Cellular Biology of Sulfur and Its Functions in Plants

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Cellular Biology of Sulfur and Its Functions in Plants

Rüdiger Hell, M. Sayyar Khan, and Markus Wirtz

Abstract Sulfur is one of the most versatile elements in life. It functions in fundamental processes such as electron transport, structure, and regulation. In plants, additional roles have developed with respect to photosynthetic oxygen production, abiotic and biotic stress resistance and secondary metabolism. Sulfate uptake, reductive assimilation, and integration into cysteine and methionine are the central processes that direct oxidized and reduced forms of organically-bound sulfur into its various functions. These steps are distributed between several cellular compartments and tightly regulated by supply, demand, and environmental factors in a network with assimilation of carbon and nitrogen. Signaling cues such as sulfate availability and thiol-based redox homeostasis via glutathione and their integrating by sensing systems will be presented in this chapter and analyzed.

1 Sulfur is an Essential Mineral Element

1.1 Physiological Functions of Sulfur

Sulfur is of elemental importance for life due to its versatility and reactivity in different oxidation and reduction states. In phototrophic organisms in general, the redox properties of sulfur in proteins and in sulfur-containing metabolites are important as mediators between the reductive assimilation processes of

R. Hell (✉) and M. Wirtz
Heidelberg Institute for Plant Sciences, University of Heidelberg, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany
e-mail: rhell@hip.uni-heidelberg.de
M.S. Khan
Department of Plant Breeding and Genetics, NWFP Agricultural University, Peshawar, Pakistan

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photosynthesis and reactive oxygen species that arise as byproducts of electron transport chains. Reduced sulfur compounds have been assumed as early energy donors and, together with iron, may have contributed to early electron transport processes in proto-life (Martin et al. 2003). The reductive conditions before the invention of oxygenic photosynthesis probably allowed the evolution of processes based on the redox properties of sulfur. These sulfur-based reduction systems remained when the atmospheric environment became oxidative as a consequence of the effective development of photosynthesis in bacteria and later in algae and plants. It was suggested that this transition promoted the evolution of oxygen-dependent pathways as basis of subsequently complex organismal development (Falkowski 2006).

In addition to iron-sulfur clusters, the catalytic abilities of reduced sulfur are documented in the cofactors and ligands coenzyme A, biotin, thiamin, lipoic acid, and others. They are of fundamental importance to all cells since the very origin of life and have essential functions as vitamins in mammals. In proteins, the structural role of disulfide bridges is well established and may be renewed in this chapter by the discovery of two functionally important disulfide bridges in plant γ-glutamylcysteine ligase (GSH1), the first enzyme of GSH biosynthesis (Jez et al. 2004; Hothorn et al. 2006; Gromes et al. 2008). Regulatory functions of disulfide bridges in plants are prominent in enzymes of the Calvin Cycle but are found in increasing numbers in control switches such as NPR1 in pathogen defence (Tada et al. 2008) and transcription factors (Ndamukong et al. 2007). Monothiol- and dithiol mediated transfer processes are essential in these highly specific and directed processes that include GSH, NO, thioredoxins and glutaredoxins (Meyer and Hell 2005; Meyer et al. 2008).

These primary cellular functions are, in plants, complemented by the so-called secondary sulfur compounds. Many of them have roles in plant defence processes against phytopathogenic microbes and pests, such as the phytoalexin camalexin, sulfur-rich peptides of the thionin and defensin groups, and glucosinolates in the Brassicaceae family (Rausch and Wachter 2005). Lack of these compounds reduces the defence capabilities, while their formation upon attack goes along with an activation of primary sulfur metabolism. Since the synthesis of these defence compounds seems to partially depend on optimal sulfate supply, the term "sulfur-enhanced defence" has been coined (Kruse et al. 2007). The many aspects of sulfur function in phototrophic organisms have recently been comprehensively reviewed (Dahl et al. 2008).

1.2 Symptoms of Sulfur Deficiency

The numerous functions of sulfur in plants give rise to a characteristic long-term deficiency phenotype: chlorosis of interveinal sections of young leaves. The lack of reduced sulfur for iron-sulfur clusters in photosynthesis is presumably one reason that gives rise to chlorophyll oxidation; degradation of photosynthetic proteins to

regain reduced sulfur is another (Ferreira and Teixeira 1992; Gilbert et al. 1997). In contrast to nitrate metabolism, where mature (source) leaves under nitrogen starvation turn chlorotic because they export nitrate to young sink leaves for growth, the young (sink) leaves react first and strongest to prolonged sulfate deficiency. The reason for this difference is seen in a slow release of sulfate stored in the vacuoles of mature leaves (Bell et al. 1994). In comparison, the mobility of nitrate between vacuole and cytosol upon demand is much higher (Miller et al. 2009). The activation rate of sulfate seems to be connected to nitrogen availability and growth rate (Blake-Kalff et al. 1998; Dubousset et al. 2009). This physiological feature gives rise to problems with early diagnosis of sulfur deficiency in agriculture. Determination of free sulfate in leaves is not indicative of the sulfate supply of a crop. Leaf sulfate contents may still be high, but are of only limited use, while the plant already suffers from sulfate deficiency in the soil solution (Blake-Kalff et al. 1998). As observed for many nutrient deficiency responses, the shoot:root ratio decreases during prolonged sulfate starvation. Interestingly, the morphological phenotype also includes changes in root architecture, where reactive oxygen species are produced in response to sulfate deprivation (Schachtman and Shin 2007). The precise developmental response depends on the plant species, but in Arabidopsis lateral root initiation is enhanced but not elongation (Kutz et al. 2002). At least Arabidopsis roots are also able to grow toward sulfate-rich zones as has been observed for Arabidopsis and several crop plants in response to nitrate, phosphate or potassium rich patches of soil (Robinson 1994).

Since sulfate belongs to the six essential plant nutrients, its availability has strong impact not only on plant growth and development, but also on crop yield and quality. Optimized sulfate fertilization that is in equilibrium with nitrogen application is of great concern in agriculture (Hell and Hillebrand 2001; Howarth et al. 2008; Dubousset et al. 2009). Since the decline of atmospheric intake of SO_2 due to clean air acts in the 1980s, the recognition of sulfate deficiency and precise fertilization regimes for sulfate has not only become a requirement in high yield agriculture, in particular for oilseed rape, but also cereals and sugar beet. Today, addition of 20–40 kg S/ha are regular measures to optimize yield in sulfur deficient soils. The timing of application is important, for example, before the onset of seed filling, because, in marked difference to nitrogen metabolism, the current availability of stored sulfate and less its reactivation defines the availability to the seeds (Walker and Boothe 2003).

1.3 Acclimatory Responses to Sulfur Starvation

In addition to morphological changes in response to long-term sulfate starvation, plants also respond to short-term deprivation at several levels. Marine algae live in an environment with about 30 mM sulfate and hardly experience a deficiency situation. However, sweet water algae and land plants retrieve sulfate from solutions in the micromolar range (Giordano et al. 2005). They are equipped with

high-affinity sulfate transporters that operate with half-maximal activity in the low micromolar range and are rapidly de-repressed upon sulfate removal from the solution (see Sect. 2). The short-term response consists of characteristic adaptive reactions in plants and algae: de-repression of genes of sulfate uptake and increased uptake rates at the plasmalemma (Hawkesford and De Kok 2006); induction of genes of sulfate assimilation and several marker genes (*Nit3*, Kutz et al. 2002; *UP9*, Wawrzynska et al. 2005; *Sdi1*, Howarth et al. 2009); decreased levels of cysteine and glutathione (GSH) and enhanced concentrations of *O*-acetylserine (OAS), the intermediate of cysteine synthesis (see Sect. 4). This characteristic response has been widely investigated (Lewandowska and Sirko 2008) and is the subject of comprehensive bioinformatics and system biology approaches (reviewed in Hirai and Saito 2008; Hoefgen and Nikiforova 2008; Amtmann and Armengaud 2009; Amtmann and Blatt 2009); After about 24–72 h of depletion, depending on the species and growth regime, the long-term response develops (Nikiforova et al. 2005): secondary sulfur compounds such as glucosinolates are degraded as well as proteins of the photosynthetic apparatus, genes of hormone synthesis and signalling begin to change the morphology. If the generative stage is affected by sulfur deficiency the seed developmental program is changed. Again depending on the current nitrogen status (Howarth et al. 2009), seed storage protein composition is shifted by transcriptional and posttranscriptional changes in gene expression of sulfur-rich proteins (zein, PA1) and sulfur-poor proteins (vicilin, β-conglycinin; Kim et al. 1999; Tabe et al. 2002). This shift is enhanced by posttranslational processes including proteolysis in ripening seed to optimize seed viability for germination (Higashi et al. 2006).

