Chapter 15
Sulfur Metabolism as a Support System for Plant Heavy Metal Tolerance

Laura A. Hardulak, Mary L. Preuss, and Joseph M. Jez

15.1 Introduction

Sulfur is a critical nutrient for the growth and development of plants, as well as all living organisms, and it plays a central role in plant defense responses against biotic and abiotic stresses. It is a component of the amino acids cysteine and methionine, cofactors, metal clusters, and a diverse range of primary and secondary metabolites, such as glutathione, phytochelatins, and glucosinolates that protect plants from oxidative and environmental stresses (Rausch and Wachter 2005). Sulfur is required for chlorophyll production and the conversion of inorganic nitrogen into protein. As an anionic solute, plants take up sulfur from the soil through sulfate transporters in their roots, which have differential affinities for sulfate (Bick and Leustek 1998). From the atmosphere, sulfur enters leaves by passive diffusion of sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) (Saito 2000). Once internalized, sulfate must be converted into sulfide and then incorporated into various metabolites; it is either accumulated and stored in a vacuole, or incorporated into organic compounds (Bick and Leustek 1998). Sulfur can be assimilated in either the oxidized or the reduced form (Rotte and Leustek 2000). In the oxidized form, the nonreductive pathway directly incorporates sulfate into metabolites such as glucosinolates, hormones, flavonoids, and jasmonates (Varin et al. 1997; Mugford et al. 2010). Metabolically, sulfur metabolism is a core pathway for the synthesis of molecules required for heavy metal tolerance in plants.

L.A. Hardulak • M.L. Preuss (✉)
Department of Biological Sciences, Webster University, 470 East Lockwood Avenue, Webster Groves, MO 63119, USA
e-mail: marypreuss34@webster.edu

J.M. Jez (✉)
Department of Biology, Washington University, One Brookings Drive, Campus Box 1137, St. Louis, MO 63130, USA
e-mail: jjez@biology2.wustl.edu

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15.2 Overview of Sulfur Metabolism

In plants, the reductive sulfate assimilation pathway consists of two phases: the reduction of sulfate to sulfide and the assimilation of sulfide into cysteine (Fig. 15.1). These phases also occur in distinct organelles. Sulfate reduction takes place in the plastids with cysteine synthesis occurring in the plastids as well as in the mitochondria and cytosol (Wu et al. 2010). After uptake from the environment, sulfate is first activated by the enzyme ATP sulfurylase (ATPS) via conversion of ATP to adenosine-5′-phosphosulfate (APS) and inorganic pyrophosphate (Lunn et al. 1990). The high-energy compound APS is then reduced to sulfite and AMP by adenosine 5′-phosphosulfate reductase (APSR), which transfers two electrons to APS to yield sulfite (Setya et al. 1996). Next, sulfite reductase (SiR) converts sulfite to sulfide (Saitoh et al. 2006). In second phase of the assimilatory pathway, sulfide is the source of sulfur for cysteine synthesis. Serine acetyltransferase (SAT) forms O-acetylserine from serine and acetyl-coenzyme A (Saito et al. 1995). The final step of cysteine synthesis, combining O-acetylserine and sulfide, is catalyzed by O-acetylserine sulfhydrylase (OASS; also known as O-acetylserine(thiol)lyase (Bonner et al. 2005). Cysteine can then be used for the synthesis of glutathione and phytochelatin peptides in response to oxidative stresses like heavy metal exposure (Cobbett and Goldsbrough 2002).

