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Sulfate Metabolism

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THE BIOLOGICAL HISTORY OF SULFUR

Sulfur is an essential nutrient for all organisms including plants. The biological role of sulfur traces back to the initial events in the origin of life, which might have arisen as catalytic reactions on iron sulfide surfaces under anaerobic, hydrothermal conditions (Wächtershauser, 2000). The aerobic atmosphere of the modern Earth ensures that sulfur exists predominantly in the +6 valence state in the form of sulfate. But sulfur in its other oxidation states (Table 1) exists in anaerobic or volcanic environments and within living cells. Plants and microorganisms reduce sulfate, changing the valence to -2 (sulfide) through the process of assimilative reduction. The process is assimilative because sulfide is used exclusively for the synthesis of cysteine, methionine, and other metabolites. This concept is best understood by contrasting it with the dissimilatory process carried out by certain anaerobic bacteria, which use sulfate as the terminal electron acceptor for respiration. The bulk of the sulfide they produce is released as a waste product. The analogous function in aerobic organisms is the respiration of oxygen (O₂) and release of carbon dioxide (CO₂). On Earth sulfur is in constant flux between oxidized and reduced states through the action of living organisms and chemical processes. The cycle is termed the bio-geochemical sulfur cycle.

Table 1. THE VALENCE STATES OF INORGANIC SULFUR

Sulfur Form	Chemical Formula	Valence
sulfate	SO ₄ ²⁻	+6
sulfite	SO ₃ ²⁻	+4
elemental sulfur	S ⁰	0
hydrogen sulfide	H ₂ S	-2

THE FUNCTIONS OF SULFUR

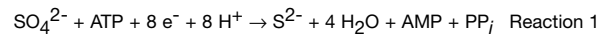
The range of biological compounds that contain sulfur is vast. Sulfur is found in the vitamins biotin and thiamine; the cofactors *S*-adenosyl-L-methionine, coenzyme A, the molybdenum cofactor (MoCo), and lipoic acid; the chloroplast lipid sulfolipid diacylglycerol; and many secondary compounds. It also serves important structural, regulatory, and catalytic functions in the context of proteins, and as a major cellular redox buffer in the form of the tripeptide glutathione and certain proteins such as thioredoxin, glutaredoxin, and protein disulfide isomerase. A feature of many sulfur-containing compounds is that the sulfur moiety is often directly involved in the catalytic or chemical reactivity of the compound. A superb example is the way in which cysteine residues in proteins sometimes form covalent disulfide bonds. Disulfides can, in turn, be reduced to the thiol form by glutathione or redox proteins like thioredoxin. For some enzymes, disulfide bond formation serves to regulate activity. Many enzymes of carbon dioxide fixation are regulated in this way as a means to coordinate their activity with the light reactions of photosynthesis. The regulatory molecule in this case is thioredoxin, which reduces target enzymes using electrons from ferredoxin.

OVERVIEW OF SULFATE ASSIMILATION IN PLANTS

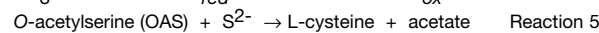
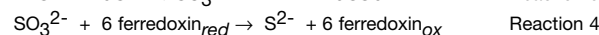
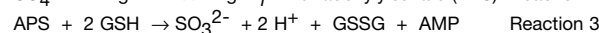
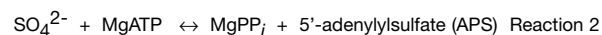
Sulfate is the primary source of sulfur for plants. Roots take it up from the rhizosphere. When available, other forms of sulfur can also be utilized. An example is sulfur dioxide, a gaseous pollutant, which enters plants via stomata. Under the alkaline condition of the cytoplasm sulfur

dioxide is chemically converted into sulfite, so it feeds into the reduction pathway after the APS reductase step (see discussion that follows).

Sulfate is reduced with 8 electrons to sulfide (reaction 1).



Metabolism of sulfate is initiated by an adenylation reaction catalyzed by ATP sulfurylase (EC 2.7.7.4) (Reaction 2). Sulfate reduction is carried out in two steps. In the first, APS reductase (EC 1.8.4.9) transfers two electrons to APS producing sulfite (Reaction 3). The source of electrons is most likely reduced glutathione. In the second step, sulfite reductase (EC 1.8.7.1) transfers 6 electrons to sulfite producing sulfide (Reaction 4). The reaction uses reduced ferredoxin. The assimilation step occurs when sulfide reacts with O-acetylserine (OAS) to form cysteine catalyzed by OAS thiol-lyase (EC 4.2.99.8) (Reactions 5). OAS is formed by the acetylation of serine with acetylCoA, catalyzed by serine acetyltransferase (EC 2.3.1.30) (Reaction 6).



Cysteine is the substrate for the various reduced sulfur compounds depicted in Table 2. It is incorporated in its entirety into proteins and glutathione. Glutathione is an enzymatically-synthesized tripeptide consisting of glutamate, cysteine and glycine. Cysteine is the thiol donor for the synthesis of methionine. Both cysteine and methionine are unstable in the presence of molecular oxygen and readily become oxidized to the disulfide compound cystine or the sulfoxide, methionine sulfoxide. Cells maintain cysteine and methionine in the reduced form using the glutathione redox buffer system and the redox proteins thioredoxin, glutaredoxin, glutathione reductase and methionine sulfoxide reductase. A major fate of methionine is its adenylation to form S-adenosylmethionine, the primary biological methyl group donor.

Whereas most organic sulfur forms are the products of the reductive assimilation pathway. A minor pathway for sulfate assimilation involves covalent addition of sulfate to a variety of compounds. The process is termed sulfation and it also begins with APS synthesis. Afterwards, APS is phosphorylated by APS kinase (EC 2.7.1.25) (Reaction 7). It has been estimated that Arabidopsis contains 4 genes for APS kinase, based on prediction of coding sequences from the genome sequence (Table 3). cDNA's corresponding to two of them have been cloned and their function demonstrated by complementation of *E. coli* and *Saccharomyces cerevisiae* APS kinase mutant strains and by analysis of the catalytic properties of the proteins

derived from the genes (Lee and Leustek, 1998; Schiffman and Schwenn, 1998; Lillig et al., 2001).



PAPS is used as a sulfonyl donor by a variety of different sulfotransferases (EC 2.8.2.-). These enzymes have highly conserved domains, which have allowed 18 putative sulfotransferase genes to be identified in the Arabidopsis genome. The subject of plant sulfotransferases is beyond the scope of this paper, but readers can learn about them from a recent review (Varin et al., 1997). Typically, sulfotransferases add sulfate to an oxygen moiety forming a sulfate ester bond. One example is choline-O-sulfate, an osmoprotective compound accumulated by species in the Plumbaginaceae family (Table 2). Another example is a class of compounds known as glucosinolates (Table 2), which function as insect feeding deterrents and are produced by Arabidopsis and other species in the Brassicales. Interestingly, glucosinolates contain two forms of sulfur in different oxidation states. The reduced form is a thioether and is derived from cysteine, whereas the oxidized form is a sulfamate and is derived from the sulfation pathway.