2 The Acquisition and Allocation of Sulfur Compounds

2.1 Sulfate Acquisition by Plant Roots

Plants take up sulfur from the soil mainly in the form of sulfate (SO_4^{2-}), although they are also able to use reduced sulfur compounds from the atmosphere such as sulfur dioxide or hydrogen sulfide (Leustek et al. 2000). After being actively transported into the roots, sulfate is distributed throughout the plant. The suggested mechanism for sulfate transport is a coupled H^+ co-transport with a probable $3H^+$:$1SO_4^{2-}$ stoichiometry as shown in *Lemna gibba* (Lass and Ullrich-Eberius 1983) and is driven by a proton gradient maintained by P-type ATPase. Plant sulfate transporters have been analyzed for a number of species, including crop plants and described in several recent reviews (Buchner et al. 2004a; Hawkesford and De Kok 2006; Hawkesford 2008; Miller et al. 2009). In general sulfate transporters are encoded by gene families with more than ten members, which are subdivided into five groups according to amino acid similarity and function. The best investigated organism in this respect is *Arabidopsis thaliana* with 14 putative sulfate transporter (*Sultr*) genes.

The size of the encoded proteins of the SulP type transporters ranges from 500 to 700 amino acids. The secondary structure consensus predicts 10–12 transmembrane spanning helices that constitute the catalytic part of the protein. A short linker joins a C-terminal region with similarity to bacterial anti-sigma factor antagonists such as the *Bacillus subtilis* SpoIIAA. This domain is termed STAS (sulfate transporter and antisigma factor antagonist) and thought to enable protein-protein interactions. All plant sulfate transporter types possess this domain except group 5 transporters (Hawkesford 2008). Expression of chimaeric transmembrane and STAS domain constructs had a deleterious effect on transport kinetics and deletions of the STAS domain prevented trafficking to the plasma membrane (Shibagaki and Grossman 2004). Mutations of amino acids in the *AtSultr1;2* STAS domain with analogy to the phosphorylated serine of SpoIIAA resulted in a complete loss of activity of sulfate transport (Rouached et al. 2005). Thus, the STAS domain is likely to contribute to sulfate transport control but the precise mechanism of action is unclear.

Sulfate transporter group 1 is best characterized and encodes plasmalemma transporters with high affinity for sulfate (K_m = 1.5–10 μM; Hawkesford 2003). They are expressed predominately in roots and responsible for uptake of sulfate from soil solution into the root cells (Shibagaki et al. 2002; Yoshimoto et al. 2002). The steady levels of their mRNA increases rapidly, upon sulfur starvation, leading to an increased capacity of the roots for sulfate uptake. Detailed comparison of *AtSultr1;1* and *AtSultr1;2* showed similar expression patterns in the cortex, epidermis, and root hairs based on GFP fusion approaches (Yoshimoto et al. 2002). However, *Atsultr1;1* is also expressed specifically in leaf hydathodes and *AtSultr1;2* in guard cells, suggesting individual roles in these specialized cells. It has been suggested that *Atsultr1;2* is responsible for constitutive uptake, whereas *AtSultr1;1* is inducible under stress (Yoshimoto et al. 2002).

Group 2 comprises low-affinity sulfate transporters (K_m for AtSultr2;1 = 0.41 mM and AtSultr2; 2 ≥ 1.2; Takahashi et al. 2000) that are responsible for translocation of sulfate within the plant. After the uptake of sulfate into the symplast, it is transported in the cytoplasm from peripheral root cells radially through the cortex and endodermis into the root stele. The delivery of sulfate from the symplast to the xylem vessel apoplast is thought to occur as efflux of sulfate from neighboring xylem parenchyma cells (Smith and Diatlof 2005). Apoplastic sulfate may be picked up by Sultr2;1, since its expression was found in xylem parenchyma cells and pericycles of roots, and xylem parenchyma and phloem cells of leaves, leading to the conclusion that it is responsible for uptake from the apoplasm within the vascular bundle and therefore involved in root to shoot transport (Takahashi et al. 2000). Once reached to the fine leaf veins, sulfate is again taken up into the leaf symplasm by sulfate transporters present in the bundle sheath cells that surround the vascular bundles. *Sultr2;1* and *Sultr2;2* are expressed throughout the plant (Buchner et al. 2004a), but tend to localize in vascular tissues (Takahashi et al. 2000). It is therefore likely that both contribute to translocation of sulfate between cells of the vascular tissue. AtSultr2;1 may also be involved in the transport of

sulfur to the seed according to expression in the base of the silique and in the funiculus (Awazuhara et al. 2005).

Group 3 represents a diverse group that may be further subdivided into three or possibly four small clusters (Hawkesford 2008), each containing both Arabidopsis and rice examples and indicating relatively ancient gene duplications. Evidence for sulfate uptake using yeast complementation assays is missing in group 3. However, AtSultr3;5 has been co-localized with AtSultr2;1 in xylem parenchyma and pericycle cells in roots (Kataoka et al. 2004a). Sulfate uptake was hardly detectable with Sultr3;5 alone in the yeast sulfate uptake mutant system, but cells coexpressing both Sultr2;1 and Sultr3;5 showed three-times enhanced uptake activity (v_{max}) compared to Sultr2;1 expression alone, suggesting a role in root-to-shoot transport *in planta* (Kataoka et al. 2004a).

Group 4 sulfate transporters, although first reported to be plastid localized (Takahashi et al. 1999), appear to be tonoplast located according to GFP fusion studies (Kataoka et al. 2004b). In Arabidopsis and Brassica the two group 4 isoforms show highest expression in roots (Buchner et al. 2004b; Kataoka et al. 2004b) and were inducible by sulfur deficiency. Analysis of Arabidopsis double knockout plants indicated their role in sulfate efflux from the vacuole tissue. Nevertheless, import of sulfate into plastids for assimilatory reduction is absolutely necessary. It is believed to be constituted by a minor activity of the triose-phosphate translocator (Gross et al. 1990) or possibly of an ATP binding cassette transporter system in the envelope (Hawkesford 2008). In the moss *Marchantia polmorpha* and green alga *Chlamydomonas reinhardtii* genes encoding sulfate permeases in the chloroplast membrane have been identified. However no such genes have been found in vascular plants (Melis and Chen 2005).

Group 5 sulfate transporters are quite different from the rest with respect to primary sequence and the absence of the C-terminal STAS domain There are typically two isoforms for any given species which are also quite distinct from one another. Group 5 transporters may be functionally different since Sultr5;2 appears to be involved in molybdate metabolism. Natural variation across Arabidopsis ecotypes associated low expression of *Sultr5.2* gene with low moldybdate content while the protein was shown to be localized in the mitochondrial envelope (Baxter et al. 2008). Plasmalemma uptake of molybdate using a yeast sulfate uptake mutant assay was also shown for the same gene (Tomatsu et al. 2007) and the orthologue from *Chlamydomonas*. Affinity for molybdate was in the low nanomolar range (Tejada-Jimenez et al. 2007), resulting in renaming of Sultr5;2 into Mot1. However, the latter reports used GFP fusions to locate Mot1 in the endomembrane system or indirectly in the plasmalemma, leaving the exact function and cellular localization of this protein unsettled. Plasmalemma uptake of molybdate may in fact be a side function of group 1 transporters as shown for SHST1 from *Stylosanthes hamata* in the yeast sulfate uptake mutant assay (Fitzpatrick et al. 2008). No positive indications of sulfate or molybdate transport were observed for Sultr5;1 (see also the chapter on molybdate by Bittner and Mendel in this book).

2.2 Whole Plant Allocation of Sulfur Compounds

Sulfate is the major transport form of sulfur in vascular plants. Its allocation is orchestrated by the tissue-specific expression of the *Sultr* family as described above (Miller et al. 2009). Once sulfate has been taken up by the plant and reached the leaf symplast as described above, it is moved via plasmodesmata to the leaf mesophyll cells to the sites for reductive assimilation. Sulfate constitutes 70%–90% of total sulfur in mature leaves of oilseed rape (*Brassica napus* L.), while less than 1% is found in GSH and glucosinolates. In contrast, free sulfate (42%) is less dominant in young leaves of oilseed rape, where approximately 50% of the total sulfur was incorporated into insoluble (protein) sulfur fraction, 2% into GSH, and 6% into glucosinolates (Blake-Kalff et al. 1998). From shoot to root, sulfate is mobile in the phloem (Rennenberg et al. 1979; Lappartient and Touraine 1996), to redistribute either excess sulfate or stored sulfate pools in the vacuoles under long term sulfur stress, albeit too slow to efficiently support new growth (Clarkson et al. 1983; Bell et al. 1995). In Arabidopsis, Sultr1;3 has been shown to be localized in the phloem (Yoshimoto et al. 2003) where it probably plays a role to gather and keep sulfate within the phloem tissues for long distance recycling to other organs of the plant.