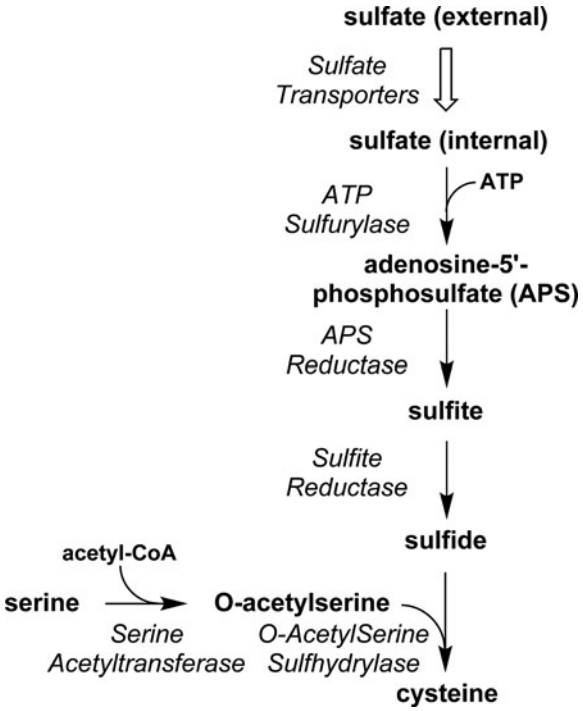

Fig. 15.1 Overview of sulfur metabolism in plants

Toxic heavy metals and metalloids are major environmental pollutants that cause abiotic stresses in plants. Lead and cadmium, for example, decrease the photosynthetic ability and biomass of soybeans (Huang et al. 1974). Flowering has been shown in *Hieracium piloselloides* to be very sensitive to heavy metal contamination of the soil with reductions in leaf life span and delayed reproduction (Ryser and Saunder 2005). Adaptation of the sulfur metabolic circuit is a crucial component of plant survival under stress (Nocito et al. 2007). Plants grown in these conditions concomitantly increase sulfate uptake and increase synthesis of low molecular weight thiols, which bind heavy metals. Cysteine is a precursor of many other cellular compounds, the most widely studied of which is the major cellular redox buffer glutathione (Meister and Anderson 1983). This tripeptide also forms the repeating subunit constituent of phytochelatins, the cysteine-rich peptides that act as heavy-metal chelators in plants and fungi (Grill et al. 1985). Phytochelatin synthesis is part of the cellular heavy metal detoxification network (Cobbett and Goldsbrough 2002). Considering the vast amount of research that has been published on the subject of glutathione and phytochelatins, in this chapter we only consider the reductive sulfur assimilatory pathway prior to the production of glutathione, as it applies to the heavy metal support system of plants.

Sulfur metabolism is profoundly affected by heavy metals. The interplay of a wide range of adaptive responses involving sulfur and sulfur-containing molecules supports plant survival under heavy metal and other oxidative stresses. Exposing sulfur-deficient maize (*Zea mays)* to cadmium stimulated expression of ATPS and OASS (Astolfi et al. 2004). Expression profiling of *Crambe abyssinica* under arsenic stress shows that genes encoding SiR and ATPS were among those highly upregulated in response to arsenic exposure (Paulose et al. 2010). OASS was also upregulated in arsenic-stressed rice (Ahsan et al. 2008). Expression of APSR, SiR, and OASS is elevated in cadmium-treated Indian mustard (*Brassica juncea*) (Minglin et al. 2005; Alvarez et al. 2009). Because glutathione is crucial as an antioxidant and as a precursor to phytochelatins, its cellular concentrations can be depleted under stress. If either sulfur supply or assimilation becomes limiting, depletion of cysteine will limit the biosynthesis and replacement of glutathione. However, if sulfur is not limiting, the challenge is to increase the flux through the sulfur metabolic circuit during heavy metal stresses.

15.3 Sulfur Transporters

The first step in the assimilation of sulfur into a biologically useful molecule for a plant is uptake via sulfate transporters. The processes of uptake and assimilation are energy dependent, driven by a proton gradient across the plasma membranes of root epidermis and root hair cells containing these transporters (Takahashi 2010). Hydrolysis of ATP maintains the proton gradient, as proton-ATPase moves protons from the cytosol into the extracellular space (Lass and Ullrich-Eberius 1984; Hawkesford et al. 1993). Movement of sulfate from the cytoplasm into the apoplast

is favored because of the lower apoplastic pH. Reduction from sulfate to sulfide occurs via a series of enzymatic reactions in plastids.