A fascinating class of sulfur compounds is the sulfonic acids in which a carbon atom is linked to an oxidized sulfur atom. One example is cysteic acid, which forms through oxidation of cysteine (Table 2). The chloroplast sulfolipid, sulfoquinovosyl diacylglycerol also contains a sulfonic acid bond. But it is not derived from cysteine, rather, it appears to form by direct addition of sulfite to UDP-glucose forming UDP-sulfoquinovose catalyzed in Arabidopsis by the SQD1 enzyme (Sanda et al., 2001). SQD1 is the only enzyme known that can catalyze sulfonic acid formation from inorganic sulfur.

SULFATE TRANSPORT ACROSS MEMBRANES AND LONG-DISTANCE TRANSPORT

Plants take up sulfate from the soil and distribute it throughout the plant body. The mechanism of sulfate transport across the plasma membrane into roots has been well studied. It is driven by an electrochemical gradient established by a proton ATPase. The sulfate transporter uses the gradient to transport sulfate into the cell along with protons at a ratio of 1 sulfate to 3 protons. The dynamics of transport across other membrane systems such as the tonoplast or chloroplast membrane is not well understood.

Sulfate is taken into root cells and is then distributed throughout the plant. The process is multifaceted because

TABLE 2. EXAMPLES OF ORGANIC SULFUR COMPOUNDS IN PLANTS

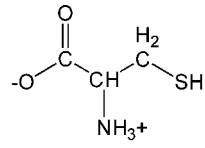
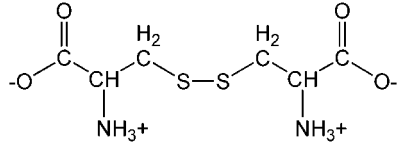
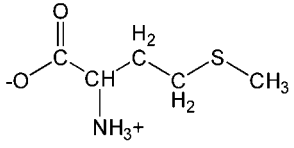
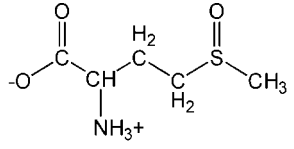
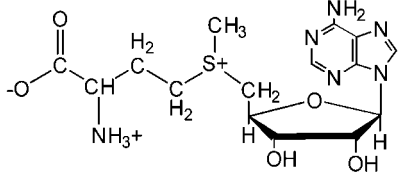
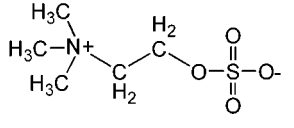
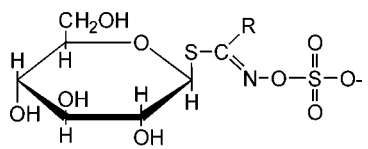
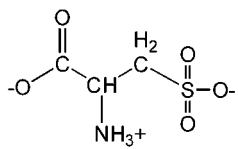
Form of Sulfur	General Structure	Example	Structure
Thiols	RSH	cysteine (others- coenzyme A, reduced glutathione, protein thiols)	
Disulfide	RSSR	cystine (others- oxidized glutathione, protein disulfides)	
Thioethers	R1SR2	methionine (others- biotin, thiamine pyrophosphate)	
Sulfoxides	R1SOR2	methionine sulfoxide (others- allicin the onion flavor compound)	
Methylsulfonium	(H3C)S+(R)2	S-adenosylmethionine (others- dimethylsulfoniopropionate, S-methylmethionine)	
Sulfate Esters	R-O-S(=O)2-O-	choline-O-sulfate (others- sulfated brassinosteroids)	
Sulfamates	R=N-O-S(=O)2-O-	glucosinolate- flavor compound of Brassica	
Sulfonic Acids	R-C(=O)-CH2-SO3H	cysteic acid (others- sulfoquinovosyl diacylglycerol)	

TABLE 3. INVENTORY OF ARABIDOPSIS GENES ENCODING SULFATE ASSIMILATION PROTEINS

Protein	Chromosome	Genomic Locus and Associated pid#	Corresponding cDNA and Associated pid#	
SULT	CHR1	At1g22150/F2E2.22 NP 173634	Sultr1;3 BAB16410	
		At1g77990/F28K19.21 NP 177922	AST56/Sultr2;2 BAA12811	
		At1g78000/F28K19.22 NP 177923	Sultr1;2 BAA95484	
		At1g23090/T26J12.13 NP 173722	AST91/ Sultr3;3 BAA75015	
		At1g80310/F5I6.6 NP 178147		
	CHR2	At2g25680/F3N11.13 NP 180139		
	CHR3	At3g15990/MSL1.3 NP 188220	Sultr3;4 BAB21264	
		At3g51895/AEM1.15 NP 190758 also At3g51900/F4F15.10 CAB41310 At3g12520/T2E2.117 NP 187858	ATST1/AST12/Sultr3;1 BAA21657	
	CHR4	At4g08620/T3F12.7 NP 192602 At4g02700/T10P11.3 NP 192179	AST101/Sultr1;1 BAA33932 AST77/Sultr3;2 BAA20282	
	CHR5	At5g13550/T6I14.80 NP 196859 At5g10180/T31P16.170 NP 196580 At5g19600/T29J13.20 NP 197461	AST82/Sultr4;1 BAA23424 AST68/Sultr2;1 BAA20085	
	APS	CHR1	At1g19920/F6F9.2 NP 173421	APS2 S44943
		CHR3	At3g22890/F5N5.6 NP 188929	APS1 AAF19185
		CHR4	At4g14680/dI3380c NP 193204	APS3 S68201
CHR5		At5g43780/MQD19.13 NP 199191	APS4 AAD26634	
APR	CHR1	At1g62180/F19K23.11 NP 176409	APR2/PRH43 AAC26980	
	CHR4	At4g04610/F4H6.13 NP 192370	APR1/PRH19 AAC26979	
	CHR4	At4g21990/F1N20.90 NP 193930	APR3/PRH26 AAC26981	
APK	CHR2	At2g14750/F26C24.11 NP 179082	APK1/AKN1 Q43295	
	CHR3	At3g03900/F20H23.5 NP 187040		
	CHR4	At4g39940/T5J17.110 NP 195704	cDNA AKN2 AAC39520	
	CHR5	At5g67520/K9I9.8 NP 201553		
SIR	CHR5	At5g04590/T32M21.190 NP 196079	Fd:SiR CAA89154	
OASTL	CHR2	At2g43750/F18O19.37 NP 181903	OASTL-B P47999	
	CHR3	At3g59760/F24G16.30 NP 191535	OASTL-C Q43725	
	CHR3	At3g22460/F16J14.2 NP 188885	OASTL-A2 AJ272028	
	CHR4	At4g14880/dI3480c NP 193224	OASTL-A1 P47998	
SAT	CHR1	At1g55920/F14J16.18 NP 175988	SAT-p/SAT1 S71181	
	CHR2	At2g17640/T17A5.1 T19E12.2 NP 179355	SAT2 AAD45614	
	CHR3	At3g13110/MJG19.1 MJG19.6 NP 187918	SAT-m/SAT 1-a S69192	
	CHR4	At4g35640/F8D20.150 NP 195289		
	CHR5	At5g56760/MIK19.23 NP 200487	SAT-c/SAT52 S71207	
βCS	CHR3	At3g61440/F2A19.40 NP 191703	BAA78560	