The smaller part of sulfur is allocated or moved between cells in reduced forms. In maize leaves assimilatory sulfate reduction is restricted to bundle-sheath cells, whereas the formation of GSH takes place predominantly in the mesophyll cells (Kopriva and Koprivova 2005). Here, cysteine acts as the transport metabolite of reduced sulfur between the two cell types (Burgener et al. 1998). However, maize may be a special case, since this is the only plant where a direct role of cysteine as metabolic repressor of sulfate uptake has been reported (Bolchi et al. 1999). GSH has long been assumed to be the main reduced long-distance sulfur compound (Rennenberg 1976; Brunold and Rennenberg 1997). GSH and/or its precursor γ-glutamylcysteine (γ-EC) have been shown to be transported over long-distance along the phloem to the roots (Fig. 1, Lappartient and Touraine 1996; Li et al. 2006). GSH must also be transported from maternal tissue into the embryo. The embryo is symplastically isolated and elimination of glutathione synthesis in the embryo causes lethality (Cairns et al. 2006).

More recently S-Methylmethionine (SMM) has also been shown to contribute up to 50% of the sulfur moving to developing grains in wheat, albeit the contribution was less in other species (Bourgis et al. 1999). SMM can be formed by methionine methyltransfease (MMT) and converted to methionine by homocysteine *S*-metyltransferase (Fig. 1). Both would form a futile SMM cycle if expressed at the same time and place, leaving its function at least in part unclear. SMM arrives in the phloem at a level of ~2 mol% of amino acids and could readily provide the methionine needed to synthesize grain proteins with an average methionine content of ~2 mol%. The claim that SMM is a source of sulfur in

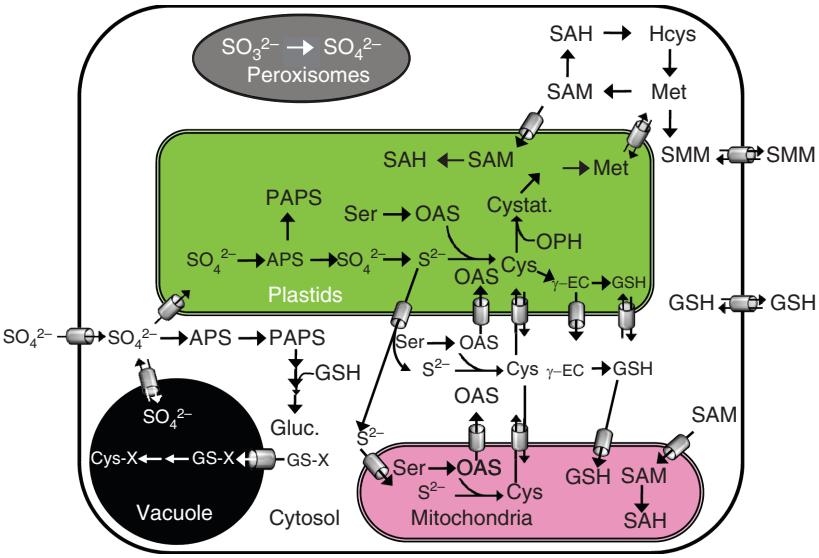

Fig. 1 *Transport of sulfur metabolites within plant cells*. Sulfur containing metabolites are actively transported between sub cellular compartments of a plant cell to separate reduction or oxidation of sulfur. E.g. sulfate is exclusively reduced in plastids will be used in its reduced form (sulfide, S^{2-}) for synthesis of cysteine in plastids, mitochondria and cytosol, but its oxidation is restricted to peroxisomes. A network of transporters (barrels) links production and use of key metabolites for primary and secondary sulfur metabolism like SAM. Finally, long distance transport of reduced or oxidized sulfur compounds crossing the plasmalemma takes place to meet demand of source and sink tissues

the seeds is based on the indirect evidence that it is essentially absent from mature grains in wheat and must be metabolized (Pimenta et al. 1998), and conversion to methionine is its only likely fate (Bourgis et al. 1999). However, the role of SMM in methylation or transport of sulfur has been investigated in *mmt* null mutants of Arabidopsis and maize that lacked the capacity to produce SMM (Kocsis et al. 2003). The normal growth and seeds sulfur contents in Arabidopsis *mmt* plants rule out the indispensable role for SMM in sulfur transport but increased methylation ratio. Thus, sulfate and GSH might play a compensatory role in long distance sulfur transport, or perhaps SMM is a minor form of pholem sulfur present in these plants or the SMM cycle function in methylation. Interestingly, not only primary sulfur compounds but also secondary sulfur compounds can be transported between cells. An example is glucosinolates. Feeding experiments with radiolabeled *p*-hydroxybenzyl-glucosinolate (*p*-OHBG) showed long distance phloem transport in Arabidopsis. When applied to the tip of detached leaves the labeled glucosinolate was rapidly transported from the application site into the whole plant and intact *p*-OHBG recovered from phloem sap and different tissues (Chen et al. 2001).

2.3 Cellular Distribution of Sulfur-containing Compounds

Secondary sulfur compounds have a very complex cell biology with respect to distribution of biosynthetic pathways between subcellular compartments, accumulation inside and outside the cell and, accordingly, transmembrane transport. Often taxonomic specialization of these compounds is observed, as for the alliins in the Alliaceae, while others such as defensins are truly ubiquitous. The reader is referred to specialized reviews with respect to sulfate-containing peptides (phytosulfokines; Fukuda et al. 2007), sulfur-rich proteins (thionins an defensins; Kruse et al. 2005) and sulfur-containing defence compounds such as phytoalexins (Glawischnig 2007), glucosinolates (Halkier and Gershenzon 2006), alliins (Burow et al. 2008) and elemental sulfur (Cooper and Williams 2004).

In this context it should be noted that many of these pathways finish with the transfer of sulfate from the activated form phosphoadenosine phosphosulfate to hydroxylgroups of the final biologically activating compound, e.g. glucosinolates or phytosulfokines. Such sulfation reactions are carried out by sulfotransferases (SULTs), a family of enzymes that is absent in basal land plants and *C. reinhardtii* (Kopriva et al. 2007a).Their substrates and physiological functions are predicted to be very diverse (Hernández-Sebastià et al. 2008). Small organic molecules such as flavonoids, steroids, glucosinolates, and hydroxyjasmonates are sulfonated by cytosolic SULTs, whereas membrane-associated SULTs sulfonate larger biomolecules such as peptides, proteins, and complex carbohydrates (Hernández-Sebastià et al. 2008).

The exception from this complexity is the sulfolipids, both with respect to pathway and distribution. Sulfolipids are ubiquitous in oxygenic photosynthetic organisms and in eukaryotes are always associated with plastids. They are sulfoquinosovyl-diacylgycerides and form a substantial part of polar lipids in plastid membranes. Their synthesis includes two steps and introduces the unusual substrate sulfite into the sugar moiety (Benning et al. 2008). Apart from many less confirmed assumptions their only known function is the replacement of phospholipids during phosphate deficiency stress.

3 Reductive Sulfate Assimilation

3.1 Subcellular Organization of Reactions

Assimilatory reduction of sulfate is predominantly a plastid localized process. However, branching points of the pathway often mark parallel enzymatic activities in other cellular compartments. A comparison of sequenced plant genomes shows that the presence of paralogous genes related to sulfur metabolism is quite common (Kopriva 2006; Kopriva et al. 2007a). After uptake of sulfate at the plasmalemma the pathway is initiated by ATP dependent activation of sulfate to adenosine

5′-phosphosulfate (APS) that is catalyzed by ATP sulfurylase (ATPS). In Arabidopsis and other plants nuclear–encoded isoforms of ATPS reside in plastids and the cytosol (Klonus et al. 1994; Rotte and Leustek 2000). All four ATPS forms in Arabidopsis carry transit peptides but which form provides in addition cytosolic activity via dual targeting is not known. In contrast to the endosymbiont theory ATPS proteins in organisms with primary plastids have been suggested to be of eukaryotic (host) origin (Patron et al. 2008). Further activation of APS with ATP is catalyzed by APS kinase (APK) and yields 3′-phosphoadenosyl-5′-phosphosulfate (PAPS). APK is also present in plastids and the cytosol to provide PAPS in both compartments for sulfation reactions (Mugford et al. 2009; see Sect. 2.3).

In plastids, APK and the assimilatory reduction pathway compete for APS/PAPS. This pathway operates exclusively in plastids in photoautotrophic as well as in heterotrophic tissues and has long been disputed (for review, see Schmidt and Jäger 1992; Leustek 1996; Hell 1997; Leustek et al. 2000; Kopriva and Koprivova 2004; Kopriva 2006). The first option refers to the so-called "bound pathway" of sulfate reduction. It starts from APS and requires the activities of APS sulfotransferase, a thiol-bound intermediate (possibly GSH) and thiosulfonate reductase (TSR) to release sulfide for cysteine synthesis. The bound pathway would avoid the production of free sulfite. Sulfite ions (HSO_3^- and SO_3^{2-}) are strong nucleophiles that can deleteriously react with a wide variety of cellular components. The bound pathway was first described for green algae (Hodson and Schiff 1971) and evidence for APS sulfotransferase activity and regulation was reported from numerous vascular plants (Schmidt 1975; Brunold and Suter 1990). Activity of TSR was described from the green alga *Chlorella* as well (Schmidt 1973).

