also predicted to contain 12 membrane-spanning domains. Plant sulphate-transporter proteins are 69-75kDa and range from 635 to 685 amino acids in length. The sulphate transporters are predicted to contain 12 membrane-spanning domains (MSDs) with C-terminal and N-terminal towards the inside of membrane. The highly co-ordinated expression of the gene family is controlled by the sulphate availability and the requirement for reduced S-compounds via feedback mechanisms on a cellular and whole plant level (Davidian et al., 2000; Hawkesford, 2000). Sulphate transporters are transmembrane proteins and these transporters are distributed in whole plant to fulfill the requirement of sulphur in different parts of the plant. The mode of the sulphur uptake and transport depends on passive and active mechanisms of ion uptake. Active mechanism dominates at
low sulphate concentrations in the external environment, while at high concentrations the passive portion of sulphate uptake increases. Sulphur is taken up to certain extent by passive movement through the plasmalemma, but the main form of the uptake and transport across plasma membrane is mediated by carriers. Sulphur is taken through 2 types of transporter, the higher affinity and lower affinity transporters. The high affinity transporter operates at low external concentration and a low affinity transporter system operates at higher external concentrations. The high affinity forms with $K_m$ for sulphate of approximately 9 μM are expressed exclusively in roots, whereas the lower affinity form with $K_m$ for sulphate of approximately 100 μM is expressed principally in leaves but also in roots. From the earlier physiological experiments on membrane vesicles and cell cultures, it is demonstrated that plants can adapt to low sulphate availability by modulating the sulphate-transport activity. The activity of the sulphate transporters has been extensively shown to be transcriptionally regulated by signal such as the GSH level, reflecting the sulphur nutritional status of the plant (Hawkesford, 2000; Maruyama-Nakashita et al., 2004). Glutathione, the end product of sulphur assimilation, essential in the storage and transport of reduced sulphur, was suggested as an important inter-organ-signal compound of the sulphur status from the shoot to the root (Lappartient et al., 1999; Davidian et al., 2000; Herschbach et al., 2000; Vauclare et al., 2002). After the initial uptake of sulphate at the root epidermis, internal distribution through the vasculature and cell to cell symplastic movement are required to
facilitate a continuous supply of sulphur to all organs and cell types. Multiple transport systems are activated to survive with a restricted sulphur source. First, plants use and induce high-affinity sulphate-uptake system in roots, facilitating the absorption of micromolar concentration of sulphate from the rhizosphere (Leggette and Epstein, 1956; Clarkson et al., 1983; Smith et al., 1995).

To facilitate the complex movements of sulphate around the plant, the sulphate transporters themselves are encoded by a gene family consisting of 14 members in *Arabidopsis*, probably with little redundancy (Hawkesford, 2003). There is clear evidence that transcription of the genes encoding the transporters involved in initial uptake at the soil-root interface, cell to cell transfer and vascular transportation as well as the vacuolar efflux transporter is controlled by plant sulphur status (Buchner et al., 2004a). The coordinated expression of this gene family facilitates optimum management of plant sulphate under varying conditions of supply and demand. The sulphate transporter family comprises 14 genes in *Arabidopsis* and probably a similar number in other species (Hawkesford, 2003). Based on sequence comparisons, these genes may be aligned into at least five clusters (referred to as groups 1-5). It has been proposed that the different groups represent functional subtypes; however, this may be an oversimplification. Broadly, group 1 represents high-affinity transporters, predominantly but not exclusively expressed in the roots (Smith et al., 1995; Smith et al., 1997). Many of this group are transcriptionally regulated in response to sulphur availability. A
unique specific localization of one isoform in this group, \textit{AtSultr1;3}, to the sieve elements–companion cell in the phloem is indicative of a specialized role in the redistribution of sulphur from source to sink tissues (Yoshimoto et al., 2003). Group 2, when expressed in yeast, has a lower affinity for sulphate. The introduction of techniques utilizing GFP-fusion proteins allowed the localization of these sulphate transporters isoforms within plant tissues, organs and plastids (Takahashi et al., 1997). Expression studies indicate a vascular tissue location, and therefore, of potential significance in considering tissue distribution of sulphate. Details of the expression patterns of the two genes found in this group differ substantially between the two plant species investigated in detail. In \textit{Brassica}, only isoform \textit{AtSultr2;1} is expressed substantially in the root, stem and leaves, while \textit{AtSultr2;2} is also expressed in roots. In \textit{Arabidopsis}, both isoforms are expressed in roots and leaves. In \textit{Brassica}, the expression of \textit{AtSultr2;1} occurs under sulphur starvation in the roots; however, in the leaves expression occurs also under sulphur-replete conditions, but is increased upon sulphur starvation (Buchner et al., 2004a, b). The \textit{AtSultr2;2} expression in the root is increased by sulphur starvation. In \textit{Arabidopsis}, \textit{AtSultr2;1} is noticeably induced by sulphur starvation in the roots, as found in \textit{Brassica} (Takahashi et al., 2000). There is little influence of sulphur nutrition on \textit{AtSultr2;2} expression. Reporter gene expression studies in \textit{Arabidopsis} indicate phloem expression of \textit{AtSultr2;2} in the leaves, but xylem-parenchyma expression of \textit{AtSultr2;1}. 
By contrast, *AtSultr2;2* was expressed in phloem in the roots but in the vascular bundle sheath cells and not in the phloem itself (Takahashi et al., 2000). The group 3 transporters are rather enigmatic. This is a rather larger group with five examples in *Arabidopsis*. An intriguing suggestion is that one isoform, *AtSultr3;5*, functions as a heterodimer with *AtSultr2;1* (Kataoka et al., 2004a). Expression of *AtSultr3;5* in yeast by itself fails to catalyse sulphate transport, but contributes to uptake when co-expressed with *AtSultr2;1*. In addition, *AtSultr3;5* is constitutively expressed in the same cells as *AtSultr2;1*, which is only expressed under sulphur-limiting conditions; the higher activity of the heterodimer being part of the adaptive response. A homologue of *AtSultr3;5* has been described for *Lotus japonicus*, which is localized on the symbiosome membrane in the N₂-fixing nodule (Krusell et al., 2005). This transporter is essential for sulphur delivery to the bacteroids and for an efficient N₂ fixation. By contrast to the *Arabidopsis* isoform, the *Lotus Sultr 3;5* is able to function when expressed alone in yeast. By contrast to the plasma-membrane location of the sulphate transporters of groups 1-3, groups 4 and 5 putative sulphate transporters have been localized to the tonoplast membrane (Kataoka et al., 2004b).

The group 4 transporters have been implicated in efflux of sulphate from the vacuole and are up-regulated by sulphur stress, thus favouring the unloading of sulphate from the vacuole. The role of the group 5 transporters has yet to be established. A greater resolution of expression patterns of individual isoforms will be informative, particularly in the light of the
complex patterns of sulphate redistribution, which are both developmentally programmed and also influenced by nutrient availability.

Plants that are grown with insufficient levels of sulphate develop symptoms of sulphur deficiency, which include chlorosis of young leaves, growth retardation, and altered root morphology (Hawkesford, 2000; Lopez-Bucio et al., 2003; Nikiforova et al., 2003). This indicates that an insufficient sulphur supply firstly results in a reduced amount of cysteine, being produced at the growing point of the plant, where proteins are synthesized at high rates.