Protein abbreviations are: SULT=sulfate transporter, APS=ATP sulfurylase, APR=APS reductase, APK=APS kinase, SIR=sulfite reductase, OASTL=OAS thiol-lyase, βCS=β-cyanoalanine synthase SAT=serine acetyltransferase.

it involves transport across many different membrane systems and involves many different transporters (Hawkesford, 2000). A family of 14 different genes has been identified in Arabidopsis that may encode sulfate transporters (Table 3, Fig 1). Of these, the expression of 10 has been confirmed (Takahashi et al., 1996; 1997; 1999; 2000; Yamaguchi et al., 1997) as evidenced by the cloning of the encoding cDNA's. The functions of 3 have been demonstrated by their ability to complement a sulfate transporter mutant of yeast and by analysis of the kinetics of sulfate transport in yeast expressing the plant transporter (Takahashi et al., 2000; Grossman and Takahashi, 2001). Plant sulfate transporter genes can be recognized by the occurrence of specific motifs in the translated sequence, including 12 membrane-spanning domains (MSD), a conserved Arg residue between MSD 9 and 10, and a potential glycosylation site between between MSD 5 and 6 (Smith et al., 2000). The precursor forms of the transporters range from 456 amino acids to 685 amino acids. The primary structure of plant sulfate transporters indicates that they belong to a superfamily of cation/proton cotransporters found in eukaryotic organisms.

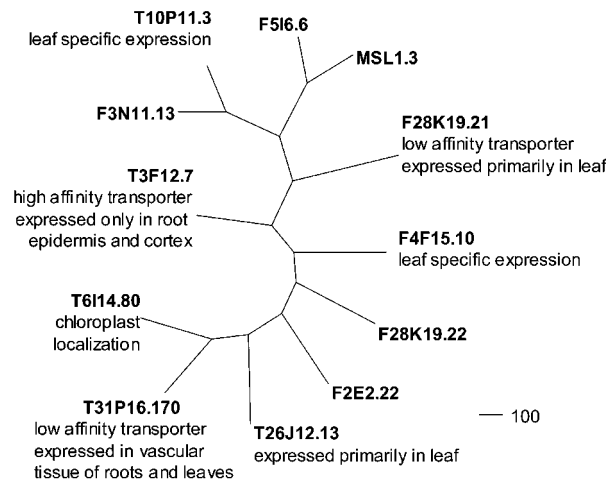


Fig 1. Dendrogram showing the amino acid sequence relationship between different Arabidopsis sulfate transporters. The sequences are referenced in Table 3, are indicated by their genomic locus designation. The dendrogram was prepared using the GCG, Inc. program PAUPSearch. The properties of transporters that have been studied are indicated. This information references the work of Takahashi et al., (1999a; 1999b; 2000).

Plants are able to take up sulfate from soil over a wide range of concentrations through the use of high affinity and low affinity transporters localized in root epidermal and cortical cells. From molecular studies it is now clear that low and high affinity sulfate transporters are the products of different genes in Arabidopsis (Takahashi et al., 2000) and other flowering plants (Smith et al., 1995; 1997; Vidmar et al., 1999). Transport throughout the plant body involves export of sulfate from root cells during the loading of xylem and then import into the cells within sink organs. Molecular studies have revealed that specific sulfate transporters, the products of distinct genes, are expressed in different tissues and organs of Arabidopsis (Takahashi et al., 1999; 2000)(Fig 1). Within cells, sulfate is transported to the chloroplast where most reduction takes place, and also to the vacuole where it serves as a major anionic constituent. The various sulfate transporters of Arabidopsis have been classified into 4 groups (*Sultr* 1 to 4) based on similarities in coding sequence, tissue and cellular localization and kinetic properties (Grossman and Takahashi, 2001). Group 1 consists of high affinity transporters expressed primarily in roots of plants after sulfate starvation, suggesting they function under sulfur-limiting conditions. Direct evidence for this hypothesis comes from analysis of mutant alleles of *Sult1;2* identified by their sensitivity to growth inhibition to selenate, a toxic sulfate analog. Sulfate uptake into roots is impaired in *Sult1;2* mutants, but not transport of sulfate to the shoot (Shibagaki et al., 2002). Similarly, antisense inhibition of *Sultr1;1* expression reduces the ability to accumulate sulfate in roots (Yoshimoto et al., 2002). Group 2 transporters have a lower affinity for sulfate and are expressed primarily in vascular tissues. They may be involved in transporting sulfate throughout the plant. Group 3 transporters are expressed primarily in leaves, but their function has not yet been characterized. Finally, group 4 consists of a transporter (*Sultr4;1*) localized to chloroplasts.

Although molecular studies have contributed significantly to the understanding of sulfate transport in plants, the fact that 14 putative transporters have been identified in Arabidopsis indicates a surprising complexity. It is fascinating to note that the number of sulfate transporter genes in Arabidopsis exceeds the number of genes for nitrate (7 genes), inorganic phosphate (9 genes), or ammonium (6 genes) transporters. The reasons for this diversity are unclear. But it emphasizes the need to characterize the functions of this gene family. It is by no means certain that the *Sult*-type transporters all transport sulfate. An alternative possibility is that they could transport other oxyanions whose physicochemical properties resemble sulfate including molybdate, selenate or chromate. Another possibility is that they could function in transport of reduced forms of sulfur. The exclusive site for sulfate reduction is plastids, yet cysteine synthesis enzymes are localized in

plastids, cytosol and mitochondria, suggesting that sulfide or some derivative thereof, such as thiosulfate, may be transported out of plastids.