The second option suggests a "free pathway" that begins with PAPS and would proceed as found in enterobacteria via thioredoxin-dependent PAPS reductase to free sulfite and via sulfite reductase (SiR) to sulfide. Both enzyme activities were found in *E. coli*, *Salmonella typhimurium,* and *Saccharomyces cerevisiae* and also in vascular plants (Schwenn and Kemena 1984; Schwenn 1989). Progress toward bound and free pathway hypotheses suffered from the reactive chemistry of sulfur in its different oxidation states and a number of biochemical ambiguities. The corresponding enzymes were never purified to homogeneity from plants. However, search for genes encoding PAPS reductase activity by functional complementation of an *E. coli cysH* mutant strain that lacked this enzyme led to the breakthrough discovery of APS reductase (APR) in Arabidopsis (Gutierrez-Marcos et al. 1996; Setya et al. 1996), favoring SiR-dependent reduction over TSR-mediated electron transfer to sulfide. The APR protein consists of a plastid transit sequence, a catalytic domain carrying a 4S–4Fe cluster with sequence homology to CysH and a glutaredoxin-like C-terminus that was shown to mediate electron transfer from GSH for reduction (Bick et al. 1998). APR from Arabidopsis and other plants strongly prefers APS instead of PAPS as substrate, its expression responds to sulfate and nitrate availability, and a number of stress factors result in activation of its activity (Leustek et al. 2000, for review). In addition, flux analysis using ^{35}S-labeled sulfate hinted that APR, after sulfate uptake, exerts strongest control over flux through the

sulfate reduction pathway in Arabidopsis (Vauclare et al. 2002) and is responsible for genetically determined variation in sulfate content in Arabidopsis ecotypes (Loudet et al. 2007). To add to the controversy, the identification of APR led to the discovery that many bacterial taxa, but not the model organism *E. coli*, prefer APS as substrate for the free reduction pathway and possess a bacterial thioredoxin-dependent APR without glutaredoxin domain (Bick et al. 2000). Furthermore, the moss *Physcomitrella patens* was shown to carry two genes encoding plant-type APR and bacteria-like APR-B. The latter lacks the plant-specific iron-sulfur cluster as ligand and the glutaredoxin domain. It preferentially reduces not only APS but also PAPS although at a slow rate (Kopriva et al. 2007b). Targeted knockout lines of the *AprA* locus in Physcomitrella were still viable, demonstrating that under nonstressed conditions APR-B was sufficient for normal growth (Koprivova et al. 2002). Phylogenetic comparisons suggest that vascular plant APR with Fe-S cluster is of endosymbiontic origin although the cyanobacterial heritage is not entirely clear, while APR-B is derived from γ-proteobacterial PAPR lacking the Fe-S cluster (Patron et al. 2008). The amino acid sequence from a native purified APS sulfotransferase of *Lemna minor* confirmed, that this protein and APR are, the same enzyme (Suter et al. 2000). This apparently solves the controversy in favor of a free reduction pathway via APR and sulfite. Nevertheless a cDNA from *Arabidopsis thaliana* encoding APS kinase was reported to confer also APS sulfotransferase activity in a nonphysiological side reaction (Schiffmann and Schwenn 1994), documenting the many difficulties of sulfur biochemistry.

The second enzyme of the free reduction pathway, SiR, is exclusively localized in plastids and consists of two 65 kDa subunits. It contains a single siroheme and (4Fe–4S) cluster as prosthetic groups, has a high affinity for sulfite ($K_m^{sulfite}$ ~10 μM; Krueger and Siegel 1982; Nakayama et al. 2000) and releases sulfide that is then integrated into cysteine in a step that is comparable to ammonia fixation into glutamine (see Sect. 4). Ferredoxin acts as physiological donor of six electrons required for sulfite reduction, while bacterial SiR uses NADPH (Yonekura-Sakakibara et al. 2000). The structure, sequence and ligands of SiR in bacteria, archea and eukaryotes are similar to nitrite reductase, which catalyzes an equivalent reduction step in nitrate assimilation, i.e. a six electron reduction of nitrite to ammonia (Crane et al. 1995; Swamy et al. 2005). SiR is able to reduce nitrite as well and substrate preference can be converted by a single amino acid mutation (Nakayama et al. 2000). Physiological relevance of SiR had thus been questioned and sulfite as a substrate was regarded as an artifact, since a supposedly SiR deficient mutant strain of *Chlorella* was reported to carry TSR instead (Schmidt et al. 1974; Schmidt 1976). Nitrite reductase may react similarly, adding to the uncertainty of the free pathway as indispensable reduction pathway (Schmidt and Jäger 1992). Phylogenetic analysis showed that both SiR and NIR arose from an ancient gene-duplication in eubacteria, before the primary endosymbiosis that gave rise to plastids (Patron et al. 2008). It should be noted that SiR was observed in association with nucleoids in plastids of pea, maize and soybean. SiR was suggested to compact nucleoids and to repress DNA synthesis (Cannon et al. 1999) and transcription (Sekine et al. 2002). It cannot be ruled out that SiR has a second

"moonlighting" function, although it appears completely soluble in biochemical experiments.

3.2 Signal Mechanisms and Homeostasis of Uptake and Reductive Assimilation

The characteristic short- and long-term responses to sulfate deficiency described in Sect. 2.2 are based on the entire inventory of regulatory mechanisms in eukaryotic cells (Amtmann and Blatt 2009; Miller et al. 2009). Induction of *Sultr1;1* expression was suggested to involve phosphorylation/dephosphorylation as regulatory part of root sulfate uptake (Maruyama-Nakashita et al. 2004). A remarkable mechanism is the enhanced sulfate transport capacity based on membrane protein interaction. The almost identical expression patterns of *Sultr2;1* and *Sultr3;5* in the vascular tissue (see Sect. 2.1) prompted co-expression analysis of both proteins in yeast and showed increased sulfate uptake rates compared to SULTR2;1 or SULTR3;5 alone. The function of this interaction was indirectly confirmed by reduced root-shoot sulfate transport in a *sultr3;5* null mutant (Kataoka et al. 2004a).

A recently discovered regulatory component in plants is miRNAs that respond to nutrient deprivation (Pant et al. 2009). In Arabidopsis miRNA395 targets *ATPS* genes *1*, *3* and *4*. and *Sultr2;1*. When miRNA395 increases during sulfate deprivation, the abundance of ATPS transcripts decreases (Kawashima et al. 2009). However, expression of genes encoding ATPS are also known to respond with moderately enhanced mRNA contents to sulfate starvation and feeding with GSH (Logan et al. 1996; Lappartient et al. 1999). Whether ATPS has a limiting role for flux control in the reduction pathway is unresolved. Over-expression experiments in tobacco cells and in *B. juncea* plants were contradictive (Hatzfeld et al. 1998; Pilon-Smits et al. 1999). The role of miRNA395 that is encoded from several loci in the Arabidopsis genome is also not clear at present and experiments with respect to short-term deprivation and long-term starvation need to be conducted to resolve these contrasting results. Interestingly, *mi395* expression is controlled by transcription factor SLIM1 (Maruyama-Nakashita et al. 2006), but not the mi395 target *Sultr2;1* due to cell-type specific patterns (Kawashima et al. 2009).

Once inside the cell, APR is in command of flux for sulfate reduction (Vauclare et al. 2002), although mutant analysis of the semi-constitutive SiR gene suggests a potentially limiting role at high flux conditions (Khan, Wirtz, Hell, unpublished). Transcriptional regulation of APR genes in response to sulfate and nitrate deficiency, environmental stresses and during day-night cycle has long been known (Brunold and Suter 1990; Kopriva 2006). Support for the major control function comes from constitutive overexpression of bacterial APR from *Pseudomonas aeruginosa* in Arabidopsis and maize that resulted in massive deregulation of primary sulfur metabolism (Tsakraklides et al. 2002; Martin et al. 2005). In addition, posttranslational control by redox processes was shown in vitro and

in vivo, treatment with ozone enhanced APR activity without changes in protein abundance and in the presence of transcription or translation inhibitors (Bick et al. 2001).