Plant sulfate transporters were first identified in the legume *Stylosanthes hamata*. Their basic structure is similar to predictions based on their orthologs in fungi (Ketter et al. 1991; Smith et al. 1995; Cherest et al. 1997). Multiple sulfate transporters have been identified, mostly in the epidermis and cortex of root systems (Takahashi 2010). The plant sulfate transporter family consists of five groups, based on their amino acid sequences, as observed in *Arabidopsis thaliana* (Nocito et al. 2007). They differ in their affinities for sulfate, rates of transport, and localization in the tissue types.

The locations and capacities of sulfate transporters are greatly influenced by the availability of sulfur in the environment (Takahashi et al. 1997). Low sulfur conditions are associated with abundant expression of high-affinity isoforms. Takahashi et al. (1997) reported the first comprehensive study on changes in gene expression in response to sulfur starvation in Arabidopsis. Transcription of *SULTR1;1* and *SULTR1;2*, the main high-affinity transporters in the roots, accumulated in response to sulfur starvation, but *SULTR1;2* is also abundantly transcribed in conditions of high sulfur. They are predominantly located in root hairs, epidermis, and cortex of roots. Coordinate transcriptional changes for several enzymes, including those involved in cysteine synthesis, occurred as well. Mutant plants defective in *SULTR1;2* are tolerant of selenate, a toxic analog of sulfate (Smith et al. 1995; Cherest et al. 1997). Growth of double mutants defective in both *SULTR1;1* and *SULTR1;2* was stunted under low-sulfur conditions, but adequate sulfur supply permitted growth. This suggests that initial sulfate uptake can be partially substituted by a lower affinity transport system (Barberon et al. 2008; Yoshimoto et al. 2007). Through genetic screening of mutant Arabidopsis plants impaired in their ability to respond to conditions of sulfur starvation, a gene was identified which plays a key role in the demand-driven regulation of sulfate uptake and assimilation (Maruyama-Nakashita et al. 2006). The Sulfur Limitation 1 gene, *SLIM1,* is responsible for transcription of *SULTR1;2*, and is critical for maintaining balance of the entire biochemical circuit of sulfur metabolism in the plant, from uptake all the way through glutathione biosynthesis.

The other three phylogenetic groups of sulfate transporters are less well understood. The group 2 transporters have low affinities to sulfate and the group 3 transporters are relatively uncharacterized, although there is evidence that the SULTR3;5 isoform may form a heterodimer with SULTR2;1 which facilitates the root-to-shoot transport of sulfur in Arabidopsis (Kataoka et al. 2004). Moreover, the group 3 proteins are expressed preferentially in the leaves (Takahashi 2010). In chromium-treated *B. juncea, sulfate* uptake rates were decreased, concomitant with repression of the low-affinity transporter BjST1 (Schiavon et al. 2008), suggesting involvement of sulfate carriers in the transport of chromate. Group 4 transporters are primarily involved in the mobilization of vacuolar sulfate pools. Increased expression of SULTR4;1 and SULTR1:1 in wheat grown on low-sulfur augmented accumulation of selenium and molybdenum in the grain, suggesting efficient mobilization of these metals (Shinmachi et al. 2010). The putative

transporters that make up group 5 have short amino acid sequences about which little is known. SULTR5;2 is thought to be involved in molybdenum accumulation in Arabidopsis, but its expression patterns in sulfur-deprived wheat do not fully explain distribution patterns of molybdenum throughout plant tissue (Shinmachi et al. 2010).

Although the basic phylogenetic groups of sulfate transporters and their putative functions have been identified, their exact mechanisms of action and regulation remain elusive. How sulfur is sensed at the molecular level and the subsequent interactions of proteins, metals, and gene expression changes is an area of ongoing research.

15.4 Sulfur Assimilation

15.4.1 ATP Sulfurylase

Once sulfate has been internalized, ATPS catalyzes the entry step into the reductive assimilatory pathway. Because this enzyme catalyzes the first committed step in sulfur assimilation, it has been targeted for the generation of transgenic plants to increase tolerance to cadmium, selenium, and other heavy metals (Khan et al. 2009). It appears to have a rate-limiting role in the accumulation of and tolerance to heavy metals.