A further complication is that it is not certain the transporters that have been identified are the only proteins that move sulfate across membranes. For example, experimental evidence indicates that sulfate transport into chloroplasts may be mediated by a triose phosphate transporter (Mourioux and Douce, 1979). In yeast an oxaloacetate/sulfate (OAC type) transporter moves sulfate into mitochondria (Palmieri et al., 1999). A number of homologs of the yeast OAC transporter are evident in the Arabidopsis genome. Sulfate transport into chloroplasts of the non-vascular plants *Marchantia polymorpha* and *Chlorella vulgaris* is suspected to be mediated by a bacterial-like ATP-dependent transport system (ABC-type transporter) (Ohyama et al. 1986; Wakasugi et al., 1997) and the genome of Arabidopsis contains many genes of the ABC-transporter type.

ACTIVATION OF SULFATE

ATP sulfurylase catalyzes sulfate activation. The enzyme hydrolyzes the bond between the alpha and beta phosphates of ATP and then adds sulfate to the alpha phosphate (Reaction 2, Fig 2). The activation step is necessary because sulfate is metabolically inert. The energy stored in the phosphoric acid-sulfuric acid anhydride bond of the reaction product, 5'-adenylsulfate (APS) allows sulfate to undergo further reactions.

The energetics of the sulfate adenylation reaction favors ATP formation. The equilibrium constant (K_{eq}) is approximately 10^{-7} M. Therefore, the reaction products, APS and PP_i , must be maintained at a low concentration by the enzymes, inorganic pyrophosphatase that hydrolyzes PP_i , and APS reductase and APS kinase that metabolize APS. APS reductase catalyzes the first step of sulfate reduction (Reaction 3). APS kinase catalyzes ATP-dependent phosphorylation on the 3'-position of APS (Reaction 7). The product, 3'-phosphoadenylsulfate (PAPS) is the substrate for sulfotransferases that catalyze sulfations.

Four different genes encode ATP sulfurylase isoenzymes in Arabidopsis (Table 3). All are expressed, as evidenced by the cloning of the encoding cDNA's. The function of the gene products have been demonstrated by functional complementation of yeast and *Escherichia coli* ATP sulfurylase mutants, and by the catalytic properties of the enzymes derived from the genes (Murillo et al., 1995, Hatzfeld et al., 2000 and unpublished results). Arabidopsis ATP sulfurylase polypeptides are approximately 52 kDa,

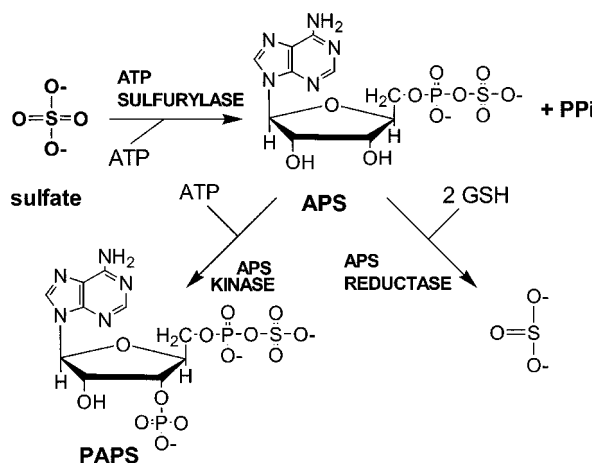


Fig 2. Reactions catalyzed by ATP sulfurylase, APS reductase and APS kinase.

and at least one of them, the product of the APS3 gene, is a homotetramer (Murillo and Leustek, 1995). The primary structure of plant ATP sulfurylases resembles that of the enzyme in other eukaryotes, but they are completely unlike the enzyme from prokaryotes.

The subcellular localization of ATP sulfurylase has been investigated in a number of plant species including Arabidopsis. The general agreement is that there are two forms localized in plastids and the cytosol (Rotte and Leustek, 2000). Plastid-localized ATP sulfurylase makes up 70% to 95% of the total enzyme in leaves. It has been speculated that plastid-localized ATP sulfurylase is involved in assimilative sulfate reduction since the subsequent enzymes, APS reductase and sulfite reductase are localized only in plastids. The absence of sulfate reduction enzymes in the cytosol suggests that cytosolic ATP sulfurylase may be responsible for another function, perhaps in providing activated sulfate for the sulfation reactions in the cytosol. The genetic origin of cytosolic ATP sulfurylase of Arabidopsis is not known. All the Arabidopsis genes encode enzymes with a characteristic plastid transit peptide (Leustek et al., 1994; Murillo and Leustek, 1995; Hatzfeld et al., 2000). By contrast, a candidate for the gene encoding cytosolic ATP sulfurylase was cloned from potato. Its coding sequence does not include a transit peptide (Klonus et al., 1994). In Arabidopsis, the chloroplast and cytosolic isoforms are very closely related antigenically, thus it is likely that one of the 4 ATP sulfurylase genes must encode the cytosolic enzyme. Hatzfeld et al. (2000) proposed that it might be encoded by APS2. The coding

sequence of this gene contains several possible translational initiation codons. The one that lies within a nucleotide context that most closely resembles the Arabidopsis translational initiation sequence would produce a protein lacking the transit peptide.

ATP sulfurylase is expressed in leaves and roots of flowering plants. The activity in roots of Arabidopsis is about 30% of the activity in leaves (Lee, 1999), indicating that both organs play a significant role in sulfur assimilation, an idea put forward by investigators working on other sulfate assimilation enzymes (Barroso et al., 1998).

THE REDUCTIVE SULFATE ASSIMILATION PATHWAY

Sulfate Reduction

Sulfate reduction is carried out by the enzymes APS reductase and sulfite reductase (Fig 2). Both are localized exclusively in plastids of leaves and roots. In the literature before 1996 APS reductase is referred to as APS sulfo-transferase (Suter et al., 2000).

Arabidopsis contains 3 genes encoding APS reductase (Table 3). cDNA's have been cloned for each of the genes and analysis of the catalytic properties of the individual gene products has been studied using protein expressed in *E. coli* (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Bick et al., 1998; and Bick et al., 2001). Arabidopsis APS reductases are polypeptides of approximately 50 kDa. They are composed of an amino terminal domain with homology to APS and PAPS reductases of bacteria (Bick et al., 2000), and a carboxyl terminal domain with homology to thioredoxin (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Despite its homology with thioredoxin, the carboxyl terminal domain functions as glutaredoxin (Bick et al., 1998). Both thioredoxin and glutaredoxin are related redox proteins that obtain electrons from different sources, thioredoxin from NADPH or NADH and glutaredoxin from reduced glutathione. APS reductase efficiently uses reduced glutathione as an electron donor and the carboxyl terminal glutaredoxin domain probably mediates this function. It is not certain that glutathione is the only source of electrons that APS reductase uses in vivo. Transgenic Arabidopsis lines with less than 5% of the glutathione content of wild-type are able to grow normally (Xiang et al., 2001), suggesting that the plants synthesize enough cysteine to support growth.