Transport rates from source organs thus seem to be insufficient to compensate for this limitation. In higher plants, reduction of chlorophyll by sulphur deficiency appears first in young leaves, in contrast to nitrogen deficiency, which causes paling of source leaves. This implicates that down regulation of photosynthesis by different mineral stresses is specifically regulated rather than a pleiotropic effect. At least partially the pale phenotype of sulphur deficiency is based on reduced cysteine synthesis caused by sulphide limitation in higher plants. Sulphur-deprived plants show accumulation of phenolic compounds, as is typical for all nutrient starvations and various other environmental stresses such as cold, drought, or high light (Nikiforova et al., 2003, 2005a,b, Hirai and Saito, 2004, Hirai et al., 2003). When a soil is deficient in S and the deficiency is not rectified, the full potential of a crop variety cannot be realised, regardless of top husbandry practices (Ahmad et al., 2008).
Furthermore, the existence of a family of sulphate transporters, with specific occurrence in different tissues, and with differential responsiveness to sulphur supply, supports the idea that sulphate transporters have an important role in whole plant sulphur-management. Systematic analysis of these transporters in terms of function and expression is in progress. The expression of genes encoding different sulphate transporters is regulated by signals that respond to the nutrient status of the plants (sulphur-supply). When the external supply of sulphate is affecting the internal concentrations of sulphate, cysteine and glutathione decline, while a rapid increase in mRNA transcripts is observed. Sulphur re-supply decreases the gene expression of sulphate transporters (Smith et al., 1997; Bolchi et al., 1999; Lappartient et al., 1999; Vidmar et al., 1999; Takahashi et al., 2000). These data suggest that the expression of sulphate transporters is induced if the intracellular sulphur status is low, giving a hint that the reduced sulphur in the form of glutathione or cysteine might act negatively on the expression of sulphate transporters and ATP-sulphurylase in sulphur-starved plants. These data support a model in which, under sulphur-sufficient conditions, metabolites such as cysteine and glutathione act as regulators of sulphur-uptake and assimilation at the level of gene expression. While under sulphur-deficient conditions the decreasing levels of these compounds remove repression and, thus, result in increased transporter activity with maximized sulphate uptake. APR activity and transcript levels were decreased if cysteine and GSH were fed in excess
(Vauclare et al., 2002). This result implies that increased internal cysteine and GSH levels might control sulphate assimilation.

On the basis of analysis of metabolite content versus sulphate uptake, it was suggested that sulphate itself, rather than thiols or O-acetyl-serine, was involved in the signaling of regulation of sulphate-uptake efficiency of roots (Buchner et al., 2004b; Hawkesford and De Kok, 2006; Yang et al., 2006). The uptake and distribution of sulphate and the expression of the sulphate transporters are generally modulated by the sulphur status of the plant (Saito, 2004; Hawkesford and De Kok, 2006). Sulphate deprivation generally resulted in a rapid increase in the sulphate uptake capacity, which varied between species, transferring plants from high-sulphate concentrations (100 μM) to sulphate-deprived conditions (0 μM) and *vice versa*. It provides only a limited insight into regulatory aspects of sulphate uptake and its distribution at a whole plant level. For instance, both at a low external sulphate concentration and upon sulphate deprivation the sulphate-uptake capacity was increased and mRNA abundance of high-affinity transporters increased. In addition, the sulphate uptake capacity (even when measured at several external sulphate concentrations) upon sulphate deprivation and the one at low-external sulphate concentrations were only slightly different, three- to four-fold at the most. However, there was a substantial difference between the degrees of accumulation of mRNAs of the sulphate transporters. At low-external sulphate concentrations, only the high-affinity sulphate transporters (Group 1) were moderately induced, whereas there was a mass-induced expression of
all sulphate transporters upon sulphate deprivation (Buchner et al., 2004b). Apparently, at a low-external sulphate concentration, only the sulphate transporters involved in the actual uptake were up-regulated, whereas upon sulphate deprivation there might be a mass-nonspecific expression of all sulphate transporters throughout the root tissue (Hawkesford and De Kok, 2006). Furthermore, a decreased shoot to root biomass allocation occurred solely upon sulphate deprivation. The relatively increased proportion of the root biomass versus that of the shoot upon sulphate deprivation, combined with the higher potential sink capacity for sulphate when sulphate content of deprived roots was quite low, might be the cause of the observed lower xylem loading upon sulphate deprivation.

Plants are estimated to possess thousands of transcription factors, each of them being considered to mediate and to control the expression of thousands of target genes, by targeting appropriate DNA-specific cis-regulatory elements (Priest et al., 2009). Such a cis-element has been identified in Arabidopsis as being involved in regulation of plant response to sulphur deficiency (Maruyama-Nakashita et al., 2005). This core cis-element, comprising 16 base pairs of a sequence, has been called Sulphur-Responsive Element (SURE). It is present in the promoter of AtSultr1;1 and several other genes of the sulphur metabolic pathway. A transcriptional regulator, Sulphur LIMitation1 (SLIM1), that regulates sulphate uptake and assimilation, has been shown to up-regulate AtSultr1;1, AtSultr1;2, and AtSultr4;2 gene expression in response to sulphate starvation (Maruyama-Nakashita et al.,
2006). However, it seems that SLIM1 does not require the SURE cis-element.

As already pointed out, AtSultr1 and AtSultr4 members are differentially expressed under –S conditions (Kataoka et al., 2004b; Rouached et al., 2008). The higher inducibility of AtSultr1;1 compared to AtSultr1;2 can be explained by the absence of the SURE element in the promoter of AtSultr1;2. This suggests that the up-regulation of the root major SO₄²⁻ transporter AtSultr1;2 is probably essentially controlled by SLIM1 when SO₄²⁻ availability in the external medium is restricted, but that an additional regulatory mechanism dependent on the SURE element drives a strong expression of AtSultr1;1 during –S conditions. The SLIM1 gene is also called EIL3, being a member of the ETHYLENE-INSENSITIVE-LIKE family, but has no identified link with the ethylene signaling pathway (Maruyama-Nakashita et al., 2006).

To date, SULTR1;1 and SULTR1;2 are the only two transporters which have been demonstrated to display high affinity in the micromolar range for sulphate (Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002). Both transporters are co-regulated by metabolites of the sulphur assimilatory pathways (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003; Hirai and Saito, 2004; Maruyama-Nakashita et al., 2004; Rouached et al., 2008) their transcription is responsive to sulphur deprivation, although the SULTR1;2 gene is also abundantly expressed under high sulphur availability (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002; Maruyama-Nakashita et al., 2003; Fizames et al., 2004; Maruyama-Nakashita et al., 2004; El Kassis et al., 2007; Yoshimoto et al., 2007; Rouached
et al., 2008) and post-transcriptional modifications have been evidenced for both of them (Yoshimoto et al., 2007). On the whole, the current data allow us to suppose that the Arabidopsis sulphate transporters SULTR1;1 and SULTR1;2 could function redundantly. However, previous genetic analyses leave the individual contributions of the SULTR1;1 and SULTR1;2 genes open to question. Indeed, using selenate as a sulphate toxic analogue, resistance screening leads to the isolation of several mutants that all belong to the same complementation group (sel1, 11 alleles) identifying the SULTR1;2 gene (Rose, 1997; Shibagaki et al., 2002; El Kassis et al., 2007). These independent screening procedures could not identify any other sulphate transporter, in particular no mutant in the SULTR1;1 gene, despite its high degree of structural and functional identity with SULTR1;2 (Yoshimoto et al., 2002; Rouached et al., 2005).