Availability and demand are discussed as driving forces for regulation of sulfate uptake and reduction. Most of the conditions and metabolites tested affect the steps of the pathway similarly, although to varying extent. Under normal conditions the genes of primary sulfur metabolism are under the same control. The rate of sulfate uptake by the root more or less equals the sulfur requirement for growth of the plant (Hawkesford and De Kok 2006). The most rapid response occurs in the classical sulfate deprivation experiment where plants are grown under regular sulfate supply and are transferred to low or zero sulfate concentrations in the growth medium. Genes encoding sulfate transporters respond first, usually within less than 1 h, suggesting sensing of sulfate outside or in the cytsol of root surface cells, although evidence for this is rather indirect at present (Rouached et al. 2008; Amtmann and Blatt 2009). A catabolite repression system as proposed for control of nitrate uptake and assimilation may operate via GSH or possibly cysteine (Hell and Hillebrand 2001; Hell et al. 2002). Feeding of cysteine repressed sulfate uptake in maize seedlings (Bolchi et al. 1999), whereas only GSH but not cysteine down-regulated uptake in Arabidopsis roots (Lappartient et al. 1999), suggesting species specific differences in regulation. OAS may have a special role, because it links nitrogen and carbon metabolism with sulfur. Feeding of OAS rapidly triggers not only *Sultr* and *APR* genes in different plants, but induces numerous genes, effectively imitating the transcriptome deficiency response (Hirai et al. 2003; Hirai and Saito 2008; Hoefgen and Nikiforova 2008). These studies also revealed network links to jasmonate signalling and auxin metabolism. Over-expression of SAT to enhance internal OAS concentrations in potato enhanced *Sultr* transcription in the roots, but in long-term studies no correlation between OAS levels and sulfate uptake activity was observed (Hopkins et al. 2005). Whether OAS accumulation during sulfate deprivation is purely a secondary process that regulates the fine-tuning of cellular cysteine synthesis (see Sect. 4) or mediates actually long-term responses remains to be elucidated.

Signal transduction research in the sulfate deficiency response identified a seven base pair *cis*-element (SURE; sulfur-responsive element) in the promoters of the group 1 transporters and other sulfur-related genes. Since the SURE element is present also in nonresponsive genes more *cis*-elements are required for a sulfur-specific response (Maruyama-Nakashita et al. 2005). A genetic screen exploited a chimeric promoter of an OAS responsive *cis*-element of the bean β-conglycinin promoter with a 35S minimal promoter fused to GFP in transgenic Arabidopsis. This approach led to the isolation of several genes none of which revealed a direct regulatory function (Ohkama-Ohtsu et al. 2004; Kasajima et al. 2007). A similar screen used the promoter of Arabidopsis *Sultr1;1* fused to GFP to isolate mutants that are nonresponsive to sulfate deficiency. This identified the transcription factor SLIM1 (sulfur-limitation1) that belongs to the family of ethylene-insensitive such as trans-factors (Maruyama-Nakashita et al. 2006). SLIM1 appears to be a major regulator and was shown to be involved in the control of the sulfur deficiency

response of numerous genes including degradation of glucosinolates, but not of *APR* genes. It is further puzzling that SLIM1 seems to be exclusively expressed in the vascular tissue and thus far away from Sultr1;1 occurrence in the rhizodermal cells (Maruyama-Nakashita et al. 2006). Other transcription factors were described for regulation of primary and secondary sulfur metabolism. R2R3 type Myb transcription factors were observed to be up-regulated during sulfate deficiency in microarray experiments but have not definitively been verified (Nikiforova et al. 2003). Glucosinolate biosynthesis and degradation are known to be regulated in response to biotic and abiotic stress (Malitsky et al. 2008). Several Myb factors seem to specifically share control of promoters of genes of aliphatic and indol glucosinolates (Gigolashvili et al. 2007; Hirai et al. 2007). It remains to be analyzed if some of these factors are able to address promoters of genes of primary sulfur metabolism to achieve coordination of assimilatory reduction and downstream demand.

4 Regulation of Sulfur Amino Acids Biosynthesis

4.1 Regulation of Cysteine Biosynthesis

Synthesis of cysteine is the entry point of reduced sulfur into metabolism. It can be subdivided into three steps: (1) the assimilatory sulfate reduction for provision of sulfide (Sect. 3), (2) the synthesis of the carbon and nitrogen containing backbone of cysteine, and (3) the incorporation of reduced sulfur into the organic backbone. The released cysteine is the pivotal compound in sulfur metabolism and the starting point for production of all compounds containing reduced sulfur like methionine and GSH (Hell 1997; Saito 2004).

As in bacteria, the synthesis of the carbon-nitrogen backbone of cysteine is catalyzed by serine acetyltransferase (SAT, EC 2.3.1.30), which transfers an acetyl-moiety from acetyl-coenzyme A to serine, leading to *O*-acetylserine (OAS) formation (Kredich and Tomkins 1966; Hell et al. 2002; Droux 2004). Then *O*-acetylserine(thiol)lyase (OAS-TL, EC 2.5.1.47) converts OAS into cysteine in the presence of sulfide (Hell 1997; Droux 2003). SAT and OAS-TL form a multienzyme complex called cysteine synthase complex (CSC; (Kredich et al. 1969; Bogdanova and Hell 1997), in which protein–protein interactions regulate activities of both enzymes (Droux et al. 1998; Wirtz et al. 2001).

SAT and OAS-TL are encoded by small nuclear gene families. Their members are ubiquitously transcribed and the gene products localized in the plastid, the mitochondria and the cytosol (Hell et al. 2002; Kawashima et al. 2005). In contrast to sulfate transporters, ATPS and APR (Sect. 1), SAT and OAS-TL isoforms are hardly regulated at their transcript levels in response to sulfur availability or exogenous application of OAS, a key regulator for cysteine synthesis (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2004; Kawashima et al.

2005). Nevertheless, transcription of OAS-TLs and SATs respond to abiotic stress conditions, which are known to perturb redox (Freeman et al. 2004; Dominguez-Solis et al. 2008; Lehmann et al. 2009). Most likely cysteine synthesis is up-regulated under these conditions to cope with the higher demand of cysteine for synthesis of GSH that is used for detoxification of reactive oxygen species (Sect. 5).

It is currently unknown why SAT and OAS-TL activities are unequally abundant in their subcellular compartments (Lunn et al. 1990; Rolland et al. 1992; Ruffet et al. 1995; Kuske et al. 1996). Purification of OAS-TL proteins in combination with reverse genetics approaches for all OAS-TL and SAT genes in Arabidopsis revealed that in leaves 90% of OAS-TL activity and protein is present in cytosol and plastids, while only a minor fraction is found in the mitochondria (Heeg et al. 2008; Lopez-Martin et al. 2008; Watanabe et al. 2008a). In contrast, 80% of total SAT activity is associated with mitochondria, while the residual activity is found equally distributed in cytosol and plastids (Watanabe et al. 2008b). None of the single knock out lines for SAT and OAS-TL show a lethal phenotype indicating that OAS and cysteine can be transported sufficiently between the subcellular compartments of the plant cell (see Fig. 2). However, the mitochondrial SAT has a pace making function in OAS net synthesis, which limits total cysteine synthesis and growth of Arabidopsis (Haas et al. 2008). The importance of mitochondrial OAS synthesis is in agreement with provision of substrates for SAT reaction, acetyl-coenzyme A and

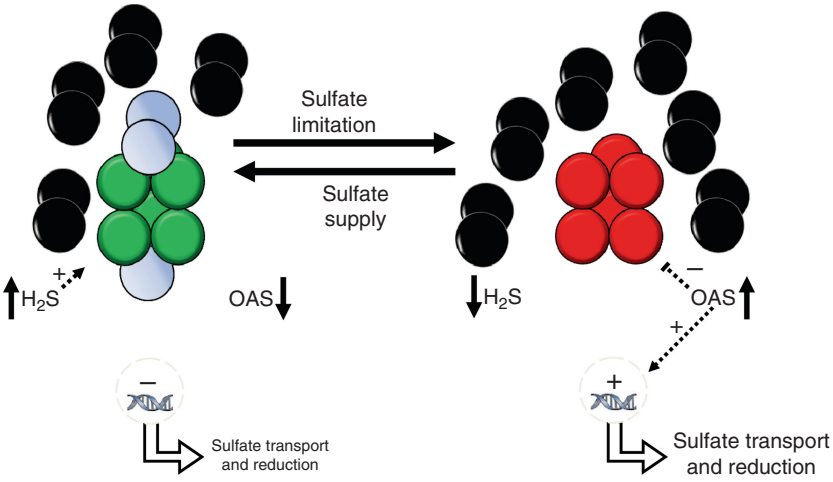

Fig. 2 *Regulatory network of the aspartate derived amino acids pathway*. The synthesis of methionine is regulated within the aspartate derived amino acid pathway. Reaction steps (black arrows) and key regulatory enzymes (grey circles) for individual branches of the pathway are highlighted. The positive (blue) and negative (red) regulatory feedback loops within the pathway are marked by dotted lines. Aspartate kinase (AK), cystathionine β-lyase (CBL), cystathionine γ-synthase (CGS), methionine γ-lyase (MGL), methionine synthase (MS), SAM-dependant methyl-transferase (MT), SAM synthase (SAMS), threonine synthase (TS)

serine, by citric acid cycle and photorespiration and the high SAT activity found in this compartment (Wirtz and Droux 2005). It also provides an elegant explanation for the unexpected growth phenotype of the mitochondrial OAS-TL knockout (Heeg et al. 2008). Only knockout of the minor abundant mitochondrial OAS-TL activity (10% of total activity) but not of plastidic (~45% of total activity) and cytosolic OAS-TL (~45% of total activity) results in reduced growth, most likely because mitochondrial OAS-TLs regulates total OAS net synthesis via formation of the CSC (see below) in mitochondria. Sulfide, which is exclusively produced in the plastid (Sect. 3), meets the OAS in the cytosol, which led to the hypothesis that under normal sulfur supply cysteine is mainly produced in cytosol (see Fig. 2). In agreement with this hypothesis the knockout of cytosolic OAS-TL most strongly affects cysteine net synthesis (Heeg et al. 2008; Watanabe et al. 2008a).