The mechanism for APS reduction may involve the formation of an S-sulfo intermediate form of the enzyme. A cysteine residue in the reductase domain of the *APR2*

enzyme forms a covalent linkage with the sulfur atom derived from APS (Weber et al., 2000). The covalently bound sulfur is released as free sulfite when incubated with thiol compounds. With these results it is possible to propose a catalytic cycle. The sulfur moiety of APS reacts with the cysteine residue of APS reductase, forming a covalent thiosulfate bond (Fig 3). Then, the thiosulfate bond is broken by reduced glutathione. An exchange reaction occurs in which free sulfite is released and glutathione forms a mixed disulfide with the enzyme. In order to regenerate the active enzyme, another reduced glutathione breaks the mixed disulfide bond, reducing the cysteine residue of the enzyme and releasing oxidized glutathione. The glutaredoxin domain could function in reducing the thiosulfate bond, or in regenerating the reduced form of the enzyme.

Recent evidence indicates that at least one isoform of Arabidopsis APS reductase, the one derived from *APR2*, and APS reductase from *Lemna minor* are metalloenzymes containing an iron/sulfur cluster (Kopriva et al., 2001). When expressed in *E. coli* these enzymes are purified in association with approximately 4 equivalents of iron and 4 equivalents of sulfide. Even though the enzymes show only a weak EPR signal, Mossbauer spectroscopy results were consistent with the presence of a diamagnetic [4Fe-4S](2+) cluster. The presence of the [4Fe-4S] in *APR2* has been confirmed by EXAFS (Thomas Leustek and Graham George, personal observation). Interestingly, Arabidopsis *APR1* and *APR3*, expressed as recombinant proteins in *E.*

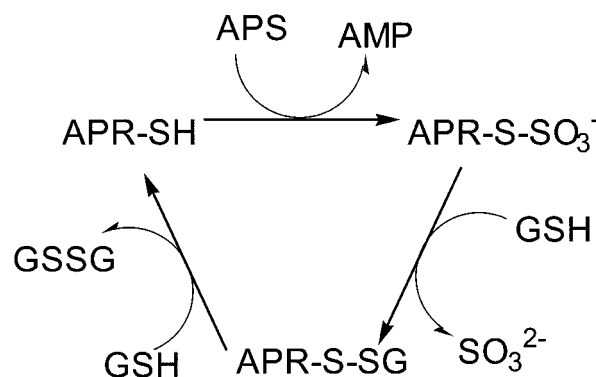


Fig 3. Proposed reaction mechanism for the catalytic cycle of APS reductase. The model is based on the work of Weber et al., (2000).

coli do not have associated iron or sulfide yet are highly active catalytically. Thus, the function of the FeS center is presently not clear.

Sulfite reductase is a homo-oligomeric hemoprotein composed of 2 or 4 identical subunits. Each subunit contains 1 siroheme and 1 iron-sulfur cluster [4Fe-4S]. Although plant sulfite 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 sulfite reductase (Table 3). The amino acid sequence of the Arabidopsis enzyme shows that it is distantly related to nitrite reductase. Both enzymes utilize siroheme as a cofactor and the enzymes show significant homology at the carboxyl terminus where the cofactor binds. Only recently has it become possible to produce an active plant sulfite 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 sulfite reductase indicates that reductant supply is a significant factor determining the rate of sulfite 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 sulfite reduction.

Sulfate Assimilation

The synthesis of cysteine represents the assimilation step of the reductive sulfate assimilation pathway. Serine acetyltransferase and *O*-acetylserine thiol-lyase catalyze the sequential reactions leading to cysteine production (Reactions 5, 6 and Fig 4).

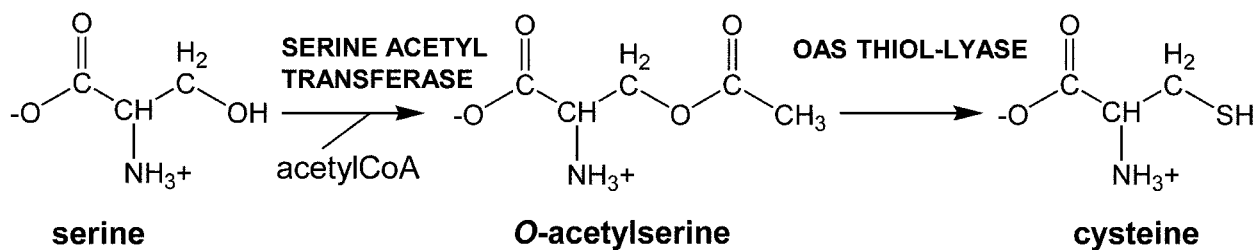


Fig 4. Reactions catalyzed by serine acetyltransferase and OAS thiol-lyase.

OAS thiol-lyase belongs to a large class of enzymes using pyridoxal phosphate as a cofactor and catalyzing β -replacement reactions. Arabidopsis contains 4 genes for OAS thiol-lyase (Table 3; Jost et al., 2000). There is still a question about whether *OASTL-A2* is a pseudogene (Jost et al., 2000) or is only weakly expressed. Even so, the others produce proteins localized to the cytosol (*OASTL-A1*), plastids (*OASTL-B*), and mitochondria (*OASTL-C*) (Jost et al., 2000). The same subcellular distribution of isoenzymes was demonstrated using subcellular fractionation studies for several different flowering plants (Lunn et al., 1990). The function for each of the isoenzymes has been established by the ability of the corresponding cDNA's to functionally complement an *OASTL* mutant of *E. coli*, and through analysis of the recombinant enzyme derived from the cDNA.

Whether all the isoenzymes function in cysteine synthesis is an open question. What was originally characterized as mitochondrial OAS thiol-lyase from spinach was recently demonstrated to function as β -cyanoalanine synthase (EC 4.4.1.9) (Reaction 8), an enzyme thought to play a role in cyanide detoxification (Warrilow and Hawkesford, 2000).

$\text{L-cysteine} + \text{HCN} \rightarrow \text{sulfide} + \text{L-3-cyanoalanine}$ Reaction 8
But, in Arabidopsis mitochondria, *OASTL-C* and β -cyanoalanine synthase are distinct enzymes. OAS thiol-lyase is encoded by the *OASTL-C* gene (Table 3) (Hesse et al., 1999), whereas β -cyanoalanine synthase is encoded by a different gene, β CS (Table 3) (Hatzfeld et al., 2000). Although the amino acid sequences of these proteins are closely related they fall into distinct subclasses. The different OAS thiol-lyases from different plant species also can be grouped into separate classes based on amino acid sequence homology. The organellar forms *OASTL-C* and *OASTL-B* from different species are more closely related than they are to the cytosolic form from the same species (Jost et al., 2000).