2.3 SULPHUR ASSIMILATION IN PLANTS

The sulphur assimilation into amino acid occurs in plastids of leaf cells. It is initiated by the uptake of sulphate by roots from the soil, proton/sulphate symporters in plasma membranes being responsible for this step. The uptake of sulphate is followed by activation of sulphate, reduction of sulphate and synthesis of cysteine (Fig. 1). This last step constitutes the unique entry of reduced sulphate into the metabolism. Before incorporation into an organic skeleton, sulphate must follow a set of reactions, which constitute this assimilatory pathway. Sulphate is then further transported into plastids, where reduction and most of the assimilation processes take place.
In the plastids, sulphate is activated by adenosine triphosphate (ATP) to form adenosine 5'-phosphosulphate (APS) in a reaction catalyzed by ATP sulphurylase. APS is a branch point intermediate, which can be employed in both reduction and sulphation reactions. APS receives two electrons from glutathione in a reaction catalyzed by APS reductase to form sulphite, which is then further reduced with six electrons from ferredoxin by sulphite reductase. The overall reduction from sulphate to sulphide requires one ATP and eight electrons. The resultant sulphide can subsequently be utilized by synthesis of cysteine from serine in a two-step process involving a complex of serine acetyl transferase and O-acetylserine (thiol) lyase (OAS-TL). This complex is called cysteine synthase. The first step catalyzed by serine acetyltransferase is acetylation of serine in the presence of acetyl-CoA to form O-acetyl serine (OAS). Provision of serine is dependent upon adequate C and N metabolism and this point of convergence of the assimilatory pathway represents an opportunity for the co-ordination of S-assimilation of C and N metabolism. The second is the formation of cysteine by O-acetyl serine thiol lyase (OAS-TL) from sulphide and O-acetyl serine (OAS), with a release of acetic acid. The two-reaction-system exits in the cytoplasm and plastids, as well as in mitochondria, although it is not clear why it is needed in each compartment. Cysteine is further utilized to synthesize methionine, proteins, glutathione etc. (Leustek et al., 2000; Saito, 2000). Cysteine (Cys), as the first carbon/nitrogen-reduced sulphur product, resulting from the sulphate assimilation pathway, serves as sulphur donor for methionine, glutathione,
vitamins, co-factors and sulphur compounds that play a major role in the growth and development of plant cells. This sulphur imprinting occurs in a myriad of fundamental processes, from photosynthesis to carbon and nitrogen metabolism.

![Sulphur assimilatory pathway](image)

**Figure 1.** Sulphur assimilatory pathway

Cysteine contains 2⁻ valence sulphur produced by reduction and assimilation of 6⁺ valence sulphate. The thiol (sulphydryl) group of cysteine in proteins takes the job of maintaining protein structure by forming disulphide bonds between two cysteine residues via oxidation. The thiol of cysteine and GSH is often involved in the redox cycle by two thiol disulphide conversions. This interchange is versatile for redox control and mitigation against oxidative stress in nearly all aerobic organisms including plants (Leustek and Saito, 1999). The versatile function of the thiol group of cysteine derives from its chemistry (acidity, lower pk and nucleophilic properties). While cysteine in
proteins has a versatile role, the only known role of methionine in proteins is associated with its hydrophobicity and thus protein folding. Regulating mechanisms are indispensable for homeostasis, facilitating and repressing sulphate uptake and reduction under sulphate-limiting and sulphur-rich conditions, respectively. The rate of sulphate assimilation is known to be controlled by allosteric regulation of enzymatic activity (Hawkesford, 2000). Metabolic effect of S-stress is a depression of root hydraulic conductivity. Karmokar et al. (1991) reported an early response, which may have role in signaling nutrient starvation from root to shoot.

The key cellular redox-buffering tripeptide, glutathione, and O-acetylserine down-regulates and up-regulates, respectively, the expression of most genes encoding root-sulphate transporters as well as enzymes of S-metabolic pathway (Hopkins et al., 2005); however, not all reports are in agreement with these hypotheses. Whether, in plants, O-acetyl serine (OAS) has a similar direct regulatory role as its counterpart N-acetylserine has in E. coli is questionable, as no clear cut correlation was found between the *Arabidopsis* root O-acetyl serine (OAS) cellular content and the mRNA levels of either SULTR1;1 and SULTR1;2 (Rouached et al., 2008). Similarly, in potato, O-acetyl serine (OAS) has been found to accumulate during -S conditions only after the sulphate transport has already been induced (Hopkins et al., 2005). Glutathione is efficient in repressing sulphate uptake after a period of sulphate starvation but also during normal S nutrition (Lappartient et al., 1999; Vauclare et al., 2002). Several studies, however, argue against the relay
model played by glutathione, and, in *Arabidopsis*, a new complex regulatory interplay between SULTR1;1 and SULTR1;2, based upon an extensive comparison of a wide set of growth conditions and metabolite signaling pathways, has been proposed (Rouached et al., 2008; Rouached et al., 2009).

The regulation of primary sulphur metabolism has so far mostly been investigated with respect to supply-dependent responses. Sulphate limitation results in the rapid transcriptional activation of a plethora of genes. Most affected are members of the sulphate transporter family, followed by genes of sulphate assimilation (Buchner et al., 2004a; Saito, 2004), but microarray analyses covering 7000 to 9000 *Arabidopsis* genes have revealed the induction of downstream genes of sulphur-related and so far considered as unrelated pathways (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). Because their internal concentrations respond rapidly to external sulphate depletion, sulphate, O-acetylserine (OAS), the intermediate metabolite of cysteine synthesis, cysteine itself and glutathione are discussed as regulatory triggers of the transcriptional response. Although local concentrations are not known, cytosolic sulphate and O-acetyl serine (OAS) levels are the most likely candidates in this respect (Leustek et al., 2000; Saito, 2000, 2004; Buchner et al., 2004b). Metabolic regulation seems to be exerted by O-acetyl serine (OAS) and sulphide via the reversible interaction of the subunits of the cysteine synthase complex (Hell et al., 2002) and by cysteine-feedback inhibition of serine acetyltransferase (SAT) that catalyses the first step of cysteine synthesis (Saito, 2000).
2.3.1 ATP Sulphurylase (ATPS)

Plant ATPS is a homotetramer of 52–54 kDa polypeptides. *Arabidopsis* ATP sulphurylase polypeptides are approximately 52 kDa (Murillo and Leustek, 1995). The primary structure of plant ATP sulphurylases resembles that of the enzyme in other eukaryotes, but they are completely unlike the enzyme from prokaryotes. ATP reacts with sulphate to form the reaction product of diphosphate and adenylyl sulphate.