Using the heavy metal accumulator *B. juncea*, Heiss et al. (1999) showed that ATPS (and APSR) are induced following cadmium treatment. The increased expression corresponded with elevated demand for thiol metabolism under this stress condition. Although analysis of transgenic *B. juncea* overexpressing ATPS showed that these plants have higher levels of glutathione and total thiols compared to wild-type plants, the accumulation of cadmium, zinc, chromium, copper, lead, and manganese showed no change versus wild type (Bennett et al. 2003). Later studies revealed that overexpression of ATPS in *B. juncea* confers a 2.5-fold increase in selenium tolerance and accumulation to levels observed in the natural selenium hyperaccumulator *Stanleya pinnata* (Van Huysen et al. 2004). Subsequent studies on metal tolerance and accumulation suggest that plants overexpressing ATPS overexpression may be promising for phytoextraction and phytoremediation (Wangeline et al. 2004).

Because photosynthesis is the source of the carbon backbones of amino acids, it might be expected that enhanced photosynthetic potential would also increase pathway flux in order to meet increased demands for cysteine and glutathione under heavy metal stress. In a study by Khan et al. (2009), two varieties of *B. juncea* (Cv. Varuna and RH30) were exposed to sublethal cadmium levels. The activity of ATPS and levels of cysteine and glutathione were increased in both plants, but were much higher in cv. Varuna with RH30 showing symptoms of

greater oxidative stress. These results also support the idea that increasing ATPS activity can enhance sulfur metabolism and hence heavy metal tolerance.

15.4.2 APS Reductase

APS Reductase (APSR), responsible for the reduction of APS to sulfite and AMP, is among a group of genes upregulated in response to cadmium stress (Minglin et al. 2005). Transcription of APSR, as well as ATPS, increases in response to depletion of cysteine and glutathione in cadmium-stressed *B. juncea* (Heiss et al. 1999). The coordinate changes resulted in increased cysteine concentrations, especially in the roots, supporting the importance of APSR in regulation of cysteine levels. Although APSR has been shown to be upregulated in response to cadmium in *B. juncea*, no reports describing overexpression of this protein and its effect on heavy metal tolerance and accumulation have been reported to date.

ATPS and APSR catalyze thermodynamically linked steps in sulfur assimilation (Yi et al. 2010). The reaction catalyzed by ATPS is energetically unfavorable in the forward reaction and requires the presence of APSR to maintain metabolic flux through the pathway (Renosto et al. 1993). Therefore, it is possible that the limited success in using ATPS to improve heavy metal tolerance and accumulation results from insufficient APSR to match elevated ATPS levels in transgenic plants. Future efforts could focus on the effect of co-expression of ATPS and APSR to overcome the energetic bottleneck in sulfur assimilation.

15.4.3 Sulfite Reductase

SiR is the last enzyme in the pathway of reduction from sulfate to sulfide. It is constitutively expressed and is not subject to allosteric effectors or posttranslational modification (Nakayama et al. 2000). Although its role in control of flux through the pathway has sometimes been neglected, it has been shown that decreasing its activity creates a bottleneck effect for the entire reductive pathway and downstream metabolites (Khan et al. 2010). In *Arabidopsis thaliana*, SiR is encoded by the only single-copy gene in primary sulfur metabolism. Arabidopsis lines with T-DNA insertions in the promoter region of *SiR* were used to downregulate the expression of the enzyme to different degrees (Khan et al. 2010). Seedlings with only 14% of the *SiR* transcript levels did not survive, which shows that the reductive sulfate pathway is essential for plant life. Seedlings expressing 44% *SiR* transcript levels were severely limited in growth, and they were also sensitive to cadmium, indicating that repression of SiR affects not only sulfur assimilation, but also downstream metabolites in the heavy metal defense network (Khan et al. 2010). No overexpression studies with SiR and analysis of heavy metal tolerance and accumulation have been reported to date.