The strong limitation of cysteine synthesis by provision of OAS is further supported by the 300-fold lower activity of SAT in comparison to OAS-TL in leaves (Ruffet et al. 1994) and the successful attempts to increase cysteine contents by over-expression of SAT (Blaszczyk et al. 1999; Harms et al. 2000; Sirko et al. 2004). In contrast, OAS-TL over-expression approaches in cytosol and plastids increase the total cysteine content only marginally because OAS is limiting (Saito et al. 1994a; Saito et al. 1994b; Sirko et al. 2004).

Consequently cysteine shows no feedback-inhibition of OAS-TL which produces cysteine, but SAT that produces the limiting precursor, OAS (Noji et al. 1998). SAT activity is inhibited by cysteine in an isoform-specific manner in plants, leading to the hypothesis that the feedback inhibition of SAT in each subcellular compartment has different functions for regulation of total sulfur metabolism. Functional analysis and structural modeling of plant SAT to their bacterial homologes revealed that the cysteine inhibition and the SAT-OAS-TL interaction domain are both located at the C-terminus of plant SATs (Wirtz et al. 2001; Wirtz and Hell 2006; Feldman-Salit et al. 2009). Recently, regulation of cysteine sensitivity of cytosolic soybean SAT in response to formation of the CSC has been demonstrated (Kumaran et al. 2009). In addition, the feedback sensitivity of cytosolic soybean SAT was shown to be regulated posttranslationally by phosphorylation (Liu et al. 2006). The phosphorylation site of soybean SAT is not conserved in cytosolic SATs from other plant species, which adds a note of caution to the universal validity of posttranslationally regulated cysteine feedback sensitivity in other plant systems.

Formation of the CSC is a general feature of all analyzed bacterial and plant SATs and OAS-TLs (see Wirtz and Hell 2006 for review). The plant CSC can be stabilized by sulfide in vitro whereas OAS is able to dissociate it (Wirtz and Hell 2006). The relevance of OAS-promoted dissociation is supported by precise concentration-dependent dissociation kinetics and revealed an equilibrium dissociation constant of 57 μM OAS (Berkowitz et al. 2002). These findings suggest that the equilibrium of association/dissociation can be effectively shifted like a switch, since fluctuations of cellular OAS concentration in this range have been observed in response to sulfate and nitrate availability (Kim et al. 1999). Inside

the CSC SAT is activated, while OAS-TL is efficiently inactivated by the C-terminus of SAT that binds in the active site of OAS-TL (Droux et al. 1998; Wirtz et al. 2001; Bonner et al. 2005; Francois et al. 2006). As a consequence, OAS leaves the CSC and will be converted by free OAS-TL to cysteine, if sulfide is present. Most likely substrate channeling is actively prohibited in the CSC to allow free OAS to act as a regulator of the transcriptional response toward sulfur availability (Kim et al. 1999; Hirai et al. 2003). During sulfate deficiency the OAS level increases as a result of missing sulfide. This leads to the dissociation of the complex by (1) the destabilizing effect of OAS and (2) the missing stabilization by sulfide. The reason for dual control of complex formation by OAS and sulfide could be the need for complex stabilization at very high cysteine synthesis rates. As a result of the strong dependence of cysteine synthesis for OAS, the most efficient way to increase net cysteine synthesis is a higher OAS level. Recently, the importance of CSC formation for regulation of cysteine synthesis *in planta* was demonstrated by over-expression of active and an inactive SAT, which was still able to enter the CSC in the cytosol of transgenic tobacco plants (Wirtz and Hell 2007).

4.2 Catabolism, Storage and Transport of Cysteine

Breakdown of cysteine can be catalyzed by two classes of enzymes. Class 1 enzymes use the reduced sulfur in cysteine to incorporate it in other metabolites, while the enzymes of class 2 break down cysteine for catabolism or release the reduced sulfur of cysteine. Cysteine desulfurases of the NifS-type (EC 2.8.1.7), which provide elemental sulfur for molybdenum cofactor synthesis in the cytosol and iron-sulfur cluster formation in mitochondria and plastids belong to class 1. Typical class 2 enzymes are: cysteine desulfhydrases (L-CDes, EC 4.4.1.15), cystine desulfhydrase (cystine lyase, EC 4.4.1.13), which breakdown cysteine or cysteine to ammonia and pyruvate (Jones et al. 2003; Bloem et al. 2004), and β-cyanoalanine synthases (EC 4.4.1.9) that use cysteine for detoxification of cyanide (Meyers and Ahmad 1991). For an update of catabolic fate of cysteine and the biosynthesis of iron sulfur clusters the reader is referred to Papenbrock et al. (2007) and Balk and Lobreaux (2005).

Long distance allocation of cysteine is mainly accomplished by phloem-specific transport of GSH (Herschbach et al. 2000, Sect. 2). The efficiency of the transport allows restoring of GSH mediated tolerance against heavy metals in roots of plants with a genetically engineered shoot-specific synthesis of GSH (Li et al. 2006). GSH can also serve as a transient storage of cysteine, which can be efficiently remobilized by degradation of GSH (see Sect. 5). Nonetheless, the multiple functions of GSH make it an unfavorable storage compound. Long term storage of cysteine is therefore achieved in proteins like the 2S-albumins in seeds (see Fujiwara et al. 2002 for review).

4.3 Biosynthesis of Methionine

Research interest in the regulation of methionine biosynthesis is driven at least in part by its economic value. Especially in legumes seeds and potato tubers, methionine is low-abundant and limits nutritional quality for feedstuff (Müntz et al. 1998; Stiller et al. 2007). For that reason, biosynthesis of methionine was first analyzed in crops like maize, potato, soybean and wheat (Müntz et al. 1998; Wang et al. 2001; Zeh et al. 2001), although recent innovations were achieved in *Arabidopsis thaliana*. In contrast to cysteine, methionine levels respond only marginally to sulfur deprivation (Hirai et al. 2004), indicating that methionine synthesis is not primarily limited by the supply of reduced sulfur. Indeed, methionine formation seems to be regulated within the regulatory network of the aspartate-derived amino acids pathway (ADAAP). The complex network for biosynthesis of lysine, methionine, isoleucine and threonine from the common precursor aspartate is depicted in Fig. 3.

One cannot overestimate the importance of allosteric regulation of key enzymes by end products of each branch of the pathway for regulation of the entire network

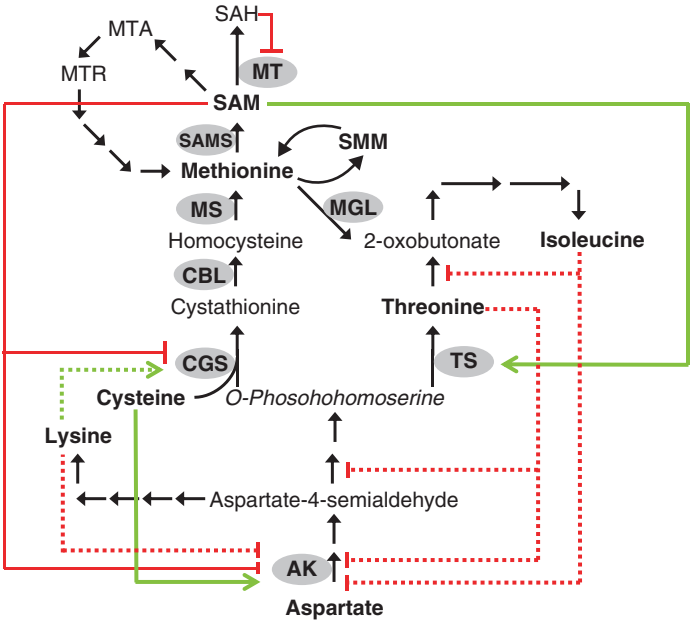

Fig. 3 *Regulatory model for the cysteine synthase complex*. Cellular sulfur availability adjusts the equilibrium of CSC, which regulates the activity of SAT and OAS-TL. Solid arrows indicate fluctuation of metabolites in response to sulfur availability, while dashed arrows represent positive (+) or negative (−) regulatory effects of these metabolites on CSC formation or gene expression. *Green circles*: active SAT, *red circles*: inactive SAT, *blue circles*: inactive OAS-TL, *black circles*: active OAS-TL

and the dependence of fluxes between the different branches. For example, the highest accumulation of methionine so far observed in plants (250-fold of wild type) is not achieved by over-expression of the key enzyme for the methionine branch of ADAAP (Cystathionine γ-synthase, CGS, EC 2.5.1.48), but by specific down-regulation of threonine synthase (TS, EC 4.2.3.1), which forces flux of aspartate into the methionine branch of the pathway (Bartlem et al. 2000; Zeh et al. 2001). TS is allosterically activated by S-adensoylmethionine (SAM) to direct flux to threonine and isoleucine formation, if methionine steady state levels are adequate (Curien et al. 1998; Laber et al. 1999; Mas-Droux et al. 2006a). Consequently, a forward genetic screen for methionine over-accumulating mutants (*mto*) using the resistance to ethionine identified *mto1-1* and *mto2-1*, which are affected in CGS and TS, respectively (Inaba et al. 1994; Bartlem et al. 2000). In summary, one can conclude that the competition of TS and CGS for their common substrate O-phosphohomoserine (OPH) is a strong determinant for methionine synthesis (Amir et al. 2002).