![ATP Sulphurylase](image)

2.3.1.1 Genes involved in regulation of ATP Sulphurylase

There are two ATP sulphurylase isoforms in most plants: a major form localized in plastids and a minor form localized in the cytoplasm. Both the enzymes have similar kinetic and structural properties. The isoenzymes are encoded by a gene family, and in *Arabidopsis* there are multiple genes for the plastid enzyme. *Arabidopsis* contains a cytosolic form of ATP sulphurylase, but the corresponding gene has not yet been identified (Rotte, 1998). The plastid enzyme exists in both leaves and roots and is responsible for initiating the reductive assimilation of $\text{SO}_4^{2-}$, since isolated chloroplasts can form cysteine from $\text{SO}_4^{2-}$ (Schürrmann and Brunold, 1980). The cytoplasmic form probably
functions by generating APS for sulphation reactions. In all organisms two isoforms of ATPS have been found except in *S. moellendorffii* with *Arabidopsis* and *Populus trichocarpa* possessing four-member gene families.

This seems to be physiologically relevant, since ATPS activity is present in the plastids as well as in the cytosol (Lunt et al., 1990; Rotte and Leustek, 2000). Indeed, spinach (*Spinacia oleracea*) and potato (*Solanum tuberosum*) contain two ATPS isoforms, specific to the cytosol and plastids (Lunn et al., 1990; Renosto et al., 1993; Klonus et al., 1994). However, the existence of cytosolic and plastidic ATPS isoforms does not seem to be universal. In other plant species including rice, poplar, and *Arabidopsis*, all ATPS genes encode proteins with putative plastid-targeting peptides (Leustek et al., 1994; Murillo and Leustek, 1995; Hatzfeld et al., 2000a). As APS reduction occurs exclusively in plastids, cytosolic ATPS may be linked to cytosolic APS kinase and play a role in provision of PAPS for secondary metabolism (Rotte and Leustek, 2000). The latest data show that *Arabidopsis* lacking cytosolic APS kinase is perfectly viable and does not show any phenotypic alterations (Mugford et al., 2009), thus indicating that the real function of cytosolic ATPS still awaits elucidation. *Arabidopsis* contains four ATPS isoforms compared to two genes in most other species, indicating some level of genetic redundancy. In contrast to several sulphate transporters or sulphotransferase genes that occupy adjacent positions in the genome, indicating their recent origin by gene duplication, the ATPS genes are located
on different chromosomes. However, the analysis of the PGDD revealed that ATPS1 and ATPS4 are present in duplicated segments of the genome.

2.3.1.2 Regulation of ATP Sulphurylase

ATP sulphurylase catalyzes the entry point of sulphate into the sulphate-assimilation pathway. Sulphate has a low reduction potential and must therefore be activated by ATP-dependent conversion to APS (Schmidt and Jager, 1992). This reaction is, however, energetically very unfavorable so that the reaction equilibrium is strongly shifted towards the reverse reaction. This is actually utilized in sulphur-oxidizing bacteria to generate ATP (Laue and Nelson, 1994). The forward reaction generating APS is facilitated in plants by coupling to inorganic pyrophosphatase, which hydrolyses the second reaction product, the pyrophosphate. In bacteria, ATP sulphurylase forms a complex with GTPase (Patron et al., 2008). ATPS is a widespread enzyme present not only in sulphate-assimilating plants, algae, fungi and bacteria, but also in Metazoa, which do not reduce sulphate. In the latter organisms, ATPS, together with APK, is necessary to synthesize PAPS, the active sulphate for sulphation reactions that are essential for many aspects of animal life (Zhu et al., 2007).

There seems to be substantial differences in regulation of the individual genes as evident in the microarray data (Matthewman et al., 2009). In addition, differential regulation and possibly different biological functions of ATPS isoforms, at least in Arabidopsis, are inducible by sulphate deficiency and, are conserved in plant species from Physcomitrella to rice (Jones-Rhoades
and Bartel, 2004; Axtell and Bowman, 2008). Kawashima et al. (2009) have recently reported that ATPS is a target of a microRNA, miR395. In Arabidopsis, miR395 was confirmed to target three of the four ATPS transcripts (ATPS-1, ATPS-3 and ATPS-4) causing their cleavage (Kawashima et al., 2009). This specific regulation strongly suggests differences in function between ATPS2 and the three miR395-targeted isoforms. Another difference in regulation has been revealed by analysis of regulation of the ATPS family by MYB factors controlling glucosinolate biosynthesis (Gigolashvili et al., 2007a,b). Only ATPS1 and ATPS3 are targets of these transcription factors and the strength of response differs depending on whether the MYB factors are involved in regulation of aliphatic or indolic glucosinolates. Thus, while some level of functional redundancy can be expected, it seems that individual ATPS isoforms have distinct biological roles. Plant ATPS genes are more closely related to ATPS genes from animals than to those in green algae (Patron et al., 2008).

2.3.2 APS Reductase (APR)

The APS reductase from plants is an enzyme with a bipartite structure, consisting of an amino terminal domain that carry the APS reductase catalytic center and a C-terminal electron transfer domain that mediates the interaction with the natural electron donor, reduced glutathione (Bick et al., 1998; Kopriva et al., 2001). Plant APS reductases contain a diamagnetic [4Fe-4S]2+ cluster that is bound by the N-domain (Kopriva et al., 2001). The structure is trigonally coordinated to three sulphur ligands and a fourth non-
sulphur ligand (C, N or O). Three of the cluster ligands have been identified as cysteine residues that are conserved with bacterial APS reductases, but are absent from PAPS reductase. Based on the conservation of these cysteine residues in a range of enzymes, some of which have been demonstrated to catalyze APS reduction, it has been proposed that the iron-sulphur cluster determines substrate specificity (Kopriva et al., 2002; Williams et al., 2002). APS reacts with ATP in the presence of enzyme catalyst APS Kinase to form reaction product of PAPS and ADP.

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\text{Adenosine 5\textsuperscript{\textprime}}^\prime\text{-Phosphosulphate} \xrightarrow{\text{APS REDUCTASE}} \text{2GSH} \rightarrow \text{sulphite}
\]

### 2.3.2.2 Genes involved in regulation of APS reductase

The evolution of the APR gene family in *A. thaliana* was explored by analysis of its genomic sequences (Chen and Leustek, 1997). APR1 is encoded by three exons that are separated by two introns. In contrast, APR2 and APR3 are composed of four exons that are separated by three introns: the salient difference being that APR1 lacks the intron that separates exons 2 and 3 in APR2 and APR3.

At the level of nucleotide homology, the coding sequence of APR1 is more closely related to APR3 (78% identity) than to APR2 (68% identity). This suggests that APR1 emerged by duplication of an ancestral APR1/APR3 gene followed by the loss of exon 2. The only PAPS reductase gene that is known to
be interrupted is from the filamentous fungus *Emericella nidulans* (GenBank accession #X82555). This gene contains a single intron that interrupts the coding sequence at a position very close to the position of intron 2 in APR1 and intron 3 in APR2 and APR3. A question of key significance in the evolution of the APR enzymes is how three distinct domains — the plastid transit peptide, the reductase domain, and the thioredoxin/glutaredoxin domain — became fused into a single polypeptide. One popular idea on gene evolution is that the domains of proteins arise through the accretion of separate exons (Traut, 1988). If this were the case in APR evolution, one might expect to find an intron separating the exons encoding individual domains. This appears to be the case with respect to the transit peptide. In all three genes it is encoded by exon 1, although the protease cleavage site is encoded at the beginning of exon 2. In contrast, the other domains are not separated by an intron, rather a portion of the reductase domain and the thioredoxin/glutaredoxin domain are fused into single exon. This finding does not help to clarify the evolutionary origin of the gene. An interesting point, however, is that in APR1 there is a nearly perfect 12 basepair sequence duplication located at the 5’end of intron 2 (gtatatttcgc) and another within exon 3 (gtatatatgc) at a position that is approximately at the border between the reductase and thioredoxin/glutaredoxin-encoding sequences. Similar, but less well conserved sequences, are present at the same position in APR2 and APR3. It is an intriguing possibility that this repeat is the remnant
of an intron that divided the last exon of these genes, isolating the thioredoxin/glutaredoxin-like domain in a separate exon.