15.5 Cysteine Synthesis

15.5.1 Serine Acetyltransferase

SAT is the first enzyme in the cysteine synthesis pathway, and is the limiting reaction in the production of this amino acid (Sirko et al. 2004; Yi et al. 2010). SAT plays important roles in heavy metal tolerance and accumulation (Freeman and Salt 2007). Gene expression studies in Arabidopsis show increased expression of SAT isoforms following exposure to cadmium (Howarth et al. 2003). The activity of SAT has also been directly linked to hyperaccumulation of nickel in *Thlaspi goesingense* (Freeman et al. 2004; Freeman and Salt 2007). Comparison of hyperaccumulator and nonaccumulator species of Thlaspi indicates that increased SAT activity correlates with the ability to hyperaccumulate nickel (Freeman et al. 2004). In transgenic Arabidopsis, heterologous expression of *T. goesingense* SAT hyperactivates sulfur assimilation, contributing to elevated shoot concentrations of glutathione, thereby conveying a high level of nickel tolerance (Freeman and Salt 2007). It also contributed to increased tolerance of zinc and cobalt, a slight increase in cadmium resistance, and increased resistance to general oxidative stress (Freeman and Salt 2007). These findings indicate excellent potential for the utilization of mitochondrial SAT for genetic engineering for the application of phytoremediation.

As a limiting enzyme in the production of cysteine, Wawrzynski et al. (2006) explored the strategy of combining overexpression of SAT with enzymes involved in glutathione and phytochelatin synthesis (i.e., glutamate-cysteine ligase and phytochelatin synthase) in transgenic tobacco. Following cadmium treatment, plants expressing the three transgenes all contained higher nonprotein thiol content and increased cadmium accumulation. Although these studies support a key role for sulfur metabolism, they also suggest that other factors, possibly transport systems, limit cadmium accumulation.

15.5.2 O-Acetylserine Sulfhydrylase (O-Acetylserine-Thiolyase)

The final step of the synthesis of cysteine from serine is catalyzed by OASS. Interaction of SAT and OASS forms the decameric cysteine regulatory complex with cellular concentrations of sulfide and *O*-acetylserine affects the reversible association of the complex (Kumaran et al. 2009). Analysis of gene expression in *B. juncea* exposed to cadmium revealed a modest increase in OASS levels, but suggested that coordinated regulation of the genes encoding the enzymes of sulfate uptake and reductive assimilation is required to increase flux through the pathway to meet increased demands due to heavy metal stress (Schäfer et al. 1998). This observation focused efforts to test the role of OASS in heavy metal protection.

Analysis of OASS gene expression in Arabidopsis showed that the cytosolic OASS isoform increased sevenfold in response to cadmium stress (Dominguez-Solís et al. 2001). This corresponded with increased cysteine and glutathione levels required to support the synthesis of phytochelatin peptides. Moreover, plants overexpressing the cytosolic OASS were ninefold more tolerant to cadmium than wild-type plants and are potential phytoremediation tools (Domínguez-Solís et al. 2004). Later studies using a knockout of the cytosolic OASS in Arabidopsis demonstrated that antioxidant activity is essential to maintain redox balance in these plants (López-Martín et al. 2008). Additional studies using OASS to generate transgenic tobacco show enhanced tolerance to cadmium, selenium, and nickel, as well as improved cadmium accumulation in the shoots (Kawashima et al. 2004; Ning et al. 2010). In *Typha latifolia* L. and *Phragmites australis*, activity of OASS increased in response to abscisic acid under stress conditions (Fediuc et al. 2005). The increased OASS activity contributed substantially to replenishing cysteine during conditions of increased demand due to cadmium and sodium chloride stress.

15.6 Alternative Sources of Sulfide Under Stress

As discussed earlier, increased flux through the sulfur assimilation pathway is necessary to maintain glutathione levels for protection against heavy metals; however, there are other ways to help meet the metal-induced demand for increased sulfide. In vitro synthesis of cysteine has been observed, using the enzyme rhodanese to transfer sulfur that has been reduced by thiosulfate reductase to OASS (Louie et al. 2003). This coupling of thiosulfate reductase to the cysteine synthetic pathway by a sulfur transferase was induced in S*chizosaccharomyces pombe* by addition of cadmium. This activity may provide an alternative pathway to provide more sulfide for increased cysteine and glutathione synthesis (Louie et al. 2003). The role of sulfur transferases in cysteine synthesis in plants is an area in which more research is needed, but studies such as this suggest that heavy metal stress may induce this link.