Serine acetyltransferase belongs to a superfamily of acetyltransferases (Murray and Shaw, 1997). Arabidopsis

contains 4 genes for serine acetyltransferase (Table 3). Each gene produces a protein localized to a specific sub-cellular compartment, the cytosol (*SAT-c*), plastid (*SAT-p*) or mitochondria (*SAT-m*). The in vivo distribution of serine acetyltransferase isoenzymes in these compartments has been studied in several different flowering plants (Ruffet et al., 1994; 1995). The catalytic function of the Arabidopsis gene products has been established by complementation of a serine acetyltransferase deficient mutant of *E. coli* and through analysis of the enzymes derived from the cloned cDNA's.

OAS thiol-lyase and serine acetyltransferase form an enzyme complex through specific protein-protein interactions that is thought to play a role in enzyme regulation (Bogdanova et al., 1997; Droux et al., 1998; Wirtz et al., 2001). In addition to its role in catalysis of cysteine formation, OAS thiol-lyase also functions as a regulatory subunit of serine acetyltransferase. When OAS thiol-lyase binds, the behavior of serine acetyltransferase changes from Michaelis-Menten-type kinetics to allosteric-type kinetics. In the free form the binding of one substrate to the enzyme does not affect the dissociation constant of the second substrate. However, when bound by OAS thiol-lyase, serine acetyltransferase shows cooperative substrate binding, namely, when one substrate binds it enhances the ability of the enzyme to bind the second substrate. The potential advantage for an enzyme showing positive cooperativity is that its catalytic velocity is much more sensitive to changes in substrate concentration than is an enzyme showing Michaelis-Menten-type kinetics. In the case of serine acetyltransferase, the change in kinetic behavior probably has a significant effect on the rate with which OAS is synthesized.

In vivo, OAS thiol-lyase is present at a 300 fold molar excess over serine acetyltransferase. The disparate equilibrium is not the sole force determining how much serine acetyltransferase is bound in the complex. OAS triggers the dissociation of the complex, whereas sulfide counteracts the action of OAS (Bogdanova et al., 1997; Droux et al., 1998). It has been proposed that the control by OAS and sulfide acts to coordinate pathways for production of sulfide and OAS (Fig 5). A lag in sulfide production would result in an accumulation of OAS, which would slow its own synthesis by disrupting the complex. Excess sulfide would stimulate formation of the complex, speeding OAS production. OAS also acts as a positive regulator of expression for sulfate assimilation genes (see the discussion that follows on Regulation of Sulfate Assimilation). An alternate hypothesis for the function of the OAS thiol-lyase/serine acetyltransferase complex is that it is involved in substrate channeling or that the complex is responsible for cysteine synthesis. The disparate equilibrium between the enzymes argues against these ideas as does the fact that the catalytic efficiency of OAS thiol-lyase is signifi-

cantly reduced when it is associated with serine acetyltransferase (Droux et al., 1998).

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). It was proposed that cytosolic serine acetyltransferase might be responsible for production of the OAS that serves in regulation of sulfate assimilation gene expression.

REGULATION OF SULFATE ASSIMILATION

A number of factors determine the rate with which cysteine is synthesized. For example, cysteine synthesis is timed to correspond with the rate of protein synthesis, which differs markedly in different plant tissues and developmental stages. Environmental conditions also have a significant impact. Oxidative stress, toxins and heavy metals induce a

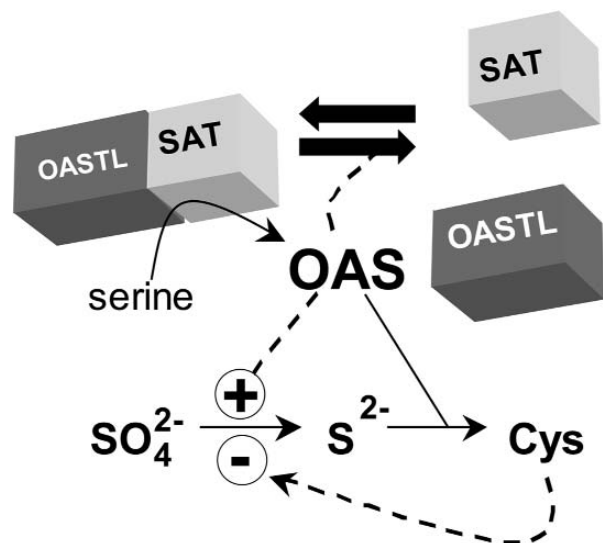


Fig 5. Hypothetical model for the role of OAS in coordination of sulfate reduction and OAS synthesis. Catalytic steps are indicated with arrows. Regulatory steps are indicated with dashed-line arrows. Protein complex formation and dissociation are indicated with block arrows.

greater demand for glutathione synthesis, which in turn increases the demand for cysteine. A constraint in the supply of sulfate or nitrogen limits the ability to produce cysteine, which plants must overcome if they are to continue growth. The reductive assimilation of sulfate and nitrogen is linked to the supply of photosynthetic reductant and photosynthetic, which fluctuates diurnally. Plants coordinate cysteine synthesis with growth and development, and respond to variations in the need for cysteine through complex regulatory mechanisms that we are only now beginning to understand.

Development

Sulfur assimilation is regulated during development. In general, the highest activity of sulfur assimilation enzymes exists at the growing points, the youngest leaves and the root tips. This also is true for Arabidopsis (Rotte and Leustek, 2000). The activity and level of chloroplast ATP sulfurylase and APS reductase was found to decline approximately 3-fold as the leaves aged. Such a developmental expression pattern suggests that sulfate assimilation is highly active in growing tissues where there is a high demand for cysteine and methionine for protein synthesis. Cytosolic ATP sulfurylase shows a pattern of expression that is different from the plastid form. Its activity and abundance increase from approximately 5 fold during the ageing of Arabidopsis leaves, suggesting that it plays a specialized function that is unrelated to sulfate reduction (Rotte and Leustek, 2000). A plausible function could be in generating APS for sulfation reactions.

High rates of sulfate assimilation may be targeted to specific cells with specialized functions. It has recently been observed that the trichomes of Arabidopsis accumulate glutathione at concentrations far above that in other epidermal cells and express to high levels serine acetyltransferase, OAS thiol-lyase and two enzyme for glutathione synthesis, γ -glutamylcysteine synthetase and glutathione synthetase (Gutiérrez-Alcalá et al., 2000; Gotor et al., 1997; Barroso et al., 1999). Trichomes are known to accumulate heavy metals and other toxins, thus it was proposed that the accumulation of glutathione might have a function in detoxification.