Sulphate reduction is initiated and carried out by the enzyme APS reductase in leaf and root plastids. The amino acid structure of plant APS reductase revealed a multidomain composition (Suter et al., 2000). The amino terminal domain of the mature protein is homologous to PAPS reductase and the carboxyl terminal domain is homologous to thioredoxin, a redox enzyme. APS reductase is able to use GSH or dithiothreitol as an electron source.

2.3.2.3 Regulation of APS Reductase
The uptake of sulphate and the reduction of APS by APr are controlled by the sulphur nutritional status of the plant and are considered to be the rate-limiting steps in the assimilation of sulphur in plants (Vauclare et al., 2002; Hawkesford and De Kok, 2006). APS reductase is thought to be one of the key regulators of the sulphate reduction pathway. Its activity and steady-state mRNA level increased markedly and coordinately in response to sulphate starvation (Gutierrez-Marcos et al., 1996; Takahashi et al., 1997; Yamaguchi et al., 1999), oxidative stress (Leustek et al., 2000), or heavy metal exposure (Heiss et al., 1999). The latter two stresses increase the demand for glutathione, and hence, the cysteine necessary for glutathione synthesis. Other sulphate assimilatory enzymes are regulated to a lesser degree (ATP sulphurylase) or are constitutively expressed (sulphite reductase) (Bork et al., 1998).
2.3.3 Sulphite Reductase

The sulphite reductase is found in plant cells and consists of homo-oligomer containing a siroheme and a iron-sulphur cluster per subunit (Schwenn, 1994). The plant enzymes contain putative catalytic domains of electron transfer that are homologous with plant nitrite reductase, using the same siroheme –iron sulphur cluster prosthetic group. Plant sulphite reductase (SiR) is a monomeric protein of 65 kDa containing a siroheme and an FeS cluster (Nakayama et al., 2000). Complete amino acid sequences, deduced from the corresponding cDNA sequences, are available for Fd-SiRs from three higher plants, maize (Ideguchi et al., 1995), Arabidopsis (Bruhl et al., 1996) and tobacco (Yonekura-Sakakibara et al., 1998). Plant sulphite reductase (SiR) is a plastidic protein that has an N-terminal cleavable extension peptide necessary for its plastid import. The mature region exhibits significant homology to cyanobacterial sulphite reductase (SiR) and the hemoprotein subunit of E. coli sulphite reductase (SiR). The extents of amino acid sequence homologies among plant sulphite reductase (SiRs) are very high (77% to 82%), while plant sulphite reductase (SiRs) are about 50% and 30% homologous to cyanobacterial sulphite reductase (SiR) and the hemoprotein subunit of E. coli sulphite reductase (SiR), respectively.

Sulphite is converted to sulphide catalyzed by sulphite reductase.

\[
\text{Sulphite reductase} \\
\begin{array}{c}
\text{O=\text{S}} \\
\text{O^-} \\
\end{array} \\
\rightarrow \\
\begin{array}{c}
\text{S^{2-}} \\
\text{O^-} \\
\end{array}
\]

\begin{center}
Sulphite \quad Sulphide
\end{center}
Sulphite reductase is a homo-oligomeric hemoprotein composed of 2 or 4 identical subunits. Each subunit contains 1 siroheme and 1 iron-sulphur cluster [4Fe-4S]. Although plant sulphite reductase uses electrons donated from ferredoxin, it is localized in plastids of both photosynthetic and non-photosynthetic tissues. In non-photosynthetic tissues the electrons come from NADPH via ferredoxin- NADP+ reductase (Nakayama et al., 2000). *Arabidopsis* contains a single gene encoding sulphite reductase. The amino acid sequence of the *Arabidopsis* enzyme shows that it is distantly related to nitrite reductase. Both the enzymes utilize siroheme as a cofactor and the enzymes show significant homology at the carboxyl terminus, where the cofactor binds. Only recently it has become possible to produce an active plant sulphite reductase as a recombinant protein in *E. coli* from the cloned cDNA. To do so it was necessary to overproduce the prosthetic group siroheme (Nakayama et al., 2000). Analysis of the catalytic requirements of maize sulphite reductase indicates that reductant supply is a significant factor determining the rate of sulphite reduction in vivo (Yonekura-Sakakibara et al., 2000). In non-photosynthetic tissues, a specific ferredoxin (Fd III) and a high NADPH/NADP+ ratio are crucial for sulphite reduction.

2.3.3.2 *Genes involved in regulation of sulphite reductase*

Sulphite reductase is the only component of sulphate assimilation, which is encoded by a single gene in *Arabidopsis* and most other vascular plants. The sulphite reductase (SiR) gene, however, still has a close paralogue in plant genomes, the nitrite reductase (NiR) (Patron et al., 2008). Plant Nitrite
reductase (NiR) and Sulphite reductase (SiR) are plastidic enzymes, catalyzing six electron reduction of nitrite and sulphite in sulphate and nitrate assimilation pathways, respectively (Nakayama et al., 2000; Swamy et al., 2005). Both the enzymes use siroheme and FeS centers as cofactors and ferredoxin as an electron donor. Indeed, they were shown to be able to catalyze reduction of both compounds, their specificity derived mainly from KM values for the respective substrates (Krueger and Siegel, 1982). The two enzymes are similar enough to conclude that they result from gene duplication (Patron et al., 2008). This duplication must have preceded the initial endosymbiotic event that gave rise to plastids, since both sulphite reductase (SiR) and nitrite reductase (NiR) from plants and all groups of algae are closely related to cyanobacterial sequences within the respective clades (Patron et al., 2008). In lower plants, surprisingly, the complexity of sulphite reductase (SiR) is greater than in the vascular plants. *P. patens* possesses three sulphite reductase (SiR) genes that are 80–90% identical. All the three gene products are, however, targeted to plastids. The three isoforms may have specific functions in sulphate assimilation, but also outside the pathway. Indeed, in vascular plants, sulphite reductase (SiR) has been identified as a DNA-packaging protein in plastidic nucleoli (Chi-Ham et al., 2002; Sekine et al., 2007)

**2.3.3.3 Regulation of sulphite reductase (SiR)**

Sulphite reductase catalyzes the transfer of six electrons from ferredoxin to sulphite to produce sulphide, $S^{2-}$. The sulphite reductase found in plant cells
consists of a homooligomer containing a siroheme and an iron-sulphur cluster per subunit. Sulphite reductase is localized in plastids of both photosynthetic and nonphotosynthetic tissues. Electrons are supplied to ferredoxin from PSI in photosynthetic cells and from NADPH in nonphotosynthetic cells. The proper combination of different isoforms of ferredoxin, ferredoxin-NADP+ reductase, and sulphite reductase is critical for efficient sulphite reduction (Yonekura-Sakakibara et al., 2000).