Glucosinolates are an important class of secondary metabolites in plants whose synthesis is linked to sulfate assimilation (Yatusevich et al. 2010). In *Arabidopsis thaliana* and other Brassicaceae, glucosinolates are broken down by the enzyme myrosinase. This degradation produces toxins that deter herbivores. Sulfur released by glucosinolate degradation can be recycled through the biochemical pathways in the roots and leaves. Synthesis of glucosinolates can be induced by biotic stresses, such as heat shock, which is highly genetically correlated to the response of plants to cadmium (Louie et al. 2003). There are two main groups of transcription factors, each regulating the synthesis of one of the two major groups of glucosinolates. The MYB28, MYB76, and MYB29 transcription factors are associated with elevated synthesis of aliphatic glucosinolates and the MYB51, MYB122, and

MYB34 transcription factors affect synthesis of indole glucosinolates. Microarray experiments have predicted that these transcription factors also regulate some of the genes involved in the primary reduction of sulfate. A trans-activation assay provided direct evidence for this link (Yatusevich et al. 2010). Overexpression of the MYB transcription factors also increased mRNA levels of ATPS and APS kinase. APS kinase catalyzes the formation of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the sulfate donor for glucosinolate biosynthesis.

15.7 Natural Hyperaccumulators

Several plant genera, as well as some microbes and fungi, are natural hyperaccumulators of heavy metals. It is suspected that their enhanced abilities to accumulate, translocate, and detoxify and sequester heavy metal ions evolved in some taxa to protect against disease and insect herbivores, similar to the function of glucosinolates (Salt 2006). The Brassicaceae family contains the largest number of hyperaccumulators with 11 genera (Prasad and Freitas 2003). *Thlaspi* species can accumulate cadmium, nickel, lead, and zinc. Cysteine and other low molecular weight thiols have been implicated in the ability of *Thlaspi caerulescens* to hyperaccumulate cadmium (Hernández-Allica et al. 2006). *B. juncea* (Indian mustard) is known for its ability to accumulate cadmium, chromium, copper, nickel, lead, and zinc. Several aquatic species, such as duckweed (*Lemma minor*) and water hyacinth (*Eichhornia crassipes*) take up metal ions from contaminated water. Sunflower has been shown to remove lead, uranium, cesium, and strontium from hydroponic solution (Prasad and Freitas 2003). Physiological and biochemical comparisons of hyperaccumulators and their nonhyperaccumulating counterparts have discovered genes at key steps in accumulation, translocation, and vacuolar sequestration (Salt 2006). By comparison with *A. thaliana, A. halleri* appears to derive its ability to hyperaccumulate zinc from overexpression of zinc transporters from the ZIP family (Becher et al. 2004). Constitutive overexpression of the CDF-family member *MTP1* contributes to the abilities of several species to compartmentalize cadmium, nickel, and zinc into vacuoles (Küpper et al. 1999, 2001; Krämer et al. 2000; Ma et al. 2005). A group of genes involved in iron balance, including *NAS2, NAS3*, *IRT1,* and FRO2 are also overexpressed in hyperaccumulators (Lombi et al. 2001). Many of the important genes have become useful in genetic engineering, in order to exploit these traits for the application of phytoremediation. The problem with using most natural hyperaccumulators for phytoremediation of polluted soils is that they are slow growing and tend to have low biomass. For these reasons there is ongoing research in the incorporation of certain genes into transgenic high-biomass crop plants.

15.8 Conclusion

The uptake, storage, and metabolism of sulfur are highly regulated both locally and at the whole plant level. The sulfate transport system responds to environmental sulfur availability, as well as to cellular levels of cysteine, and glutathione, and other compounds. Nucleophilic properties of the sulfhydryl group convey a unique ability to react with free radicals, active oxygen species, and other substances involved in oxidative stress. Transcriptional regulation of sulfate transporters and enzymes of sulfate reduction and cysteine biosynthesis controls flux through the metabolic pathways. Some plants have evolved remarkable resistances to toxic heavy metals through biochemical responses, most prominently those involving glutathione and phytochelatins. How sulfur and sulfur-containing compounds are initially sensed at a cellular level and provide a signal for the multitude of adaptive responses addresses questions for further research.

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