The question of how cysteine is supplied to the developing seeds of plants is still an open question. It could be transported to seeds from leaves. Cysteine, glutathione and S-methylmethionine are possible forms by which reduced sulfur is transported in plants (Burgener et al., 1998; Lappartient et al., 1996; 1999; Bourgis et al., 1999). In some plants, sulfur assimilation enzymes are highly

expressed in developing seeds. For example, Tabe and Droux (2001) found that in soybean the activity of ATP sulfurylase increased in seeds, whereas, at the same time, it decreased in leaves suggesting that sulfur may be assimilated directly in seeds, rather than being imported from other tissues.

Reductant Supply

The reduction of sulfate to sulfide consumes 732 kJ per mole. Sulfate assimilation requires 1 ATP, used by ATP sulfurylase, and 8 electrons for the reduction reactions. Two electrons are derived from reduced glutathione for the APS reductase step and 6 are derived from ferredoxin for the sulfite reductase step. Photosynthesis is the source of ATP and electrons in chloroplasts, whereas glycolysis and the pentose phosphate pathway are the sources in root plastids. Moreover, the synthesis of cysteine depends on the availability of serine, which is a product of nitrogen assimilation. The reduction of nitrogen is energetically similar to that of sulfate. Therefore, mechanisms exist for coordination of sulfur and nitrogen pathways with the photosynthetic light reactions. One such mechanism has been identified through analysis of circadian-regulated genes in Arabidopsis using DNA microarray analysis of mRNA expression (Harmer et al., 2000). The sulfur assimilation genes showing the most pronounced circadian changes in mRNA level, peaking just before the onset of the light period, include: *Sultr3;1* and *Sultr2;2*, two sulfate transporters expressed primarily in leaf; *APR2* APS reductase; *SAT-p*, the plastid localized form of serine acetyltransferase; and a gene for phosphoglycerate oxidase encoding the first enzyme of serine synthesis. The results of this analysis, depicted in Fig 6, show what are likely to be the major points for circadian control of cysteine synthesis. The level of *APR2* mRNA has previously been reported to be under diurnal control (Kopriva et al., 1999). In addition, the changes in mRNA were demonstrated to coincide with changes in APS reductase activity and protein level. The circadian phasing of sulfate transporters and enzymes for sulfate assimilation before the onset of the light period are thought to ensure that sulfate and the machinery for its conversion into cysteine is present in chloroplasts at a time just prior to the time when reducing power is generated by photosynthesis.

It is potentially significant that not all sulfate assimilation genes are regulated by circadian rhythm, at least not at the point of mRNA level. Other forms of regulation are possible. Some of the results can be understood by considering that sulfate reduction draws from two distinct pools of

electrons. In leaves the activity of sulfite reductase is directly linked to photosynthesis because its electron source is ferredoxin, which is reduced by the light reactions of photosynthesis. The level of sulfite reductase mRNA does not show a circadian phasing, nor have conditions been identified that regulate sulfite reductase expression. In contrast, the supply of reductant to APS reductase is not tied directly to photosynthesis. The concentration of reduced glutathione is not diurnally controlled, rather it is maintained at a constant level in plastids through the action of NADPH-dependent glutathione reductase. NADP⁺ is reduced either by ferredoxin:NADP⁺ oxidoreductase in the light or, in the dark, by the pentose phosphate pathway. Thus, circadian control of APS reductase expression is necessary to coordinate sulfite production and further reduction. The levels of both sulfite and sulfide must be stringently controlled because they are thought to be highly toxic. Synchronization of sulfate reduction with the production of OAS ensures that sulfide is efficiently assimilated.

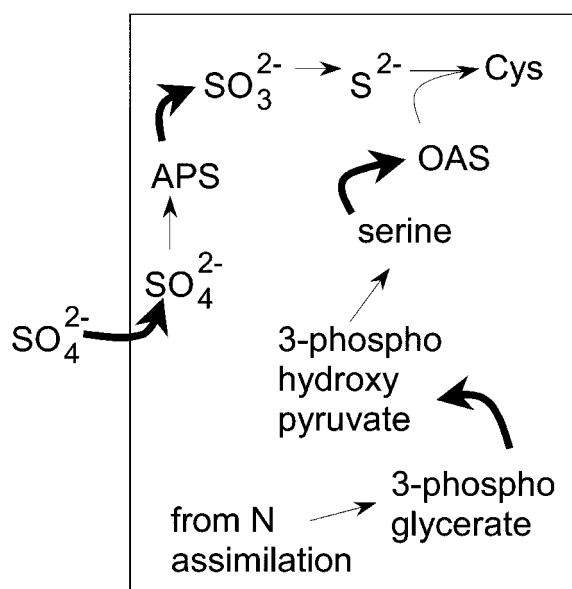


Fig 6. The circadian control of sulfate assimilation. The model depicts data obtained from global analysis of the diurnal regulation of mRNA level in *Arabidopsis* (Harmer et al., 2000) and analysis of diurnal control of APS reductase in *Arabidopsis* (Kopriva et al., 1999).

Expression of mRNA for all three *Arabidopsis* genes for APS reductase (*APR1*, *APR2*, and *APR3*) was observed to increase in roots after treating the plants with sucrose (Kopriva et al., 1999). This result suggests that photosynthate may also control expression of APS reductase.

Sulfur/Nitrogen Deficiency and Excess Reduced Sulfur

Sulfur and nitrogen deficiencies challenge plants to alter the metabolisms necessary for growth. Cysteine contains both sulfur and nitrogen. Limitations in either nutrient not only limit the ability to synthesize cysteine, but will also limit protein synthesis, and the rate with which all amino acids are incorporated into proteins. Plants that are grown at sub-optimal levels of sulfate develop symptoms of sulfur deficiency, which include chlorosis of young leaves and growth stunting (Hawkesford, 2000). The symptoms indicate that insufficient amounts of cysteine are being produced at the growing point of the plant where proteins are synthesized at high rates. The symptoms of nitrogen deficiency, chlorosis that appears first in older leaves, suggests that plants are able to mobilize nitrogen from existing protein stores more efficiently than they are able to remobilize assimilated sulfur.

Plants respond to sulfur deficiency by increasing expression of sulfate transporters, especially the high affinity form, APS reductase and chloroplast localized serine acetyltransferase. The short-term effect on expression of the mRNA for these proteins is observed primarily in roots (Takahashi et al., 1997; Smith et al., 1997). The level of mRNA and protein for other enzymes of reductive sulfate assimilation, such as ATP sulfurylase and OAS thiolylase, increase only after long-term sulfur starvation (Barroso et al., 1997; Lappartient et al., 1996; 1999; Lee, 1999). Chronic sulfate starvation is also necessary to induce expression of sulfate assimilation proteins in leaves (Barroso et al., 1997; Lee, 1999). The delayed response in leaves, compared with roots, could be due to the large store of sulfate in the vacuoles of leaf cells, which may buffer them against short-term reductions in external sulfate concentration.