Sulphite is directly utilized as the sulphur donor for the formation of UDP-sulphoquinovose (6-deoxy-6-sulpho-Glc) from UDP-Glc (Sanda et al., 2001). UDP-sulphoquinovose is the precursor of the sulpholipid, sulphoquinovosyl diacylglycerol, present in the photosynthetic membranes representing one of the few naturally occurring sulphonic acid (R-CH$_2$-SO$_3^-$) derivatives. A molybdenum enzyme, sulphite oxidase, catalyzes oxidation of sulphite to sulphate in peroxisomes (Eilers et al., 2001). This enzyme is widely distributed in higher plants and is likely responsible for detoxification of sulphite rather than for chloroplast-based sulphur assimilation.

2.3.4 O-acetyl Serine (Thiol) Lyase (OAS-TL)

The enzymatic pathway of cysteine synthesis has been characterized (Kredich et al., 1969) providing the first enzymatic data for Serine acetyltransferase (SAT) and O-acetyl serine (thiol) lyase (OAS-TL) as free homomers as well as bound in the heterooligomeric cysteine synthase complex. They revealed equal substrate affinities of serine acetyltransferase (SAT) in the complex compared with the homomer, but lower affinities for sulphide and O-acetyl
serine (OAS) for bound OAS-TL, effectively resulting in partial inactivation compared with free OAS-TL dimers. Accordingly, the earlier hypothesis of substrate channelling of OAS from serine acetyltransferase (SAT) to OAS-TL within the complex was shown to be unlikely by (Cook and Wedding, 1977). Since, OAS-TL is much less active in the complex, the intermediate OAS readily leaves the complex. In addition, OAS was shown to dissociate the complex, whereas sulphide stabilized it in vitro (Kredich et al., 1969; Cook and Wedding, 1977). These findings were largely confirmed for OAS-TL in the plant cysteine synthase complex, where SAT became more affined to its substrates and OAS-TL almost inactivated in the complex, causing OAS to leave the complex (Droux et al., 1998).

The three-dimensional structure of OAS-TL from *S. typhimurium* was corroborated (Burkhard et al., 1998). Both N- and C-terminus are involved in the formation of the functional homodimer, while the interaction site with serine acetyltransferase is still unknown. Binding of OAS induces a strong conformational shift of the dimer, allowing only the small molecules, like sulphide, to access the catalytic centres of the dimer subunits. A putative allosteric binding site for an anion, possibly hydrogen sulphide ion (HS\(^{-}\)), has been identified, but still needs confirmation (Burkhard et al., 1999; Burkhard et al., 2000; Tai et al., 2001). It is interesting to note in this context that protein sequences of the three OAS-TLs from *A. thaliana* are sufficiently similar to CysK to predict three-dimensional structures with high reliability, using
computer modelling with Swissprot software (http://swissmodel.expasy.org; Peitsch, 1995; Schwede et al., 2003).

2.3.4.2 Genes involved in regulation of O-acetyl serine (thiol) lyase

Arabidopsis thaliana contains at least nine OAS-TL-like genes. The mainly expressed genes encode the cytosolic (A1), plastid (B), and mitochondrial (C) isoforms (Hesse et al., 1999; Jost et al., 2000). Another member (AtcysC1) encodes a mitochondrial b-cyano-alanine synthase that catalyses the detoxification of cyanide with cysteine, forming b-cyano-alanine and sulphide (Hatzfeld et al., 2000b). The other family members in A. thaliana are much less characterized (Yamaguchi et al., 2000). O-acetyl serine (thiol) lyases and b-cyano-alanine synthase carry out both reactions, however, with different substrate affinities and efficiencies, enabling kinetic discrimination of the physiological function (Jost et al., 2000; Warrilow and Hawkesford, 2000; Burandt et al., 2002). In addition, the situation can be different in other plants. Spinach contains cytosolic and plastid OAS-TL activities, but only mitochondrial b-cyanoalanine synthase activity (Warrilow and Hawkesford, 2000), while b-cyano-alanine synthase activity is present in the cytosol and mitochondria of tobacco (Liang and Li, 2001). Various substrates are also accepted, such as azide (Rosichan et al., 1983) or pyrazole (Ikegami et al., 1988), which led to the nomenclature term of b-alanine substituted synthases (Hatzfeld et al., 2000b).

According to the Arabidopsis Genome Initiative (2000) the OAS-TLS are not linked to the serine acetyltransferase genes. The encoded proteins OAS-TL
A1, OAS-TL B and OAS-TL C were shown to be enzymatically “true”
cysteine-synthesizing enzymes as compared to CAS kinetics (Jost et al., 2000).
The OAS A1 (At4g14880) and OAS A2 (At3g22460) genes encode cytosolic
isoforms, whereas the OAS B (At2g43750) and OAS C (At3g59760) genes
encode the plastid and mitochondrial enzymes, respectively. Exon-intron
structures and splice site junctions are highly conserved among these two
gene pairs and to a lower extent between all four genes. In contrast, the
putative promoter areas differ in plants considerably, suggesting specific
regulatory patterns of the isogenes (Jost et al., 2000). The OAS A1/OAS A2
and OAS B/OAS C gene pairs are probably the results of segmental
duplications within the genome as suggested by the Arabidopsis Genome
Initiative (2000). According to these analyses, the OAS A precursor gene has
been duplicated from chromosome 4 to chromosome 3, or vice versa, due to
an illegitimate recombination event. As a consequence of functional
redundancy the OAS A2 gene then apparently developed into a pseudogene
that is transcribed but not translated into a functional OAS-TL protein (Jost et
al., 2000). A similar evolutionary event might have occurred in the case of the
OAS B gene on chromosome 2 and OAS C gene on chromosome 3. The OAS C
gene then turned into a mitochondrial OAS-TL, probably by acquisition of 5′
sequences to form a corresponding transit peptide. The OAS C gene product
has been shown to be a true OAS-TL with mitochondrial localisation (Hesse et
al., 1999; Jost et al., 2000). An additional intrachromosomal duplication might
have created the CAS gene (At3g61440), since it is located next to OAS C on
chromosome 3. It encodes a mitochondrial CAS protein as demonstrated by (Hatzfeld et al., 2000b) (ARAtBSas3;1; AJ010505) and (Yamaguchi et al., 2000)(AtcysC1,AB024282); whether the presence of a mitochondrial OAS-TL is unique for Arabidopsis or the Brassicaceae family in general remains a subject for further analyses. Evolutionary analysis separated these OAS genes clearly from a CAS-like group of genes (Jost et al., 2000). Interestingly, two cDNAs encoding cytosolic proteins (AtcysD1, AB024284, and cysD2, AB024283) are associated with the CAS group (Jost et al., 2000), although they appear to be even more specific for the OAS-TL reaction than for OAS-TL A1, B and C (Hatzfeld et al., 2000b; Yamaguchi et al., 2000). Such a clear substrate preference is unusual for the β-substituted alanine synthases in general, and the recombinant CYSC1/ARAtBSas3;1, CYSD1/ ARAtBSas4;1 and CYSD2 proteins display surprisingly different affinities for the substrates OAS and sulphide (Hatzfeld et al., 2000b; Yamaguchi et al., 2000). The exact function of these two proteins as well as another OAS-TL like cDNA (CS26; Nakamura et al., 1997; AB003041) is thus not entirely clear. With respect to substrate specificity, it should be noted that purified OAS-TL like proteins are also capable of catalysing other nucleophilic elimination reactions between OAS and other compounds such as pyrazole and nitriles (Ikegami and Murakoshi, 1993).