SAM controls not only TS activity but also methionine synthesis itself by (1) allosteric inhibition of aspartate kinase (AK, EC 2.7.2.4) and (2) down-regulation of CGS activity (Chiba et al. 1999; Chiba et al. 2003; Mas-Droux et al. 2006b; Curien et al. 2007). AK activates aspartate under consumption of ATP for further use in the ADAAP. One member of the AK protein family in Arabidopsis is strongly stimulated by presence of cysteine, which allows sufficient synthesis of OPH for methionine synthesis, if reduced sulfur is available (Curien et al. 2005). CGS catalyses the first committed step of methionine synthesis by formation of cystathionine from OPH and cysteine (Fig. 3). Cystathionine β-lyase (CBL, EC 4.4.1.8) subsequently cleaves cystathionine to release homocysteine, which is the substrate for methylation by methionine synthase (MS, EC 2.1.1.14). The activity of CGS is rate limiting and can be regulated by proteolytic removal of the N-terminal regulatory domain, which results in accumulation of methionine and SAM (Hacham et al. 2002; Hacham et al. 2006; Loizeau et al. 2007). In contrast to CGS and CBL, which are exclusively localized in plastids, MS is present in plastids and the cytosol (Fig. 2). Solely in the latter, S-adenosylmethionine synthase (SAMS, EC 2.5.1.6) combines methionine with ATP to produce SAM (Ravanel et al. 2004). Although SAM can be transported into plastids and mitochondria (Ravanel et al. 2004; Palmieri et al. 2006), it is the cytosolic SAM pool that regulates plastidic CGS activity by posttranscriptional destabilization of the CGS mRNA, at least in Arabidopsis (Chiba et al. 1999; Chiba et al. 2003). The latter observation points toward a complex regulatory circuit controlling methionine synthesis that includes communication between pool sizes of the key effectors, SAM and methionine, in the plastid and the cytosol (Fig. 1). Reduction of SAMS activity in the *mto3-1* and *mto3-2* mutants led to a 200-fold increase of methionine levels in Arabidopsis, which is probably caused by reduced incorporation of methionine in SAM and loss of feedback control via SAM (Goto et al. 2002; Shen et al. 2002). SAM levels can also be reduced by exogenous application of lysine, which down-regulates SAMS transcription and activity, allowing an

efficient communication between the methionine and lysine branch of ADAAP (Hacham et al. 2007).

4.4 Catabolism, Storage and Transport of Methionine

SAMS directs about 80% of the metabolic flux of methionine to SAM, which is used by SAM-dependent methyltransferases (MTs) to methylate nucleic acids, proteins and cell wall components like lipids, lignins and pectins (see Lu 2000 for review; Shen et al. 2002; Yang et al. 2006). SAM is also substrate for nicotianamine synthase (EC 2.5.1.43), SAM-decarboxylase (EC 4.1.1.50) and 1-aminocyclopropane-1-carboxylate synthase (EC 4.4.1.14) to produce nicotianamine, polyamines and ethylene, respectively (Hesse et al. 2004). These results demonstrate that SAM is (1) the second most frequently used cofactor in nature, after ATP (Cantoni 1975; Lu 2000) and (2) mandatory for proper development of plants by influencing metal homeostasis and hormone function (Burstenbinder et al. 2007; Klatte et al. 2009). Interestingly, SAMS1 activity can be efficiently inhibited by S-nitrosylation by the natural NO donor S-nitrosoglutathione, which may mediate cross-talk between NO and ethylene signaling pathways in plants (Lindermayr et al. 2005)

Two cytosolic cycles regenerate methionine and SAM. First, the transfer of the methyl group from SAM generates S-adenosylhomocysteine (SAH), a potent inhibitor of MTs. To mitigate the toxic effects of SAH on MT activity and to recycle methionine, SAH is removed by SAH hydrolase (EC 3.3.1.1) in a reaction generating adenosine and homocysteine (Hesse et al. 2004). The latter can be used as building block for synthesis of methionine by MS as described above. Second, recycling of methionine at high rates of ethylene production is achieved by the Yang- or Met-cycle (Adams and Yang 1977, Fig. 2), which converts methylthioadenosine, the byproduct of SAM dependent ethylene formation in four steps to methionine. Recently, analysis of methylthioribose kinase (EC 2.7.1.100) revealed the significance of methionine recycling under sulfur limiting conditions in Arabidopsis (Burstenbinder et al. 2007).

Besides consumption of methionine in form of SAM, methionine can be catabolized in the cytosol by the activity of methionine γ-lyase (MGL, EC 4.4.1.11), which produces ammonia, 2-oxobutanoate and methanthiol (Rebeille et al. 2006). Methanethiol can be incorporated into cysteine, while 2-oxobutanoate can serve as a precursor for isoleucine synthesis (Rebeille et al. 2006; Goyer et al. 2007). Under normal growth condition a knockout of methionine γ-lyase activity in Arabidopsis results in no visible phenotype, but a tenfold accumulation of methionine under sulfate-limiting conditions (Goyer et al. 2007).

Long distance transport of methionine is achieved via the phloem after conversion of methionine to S-methylmethionine (SMM, see Sect. 2.2), which is also assumed to function as temporary storage of methionine in leaves (Bourgis et al. 1999). Methionine methyltransferase (EC 2.1.1.12) uses SAM as a methyl donor to

form SMM from methionine and is unique in plants although it is nonessential (Bourgis et al. 1999; Ranocha et al. 2000; Kocsis et al. 2003).

5 Roles of GSH in Redox Homeostasis and Detoxification

5.1 GSH Biosynthesis and Functions

The tripeptide GSH (γ-glutamylcysteinylglycine) is ubiquitous in cells and organisms, among the few exceptions are trypanosomes that contain GSH-derived compounds (i.e., trypanothione). In plants, members of the Poaceae and Fabaceae carry GSH variants with C-terminal amino acids other than glycine (references in Meyer and Hell 2005). In all cases GSH biosynthesis is a two step, ATP-dependent process that is catalyzed by γ-glutamylcysteine ligase (GSH1) and GSH synthetase (GSH2). After the evolutionary invention of photosynthesis about 2.8 million years ago, cells encountered the problem of an increasingly oxidizing environment. The rapid oxidation of cysteine required the development of a reduced internal redox state. Indeed comparison of protein composition derived from sequenced genomes shows that today cysteine is one of the most rarely used amino acids (Pe'er et al. 2004). For maintenance of these thiol groups and cellular redox state, GSH may have evolved. The presence of GSH opened the additional possibility to develop redox-based sensing mechanisms that mediate between cell and environment. An emerging part of this role is the formation of mixed disulfides between GSH and thiols of cysteine residues in proteins. This S-glutathionylation or thiolation has originally been seen as reversible protection against the irreversible oxidation of protein thiols to sulfinic or sulfonic acids or reaction with NO to S-nitrosylated proteins, but is now considered as posttranslational modification for redox-driven signal transduction (Rinalducci et al. 2008). GSH itself freely reacts with NO to form S-nitrosoglutathione that is able to modify protein thiol groups by both protein S-nitrosylation and S-glutathionylation. The reader is referred to specialized reviews on this aspect (Foyer and Noctor 2005; Meyer and Hell 2005; Meyer 2008; Rouhier et al. 2008).

In addition, GSH acquired numerous other functions that are often specific for organism types. In plants, GSH has been suggested as a long-distance transport form of reduced sulfur, as an intermediate storage for reduced sulfur and scavenger of xenobiotics and reactive oxygen species (Fig. 1, May et al. 1998). The superfamily of glutathione-*S*-transferases (GSTs) with multiple activities toward S–C conjugation, peroxidation and secondary metabolism transport is indicative of these additional roles (Dixon et al. 2009). Among those, detoxification of xenobiotica by is one of the most widespread tasks of GSTs in eukaryotic organisms. Detoxification phase I is catalyzed by P450 enzymes, followed by phase II GSTs that form glutathione-S-conjugates to xenobiotics for either removal from potential

susceptible target sites of intoxication or metabolic degradation or both. Reactive oxygen species are detoxified either by direct thiol oxidation or within the ascorbate-GSH cycle (Foyer et al. 2009b). GSH also serves as substrate for the synthesis of phytochelatins, γ-glutamylcysteine polymers with a terminal glycine residue, with high affinity to heavy metals such as cadmium via metal-sulfide chelation (Grill et al. 1985). They had long been suspected as principal heavy metal detoxification system in plants but more recently are believed to contribute homeostasis of metal micronutrients such as zinc (Tennstedt et al. 2009).