The response to sulfur starvation is attenuated if plants are at the same time also limited for nitrogen. Nitrogen limitation blocks the accumulation of transcripts for ATP sulfurylase and APS reductase normally induced by sulfur limitation of *Arabidopsis* (Yamaguchi et al., 1999; Lee 2000; Koprivova et al., 2000). This result suggests that some nitrogen-containing compound is necessary for de-repression of sulfur assimilation genes. One exception is that the

mRNA level for the mitochondrial form of OAS thiol-lyase was observed to increase in tobacco (Takahashi and Saito, 1996).

Exposure of plants to reduced sulfur compounds has a repressive effect on expression of sulfur assimilation genes, including sulfate transporters and ATP sulfurylase (Blake-Kalff et al., 1998; Lappartient et al., 1996; 1999).

Starvation of Arabidopsis for sulfate results in a decline in the tissue concentration of pathway end-products, cysteine and glutathione (Smith et al., 1997; Blake-Kalff et al., 1998; Lappartient, 1996; 1999), and an increase in the level of OAS (Kim et al., 1999). However the increase is blocked if Arabidopsis is simultaneously limited for nitrogen (Kim et al., 1999). OAS and the end-products of sulfate assimilation have opposite effects on regulation of the pathway. Application of OAS to Arabidopsis and other plant species causes the steady state mRNA level to increase for sulfate transporters, ATP sulfurylase, and APS reductase (Saito, 2000; Smith et al., 1997; Koprivova et al., 2000).

In total, the results from experiments on sulfur/nitrogen starvation and feeding of reduced sulfur compounds can be integrated into a model for regulation of the sulfur assimilation pathway (Fig 5). The section on Cysteine Synthesis described the negative effect of OAS on its own synthesis, mediated by the dissociation of the serine acetyltransferase/OAS thiol-lyase complex. By activating expression of sulfate reduction genes, OAS serves to increase the rate of sulfate reduction. The accumulation of cysteine and its metabolite glutathione serves as a negative control of sulfate reduction.

Heavy Metal Stress

Plants that are exposed to heavy metals produce metal ion chelators termed phytochelatins that are derived from glutathione (Zenk et al., 1996). Soon after exposure to heavy metals the levels of glutathione and cysteine decline as the synthesis of phytochelatins ensues. After heavy metal stress has been mitigated the pools of cysteine and glutathione are restored (Meuwly and Rauser, 1992). The regulation of sulfate assimilation enzymes in response to heavy metals has been explored in Arabidopsis and related species. Exposure to heavy metals induces the accumulation of ATP sulfurylase, APS reductase and OAS thiol-lyase (Heiss et al., 1999; Lee and Leustek, 1999; Barroso et al., 1999; Dominguez-Solis et al., 2001). Indeed, overexpression of certain sulfate assimilation enzymes increases tolerance to heavy metals (Pilon-Smits et al., 1999; Dominguez-Solis et al., 2001).

Oxidative Stress

Plants are negatively affected by reactive oxygen generated by oxygenic photosynthesis, carbon dioxide fixation, and environmental conditions. To combat oxidative stress plants have evolved an antioxidant system in which glutathione plays a pivotal role. Glutathione functions directly as an antioxidant, and indirectly by maintaining the level of another antioxidant, ascorbic acid (May et al., 1998). Glutathione has long been known to accumulate in plants following oxidative stress and this is true also for Arabidopsis (May and Leaver, 1998). Cysteine availability is known to limit glutathione synthesis under certain conditions (Noctor et al., 1996; 1998). Recently it was demonstrated that transgenic tobacco plants expressing a bacterial serine acetyltransferase targeted to plastids accumulate glutathione and were more resistant to hydrogen peroxide than control plants (Blaszczuk et al., 1999). Harms et al., (2000) observed that overexpression of plastid localized plant serine acetyltransferase in potato also caused the glutathione level to increase. These results indicate that serine acetyltransferase has a role in controlling glutathione synthesis. Although there are no reports on the effect of oxidative stress on serine acetyltransferase expression, this should be investigated in light of the transgenic plants studies.

APS reductase also plays a role in the response of Arabidopsis to oxidative stress. The *APR1* gene product is post-translationally activated by oxidation, probably through the formation of a disulfide bond at the enzyme regulation site (Bick et al., 2001). It was proposed that the regulation site provides *APR1* with the ability to respond to changes in the redox state of the chloroplast stroma that acts as a signal for the synthesis of additional glutathione (Fig 7). The stromal environment is usually reducing when chloroplasts are actively photosynthesizing. The glutathione pool is reduced as is the pool of thioredoxin. Under this condition the regulation site of *APR1* APS reductase is reduced and the enzyme is inactive. However, after oxidative stress the level of oxidized glutathione increases when reduced glutathione is consumed for stress mitigation. Oxidized glutathione activates *APR1*, which drives the synthesis of additional cysteine destined for glutathione synthesis. Thus, *APR1* may function specifically under oxidative stress conditions. Bick et al., (2001) also report that *APR2* and *APR3* are insensitive or less sensitive to redox regulation. These isoforms may function for cysteine synthesis when plants are not under oxidative stress.

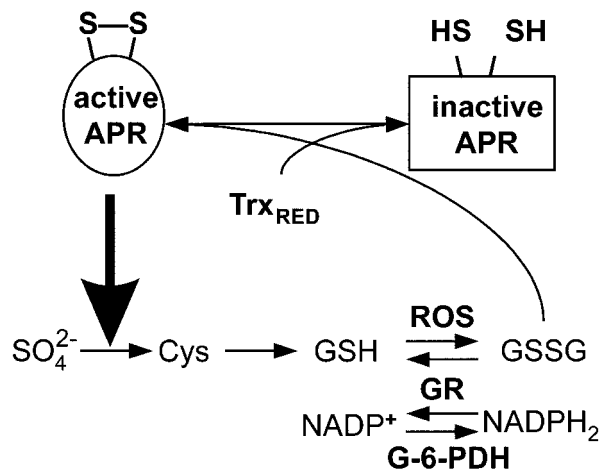


Fig 7. Hypothetical model for regulation of APS reductase and sulfate assimilation by oxidative stress. The diagram depicts the activation state of APS reductase being a function of the ability of reduced thioredoxin (Trx) to inactivate APS reductase and the ability of oxidized glutathione (GSSG) to activate the enzyme. Glutathione is normally maintained in a reduced state by glutathione reductase (GR), which depends on NADPH produced by the pentose phosphate pathway under control of glucose-6-phosphate dehydrogenase (G-6-PDH). But GSSG accumulates in plant cells when ROS are produced during oxidative stress. The activation of APS reductase stimulates the production of GSH needed to mitigate ROS. The model is derived from the work of Bick et al., (2001)

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