O-acetyl serine (thiol) lyase belongs to a group of β-substituted alanine synthases (BSAS) within the superfamily of pyridoxal-phosphate binding enzymes (Hatzfeld et al., 2000b). Similar to serine acetyltransferase, OAS-TL is
present in the cytosol, plastids, and mitochondria. The OAS-TL gene family consists of nine genes in *Arabidopsis*, with one of them *BSAS1;2* (OASTLA2) being regarded as a pseudogene (Jost et al., 2000; Heeg et al., 2008; Watanabe et al., 2008). The *BSAS1;2* is a result of a gene duplication of *BSAS1;1*, as revealed by analysis of the PGDD, which also identified two more recently duplicated gene pairs, *BSAS2;1* and -2;2 as well as *BSAS-4;1* and *BSAS-4;2*. The kinetic properties of recombinant major cytosolic, plastidial, and mitochondrial isoforms of *Arabidopsis* are, however, remarkably similar (Jost et al., 2000). Other higher plant species possess six to ten OAS-TL genes, but the gene family is much smaller in the basal species *S. moellendorffii* and *P. patens*.

Some diversification in function of the different O-acetyl serine (thiol) lyase isoforms has become apparent, as several groups have identified the mitochondrial OAS-TL as b-cyanoalanine synthase (CAS), an enzyme important for cyanide detoxification (Hatzfeld et al., 2000b; Maruyama et al., 2000; Warrilow and Hawkesford, 2000). However, significant species-specific differences exist in the mitochondrial OAS-TL. While (Maruyama et al., 2000) and (Warrilow and Hawkesford, 2000) concluded that, at least in spinach and potato, the mitochondrial OAS-TL is actually CAS, both enzymes can be detected in *Arabidopsis* (Watanabe et al., 2008). The only plant species where a systematic study of OAS-TL gene family has been performed is *A. thaliana* (Jost et al., 2000; Heeg et al., 2008; Watanabe et al., 2008). The individual genes clearly differ in their level of expression, highest steady state mRNA levels
were found for the cytosolic BSAS1;1 (OASTL-A) and BSAS3;1 (CAS), with the plastidial BSAS2;1 (OASTL-B) and mitochondrial BSAS2;2 (OASTL-C) transcripts also being abundant (Watanabe et al., 2008b). Accordingly, disruption of BSAS1;1 and BSAS2;1 resulted in a significant decrease in total foliar OAS-TL enzyme activity, while knocking-out BSAS3;1 and surprisingly also BSAS1;1 affected CAS activity specifically (Watanabe et al., 2008b). Cysteine and glutathione levels were reduced only in leaves of BSAS1;1 plants and in roots of BSAS1;1 and -2:2 (Heeg et al., 2008; Lopez-Martin et al., 2008; Watanabe et al., 2008). Interestingly, similar to Serine acetyltransferase, different laboratories reported different effects of disruption of individual OAS-TL genes on Arabidopsis growth. While Watanabe et al. (2008b) did not observe any phenotypic changes in any of the eight single OAS-TL knock-out lines (BSAS1;2 was not analyzed), Heeg et al. (2008) detected a significant growth retardation of BSAS2;2 As the two sets of plants were grown under long and short days, respectively, the differences in phenotypes can most probably be attributed to the different growth conditions. The growth defect observed in bsa2;2 plants is remarkable, as it points to a particular importance of mitochondria for the maintenance of cellular cysteine and glutathione pools, as has recently been repeatedly reported in the recent past (Dominguez-Solis et al., 2008; Zechmann et al., 2008).
2.3.4.3 Regulation of O-acetyl serine (thiol) lyase

It was demonstrated that the reaction intermediate O-acetyl serine (OAS) is not channelled from the serine acetyltransferase subunit to the OAS-TL subunit in order to provide an efficient flux of the substrate (Cook and Wedding, 1977; Droux et al., 1998). In fact, O-acetyl serine (OAS) diffuses readily out of the complex, presumably because OAS-TL has strongly affinity to OAS and sulphide when bound to serine acetyltransferase. As a consequence, serine acetyltransferase apparently is active only in the complex and inactive without OAS-TL, whereas OAS-TL is almost inactive in the complex but quite active as a free homodimer (Droux et al., 1998). These results are supported by the organization of protein-protein interaction domains of serine acetyltransferase (Bogdanova and Hell, 1997) and the positive correlation of serine acetyltransferase catalytic activity with the ability to form the complex with O-acetyl serine thiol lyase (Wirtz et al., 2001). It was furthermore found that O-acetyl serine (OAS) could dissociate the cysteine synthase complex, at least in vitro (Kredich et al., 1969; Droux et al., 1998). Another important observation was the de-repressive effect of O-acetyl serine (OAS) on sulphate-transporter genes, as demonstrated by feeding-experiments (Hawkesford et al., 1997; Smith et al., 1997). These findings have been combined in a hypothesis that explains the function of the cysteine synthase complex as a metabolic sensor of the sulphide status of a plant cell (Hell and Hillebrand, 2001). Under conditions of sufficient sulphate supply the complex is associated, Serine acetyltransferase (SAT) forms O-acetyl
serine (OAS) that diffuses out of the complex and reacts with sulphide through catalysis of free O-acetyl serine thiol lyase (OAS-TL) dimers. When sulphate, and consequently sulphide, becomes limiting, the latter reaction stops and O-acetyl serine (OAS) accumulates. Upon a certain threshold, the accumulated O-acetyl serine (OAS) exerts two functions: (1) O-acetyl serine dissociates the complex, reducing serine acetyltransferase (SAT) activity and causing further consumption of acetylcoenzyme A; (2) O-acetyl serine triggers the de-repression of some genes encoding sulphate transporters. Due to the increased affinity for sulphate uptake, the process becomes reversible: sulphate is imported and reduced to sulphide that reacts with O-acetyl serine (OAS) via O-acetyl serine thiol lyase (OAS-TL) dimers. O-acetyl serine (OAS) concentrations would decline until the complex can associate again and the genes of high and low affinity sulphate transporters would be at least partially repressed. Serine acetyltransferase (SAT) can resume its activity in the complex at a rate that is adjusted to the availability of sulphide. The equilibrium of complex association and dissociation would determine the rate of O-acetyl serine (OAS) and thus of cysteine formation. At the same time, this equilibrium would be adjusted to the available sulfide concentration and coupled to the import of sulphate at the plasmalemma. According to this hypothesis, the cysteine synthase complex functions as a metabolic sensor and part of a control system of primary sulphur metabolism at the cellular level (Hell and Hillebrand, 2001). While such a system could control the metabolite flux upstream of cysteine, the feedback inhibition of Serine acetyltransferase
(SAT) might form a downstream mechanism of product control (Saito, 2000). Both regulatory models are confined to the cellular level and might form the integrating basis of source-sink interaction in a whole plant concept of sulphur metabolism. Cysteine formation is controlled through a multiple regulatory circuit involving Serine acetyl transferase and O-acetyl serine thiol lyase (OAS (thiol)-lyase). The two component enzymes form a reversible complex, comprising a homotetramer of serine acetyltransferase and two dimers of O- acetylserine thiol lyase (OAS-TL). O-acetyl serine (OAS) is not only a rate-limiting metabolite of the Cysteine biosynthetic pathway (Saito et al., 1994), but also a positive regulatory factor for gene expression (Leustek et al., 2000; Saito, 2000; Leustek, 2002). A unique regulatory mechanism operates through the formation of an enzyme complex, involving Serine acetyltransferase and OAS(thiol)-lyase (Fig 1.). OAS(thiol)-lyase concentration is far in excess of Serine acetyltransferase concentration (approximately 300-fold, ratio of OAS(thiol)lyase to Serine acetyltransferase is 300:1), indicating that only a fraction of the OAS(thiol)-lyase forms a complex with Serine acetyltransferase (Droux et al., 1998). Yet, the free form of each enzyme has catalytic activity, and the complex is not required for channeling of O-acetyl serine (OAS) (Droux et al., 1998).