GSH is essential for survival of plants (Cairns et al. 2006; Pasternak et al. 2008). It is present in major plant cell compartments except the vacuole. However, its biosynthesis takes place only in plastids and the cytosol. Using aqueous biochemical fractionation of pea and spinach leaves GSH1 72% and 61%, respectively, of activity was found in chloroplasts and the rest in the cytosol but not mitochondria (Hell and Bergmann 1990). GSH2 activity of 24% was demonstrated in chloroplasts of photoheterotrophic tobacco cells (Hell and Bergmann 1988) and, after nonaqueous fractionation, between 47% and 64% in chloroplasts of pea leaves (Klapheck et al. 1987). In contrast, *Arabidopsis thaliana* was reported to have single genes encoding each of the enzymes with GSH1 only present in plastids and GSH2, via differential splicing, in both compartments (Fig. 1). From immunolocalization an estimated less than 10% of GSH2 protein was present in plastids and more than 90% in the cytosol (Wachter et al., 2005). In Brassicaceae and several other taxa all *GSH1* genes so far have been found to encode proteins with predicted plastid transit peptide (Gromes et al. 2008). In view of these apparent discrepancies major taxon-specific differences cannot be excluded.

In Arabidopsis this distribution implies transport of γ-EC and/or GSH across the plastid envelope. Pasternak et al. (2008) used a T-DNA null mutant of GSH2 to show that plants with only γ-EC are seedling lethal. Since *gsh1* mutant Arabidopsis plants are lethal at the embryo stage, this suggests that γ-EC compensates to a very limited extent for GSH in early developmental stages. Complementation of *gsh2* mutant plants with cytosol-specific GSH2 produced phenotypic wild type-like plants. This result implies that, due to the exclusive localization of GSH1 in plastids, γ-EC can leave plastids so as to supply the cytosol with the precursor of GSH synthesis and that GSH itself can be imported into plastids. These and other data also show that feedback inhibition of GSH1 is an important regulatory mechanism for GSH synthesis (Cairns et al. 2006; Pasternak et al. 2008).

Maintenance of redox homeostasis of GSH requires continuous re-reduction of oxidized GSH (GSSG) during the detoxification of reactive oxygen species by the ascorbic acid-GSH cycle (Foyer et al. 2009a). In Arabidopsis this is achieved by two genes encoding NADPH-dependent GSH reductases (GR). GR1 is localized in the cytosol and GR2 has a bipartite transit peptide for plastid and mitochondrial localization (Chew et al. 2003). Under nonstress conditions, GR keeps GSH mainly in the reduced form with only nanomolar concentrations present as GSSG. These observations were decisively promoted by the application of an engineered reduction-oxidation sensitive green fluorescent protein (roGFP) to plants (Meyer et al. 2007). roGFP also revealed that loss of cytosolic GR1 in an Arabidopsis *gr1*

deficient mutant is partly compensated by a cytosolic backup system. NADPH-dependent thioredoxin reductase constitutes an efficient electron transfer from NADPH via cytosolic thioredoxin to GSSG to rescue *gr1* mutants at least under nonstressed conditions (Marty et al. 2009).

Since mitochondria harbor no GSH biosynthetic enzymes they must be able to import GSH (Fig. 1). Analysis of a null mutant of the ATP binding cassette (ABC) type transporter Atm1 in yeast and Atm3 (=Sta1) in Arabidopsis showed that this membrane protein contributes to maturation of iron-sulfur clusters in the cytosol (Kushnir et al. 2001). It was suggested that GSH acts as a carrier for precursors of such clusters (Balk and Lobreaux 2005). The endoplasmatic reticulum and peroxisomes have been assumed to contain GSH. In these compartments, the organelles and other possible cellular locations GSH in its reduced and/or oxidized form must be transported by a so far unknown mechanism. Membrane protein families capable of transport of GSH or GSH conjugates with rather broad substrate range have been identified by complementation of a yeast mutant deficient in plasmalemma GSH transport (Cagnac et al. 2004; Zhang et al. 2004). Whether these proteins are true GSH transporters and whether some family members mediate also intracellular glutathione transport, remains to be investigated. Clear evidence for low specificity transport of GSH and other conjugates comes from members of the superfamily of multidrug resistance-associated proteins. Several ABC transporters of this group localize to the tonoplast and are active in conjugate transport (Rea 2007).

5.2 GSH Degradation and Detoxification of Xenobiotics

Degradation of GSH in animals is part of the γ-glutamyl cycle where extracytoplasmic GSH is cleaved into its constituent amino acids that are absorbed by plasmalemma transporters followed by re-synthesis of GSH inside the cell (Meister 1995). GSH is degraded by the sequential reaction of γ-glutamyl transpeptidase, (GGT), γ-glutamyl cyclotransferase, and 5-oxoprolinase to yield glutamate and cysteinylglycine that is cleaved by peptidase. These enzyme activities are also found in plants, but studies focused on GGT protein family in Arabidopsis revealed no conclusive evidence for a plant γ-glutamyl cycle (Grzam et al. 2007; Martin et al. 2007; Ohkama-Ohtsu et al. 2007b; Ohkama-Ohtsu et al. 2007a). Three of the four *GGT* genes in Arabidopsis encode functional GGT proteins while *GGT3* apparently is a pseudogene. GGT1 and GGT2 are plasmalemma-associated and most likely exposed to the apoplast while GGT4 is tonoplast-associated and exposed to the vacuole. GGT1 is involved in the oxidative stress response by degradation of extracellular GSSG while GGT2 may contribute to GSH transport into siliques (Ohkama-Ohtsu et al. 2007b). GGT4 cleaves GSH conjugates that have been produced by GSTs in the cytosol and rapidly imported into the vacuole by ABC type transporters (Rea 2007). In vivo labeling of GSH with a fluorescent dye imitates the xenobiotics conjugation response of phase II detoxification and

allows life cell imaging of conjugate transport and breakdown (Meyer and Fricker 2002; Meyer and Rausch 2008). This approach showed that Arabidopsis mutants of GGT4 were unable to initiate breakdown and accumulated GSH conjugates in the vacuole (Grzam et al. 2006; Grzam et al. 2007; Ohkama-Ohtsu et al. 2007a). These observations are supported by localization and feeding experiments with barley roots. (Ferretti et al. 2009) concluded from their results that apolastic GGT activity is a component of the system for retrieving exogenous GSH, possibly in connection with the oxidative state of the apoplast and as part of a plant γ-glutamylcycle between apoplast and cytoplasm.

Similar reactions that recognize γ-glutamylpeptides can be carried out by other enzymes in the plant cytosol. Glucosinolate engineering recently identified glutathione as a donor for reduced sulfur in biosynthesis of benzyl glucosinolates and a γ-glutamyl intermediate as product of a γ-glutamyl peptidase with a glutamine amidotransferase domain (Fig. 1, Geu-Flores et al. 2009). Phytochelatin synthase, a metal cofactor-dependent cytosolic enzyme that synthesizes γ-glutamylcysteinyl-polymers from GSH (phytochelatins) is capable of hydrolysis of the C-terminal glycine residue of GSH conjugates as a first step of conjugate degradation (Blum et al. 2007). The in vivo function of this reaction is not fully understood since the cytosol cannot further metabolize the resulting γ-glutamtylcysteine conjugates and in comparison the GSH conjugates are very efficiently imported in the vacuole by ABC transporters in the first place and fully degraded (Grzam et al. 2006; Grzam et al. 2007).

New support for operation of a γ-glutamylcycle in plants comes from the analysis of Arabidopsis T-DNA double mutants which are deficient in both genes encoding cytosolic 5-oxoprolinase, but in a GGT independent way (Ohkama-Ohtsu et al. 2008). Such plants accumulate 5-oxoprolin from GSH and have less glutamate compared to the wild-type, suggesting a rate-limiting role of 5-oxoprolinase for cytosolic GSH degradation. From metabolite data obtained for a *ggt1*/*ggt4*/*oxp1* triple mutant without detectable GGT activity in leaves it was suggested that GGTs have no major role in GSH degradation, but that γ-glutamycyclotransferase in the cytosol is the major source of 5-oxoproline formation from GSH (Ohkama-Ohtsu et al. 2008). The molecular identification of γ-glutamycyclotransferase is thus the missing link for a complete γ-glutamylcycle in plants. With respect to turnover, plasmalemma GGTs seem to contribute mostly to GSH degradation in the apoplast and vacuolar GGT4 serves in conjugate metabolization but not degradation of free GSH.

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