The bound form of OAS(thiol)-lyase, which showed a dramatically decreased catalytic activity, acts as a regulatory subunit to positively modulate the activity of Serine acetyltransferase in the enzyme complex. The large amount of free OAS(thiol)-lyase is responsible for the actual catalytic
function of Cysteine formation. The stabilization of the complex is controlled inversely by O-acetyl serine (OAS) and sulphide. O-acetyl serine (OAS) triggers the dissociation of the complex, whereas sulphide counteracts the action of O-acetyl serine (OAS) (Bogdanova et al., 1997; Droux et al., 1998). O-acetyl serine (OAS) accumulation stimulated by sulphur deficiency promotes dissociation of the complex to attenuate the activity of Serine acetyltransferase, resulting in a reduced O-acetyl serine (OAS) formation. In turn, upon increased sulphur supply, accumulated sulphide promotes formation of the complex, leading to stimulated O-acetyl serine (OAS) formation to fulfill Cysteine synthesis. This system allows the coordination of O-acetyl serine (OAS) synthesis from Serine and sulphate reduction for the efficient production of Cysteine. A lag in sulphide production would result in an accumulation of O-acetyl serine (OAS), which would slow its own synthesis by disrupting the complex. Excess sulphide would stimulate formation of the complex, speeding the O-acetyl serine (OAS) production. O-acetyl serine (OAS) also acts as a positive regulator of expression for sulphate assimilation genes. Hence, the dissociable complex becomes a sensor. At high levels of O-acetyl serine (OAS) (which occur under sulphur-limiting conditions), the complex dissociates and Serine acetyltransferase (SAT) are inactivated, thus preventing further O-acetyl serine (OAS) synthesis. By contrast, O-acetyl serine thiol lyase (OAS-TL) has maximal activity in the undissociated state, and with the abundant O-acetyl serine (OAS) supply will
efficiently ‘mop up’ any sulphide, and maximize cysteine synthesis under the sulphur-limiting conditions.

![Diagram of sulphate reduction and O-acetylserine synthesis](image)

**Figure 2.** Hypothetical model for the role of O-acetyl serine (OAS) in coordination of sulphate reduction and O-acetyl serine (OAS) synthesis. Catalytic steps are indicated with arrows. Regulatory steps are indicated with dashed-line arrows. Protein complex formation and dissociation are indicated with block arrows.

The incorporation of $S^{2-}$ into Cysteine is the last step in reductive $SO_4^{2-}$ assimilation. The reaction is catalyzed by O-acetylserine (thiol)lyase from $S^2$ and O-acetyl serine (OAS). The synthesis of O-acetyl serine (OAS) is catalyzed by Serine acetyl transferase. Serine acetyl transferase and O-acetyl serine thiol lyase (OAS(thiol))lyase exist in an enzyme complex known as Cysteine synthase. The stability of the complex is affected by substrates (O-acetyl serine (OAS) disrupts it and $S^{2-}$ stabilizes it), and it appears to form through specific protein-protein interactions (Bogdanova and Hell, 1997). Cytosolic serine acetyltransferase from *Arabidopsis* is subject to feedback inhibition by cysteine, whereas the chloroplast and mitochondrial forms are insensitive to cysteine.
(Noji et al., 1998; Inoue et al., 1999). One clue to the function of the complex is that association with OAS(thiol)lyase changes the kinetic behavior of Serine acetyltransferase from the Michaelis-Menten type to positive cooperativity with respect to its substrates, Serine and acetyl-CoA (Droux et al., 1998). This suggests that OAS (thiol)lyase functions as a regulatory subunit that regulates Serine acetyl transferase in response to OAS and \( S^2^- \). Positive cooperativity is a form of allosteric regulation in which the velocity of a bisubstrate enzyme is highly sensitive to small changes in substrate concentration. One can think of the enzyme as having a hair-trigger control mechanism. The idea is appealing because Cysteine synthesis requires coordination of two converging pathways (Fig. 3.) If there is insufficient \( S^2^- \) resulting from low activity of \( SO_4^{2-} \) reduction, the concentration of O-acetyl serine (OAS) will increase, causing dissolution of the Cysteine synthase complex. By contrast, over activity of \( SO_4^{2-} \) reduction results in over abundance of \( S^2^- \) and a shortage of O-acetyl serine (OAS), a condition that would stabilize the complex. Serine acetyltransferase activity would be regulated, its velocity becoming less or more sensitive to its own substrates. Another possible form of regulation is an increase in the steady-state mRNA level for the plastid form of Serine acetyltransferase after sulphur starvation (Takahashi et al., 1997; Noji et al., 1998). Unlike the earlier steps in the pathway in which mRNA regulation occurs primarily in roots, plastid Serine acetyltransferase mRNA increases primarily in leaves. A third possible regulation mechanism is the feedback inhibition of Serine acetyltransferase by Cysteine. However, only the cytosolic
isoform appears to be regulated in this way (Noji et al., 1998). Serine acetyltransferase and OAS(thiol)lyase are the only sulphur-assimilation enzymes localized in three compartments: the plastids, cytosol, and mitochondria. The signal-transduction pathway in the regulation of the uptake and distribution of sulphate in plants and the role of shoot to root signaling therein involved are still ambiguous. The regulation may occur at the transcriptional, translational and allosteric level (Hawkesford and Wray 2000; Hawkesford and De Kok, 2006). It was observed that an up-regulated sulphate uptake was accompanied by a decreased content of thiols and sulphate, which led to the model of substrate repression of expression and activity (Hawkesford et al., 2003). The products of sulphate assimilation (sulphate, sulphide, cysteine, glutathione) under sufficient sulphur supply would act as repressors in the process of negative feedback inhibition controlling the sulphate uptake. Upon sulphate deprivation a decreased level of repressors and therefore a derepression (up-regulation) of sulphate uptake would occur.
**Figure 3.** Regulation of cysteine synthesis flux through the cysteine synthesis complex. The CSC refers to the multimeric enzymatic complex composed of Serine acetyltransferase (SAT) and O-acetyl serine thiol lyase (OAS–TL). Reversible protein-protein interaction in CSC is based on changes in cellular O-acetyl serine (OAS) and sulphide concentration as a consequence of sulphur supply of the cell. As result of this interaction, activities of the corresponding enzymes that composed the cysteine synthesis complex are regulated. Active Serine acetyltransferase (SAT) and O-acetyl serine thiol lyase (OAS-TL) are shown as white ball cubes, respectively whereas black filling indicates less efficient enzymes in catalysis.