Thiolomics: Molecular Mechanisms of Thiol-Cascade in Plant Growth and Nutrition

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Abstract

Growing plants have a constitutive demand for thiol (sulfur) to synthesize protein, sulfolipid, and other essential sulfur (S)-containing molecules for growth. The uptake and subsequent distribution of sulfate is regulated in response to demand and environmental factors. Sulfate transport consists of both constitutive and sulfur nutrition-dependent regulated transport. The acquisition of sulfur by plants has become an increasingly important concern

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for the agriculture due to the decreasing trends of S-emissions from industrial sources and the consequent limitation of inputs from deposition. The recognition of the importance of sulfate for plant growth and vigor and hence crop yield, as well as the nutritional importance of sulfur for human and animal diets, has increasingly been recognized. Cysteine synthesis in plants is a fundamental process for protein biosynthesis and all anabolic pathways that require reduced sulfur. Cysteine is the first committed molecule in plant metabolism that contains both sulfur and nitrogen, and, thus, the regulation of its biosynthesis is of utmost importance for the synthesis of a number of essential metabolites in plant pathways. Cysteine is incorporated into proteins and glutathione directly or serves as a sulfur donor for the synthesis of S-containing compounds such as methionine and its derivatives *S*-adenosylmethionine and *S*-methylmethionine and many secondary compounds. Furthermore, cysteine acts as a general catalyst in redox reactions through the nucleophilic properties of its sulfur atom, utilizing dithiol–disulfide interchange, as displayed in the thioredoxin and the glutaredoxin systems. Molecular characterization involving transcriptomics, proteomics, and metabolomics profiling in major crops like rice, barley, wheat, maize, and legumes along with model plant *Arabidopsis thaliana* revealed that sulfate uptake, distribution, and reductive assimilation are regulated in fine-tune depending on sulfur status and demand and that this cascade is integrated with plant photosynthesis, nutrient transports, antioxidant defense system, hormonal signaling, kinase cascades, carbohydrate metabolism, and during plants' experiences with different biotic and abiotic stresses. This cascade can be manipulated in favor of enhanced plant growth and nutritional benefits—as, for example, effort has been initiated in food and feed legumes (chickpeas, narrow-leafed lupin, soybeans) and other plants with enhanced S-containing amino acids, threonine, glutathione, protein quality, protease inhibitors, and trace elements and with lysine, protein content, and compositions in cereal grains. This emerging prospect can be ushered by using latest cutting-edge functional genomics tools and better understanding of plant thiol-metabolism from source (soil) to sink (grains) in diverse arenas of "thiolomics." In this chapter, the comprehensive knowledge generated in this area has been compiled and analyzed.

Keywords
Antioxidant defense • Cross talk • Functional genomics • Glutathione • Hormone signaling • Nutrition • Plant stress • Regulations • Sulfur metabolisms

Introduction

Sulfur (S) is a critical nutrient for metabolism, plant growth, and development. It represents the ninth and least abundant essential macronutrient in plants (Höfgen and Hesse 2008). The importance of S as a plant nutrient has been recognized since time immemorial, but active research started in the second half of the twentieth century when widespread S deficiencies were observed. S plays an inevitable and imperative role in the formation of amino acids, methionine (Met; 21 %) and cysteine (Cys; 27 %), and synthesis of protein, chlorophyll, and oil in the oilseed crops. Numerous other plant metabolites are also formed from S. Plant S nutrition not only affects crop yield but also quality (Tabe et al. 2003; Chiaiese et al. 2004; Taylor et al. 2008). Therefore, increasing the S utilization efficiency (SUE) of plants is becoming an important issue. SUE was described as "improved capture of resources, the accumulation of greater reserves of S, and improved mechanisms for the remobilization of these reserves" (Hawkesford 2000). The importance of SUE has nicely been demonstrated in two gain-of-function *Arabidopsis* mutants, *sue3* and *sue4*, exhibiting low S tolerance equipped with well-developed root systems, tolerance to heavy metals, and tolerance to oxidative stress (Wu et al. 2010). Cysteine (Cys) is the first stable and committed molecule in plant metabolism that contains both S and nitrogen (N). It is the metabolic precursor for vital cellular

components containing reduced S, including glutathione (GSH), homoglutathione, iron–sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites. GSH is the most abundant low-molecular-weight thiol-buffer with a plethora of functions in plant stress defense, hormone signaling, redox regulation, sexual plant reproduction, and S homeostasis (Noctor et al. 2012). Thus, the regulation of S biosynthesis is of utmost importance for the synthesis of a number of essential metabolites in plant pathways.

A fair part of S incorporated into organic molecules in plants is located in thiol (–SH) groups in proteins (Cys residues) or nonprotein thiols (GSH). The thiol group of Cys in proteins maintains protein structure by forming disulfide bonds between two Cys residues via oxidation. The thiol of Cys and GSH is often involved in the redox cycle by two thiol–disulfide conversions. This interchange is versatile for redox control and mitigation against oxidative stress in nearly all aerobic organisms including plants (Leustek et al. 2000). Sulfate uptake and assimilation share approximately equal control over cellular thiol flux (Vauclare et al. 2002).

Studies in the model plant *Arabidopsis thaliana* (thale cress) provide significant insights on the molecular processes and regulation of thiol-metabolism (Leustek et al. 2000; Saito 2000; Kopriva 2006; Meyer and Rausch 2008; Höfgen and Hesse 2008; Kopriva et al. 2009; Yi et al. 2010). However, the depth of understanding these same pathways in various crop plants is somewhat limited. In general, acquisition of S from soil, its transport, reductive assimilation, formation of downstream metabolites via Cys, and highly versatile functions of different thiolic compounds in diverse events of plant growth and development encompass huge interactive and integrated multilevel networks of thiol-cascade which starts from upstream events of S-metabolisms to produce downstream thiol-metabolites GSH and GSH-mediated entire antioxidant defense within ascorbate (AsA)–GSH cycle and outside it. With the miraculous progress of different "omics" technologies, the biology of plant thiol-metabolism has entered into a new era, reflecting successful utilization of functional genomics in dissecting molecular mechanisms of thiol-cascade in plant growth and nutritional quality through understanding of a new concept, "thiolomics." Transcriptome, proteome, and metabolome analysis of any organism reflects the total biological activities at any given time which are responsible for the adaptation of the organism to the surrounding environmental conditions. *Arabidopsis* is the plant model of choice for global analysis of transcriptome, proteome, and metabolome. S deficiency, the major physiological problem connected with S-metabolism, is being investigated by expression profiling (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003) and combined transcriptome and metabolome analysis (Hirai et al. 2005; Nikiforova et al. 2005a). More than 2,700 genes were found to be affected by S starvation. The genes induced by S deficiency included those coding for sulfate transporters, reduction, and assimilation to downstream thiol-metabolites. Metabolome analysis revealed that from approximately 6,000 analyzed metabolites, 11.5 % were significantly affected by 13 days of S starvation (Nikiforova et al. 2005a, b). The power of the global study of metabolite and transcript networks was demonstrated by Hirai et al. (2005), who used a batch-learning self-organizing mapping analysis to reveal clusters of genes and metabolites regulated by the same mechanism. Altogether, transcriptome, proteome, and metabolome analyses revealed the complexity of the interactions between S, N, and C metabolism and created new domains of the molecular mechanisms of thiol-metabolisms, and the discovery of 49 transcription factor genes responding specifically to S deficiency is of greatest importance in this regard (Nikiforova et al. 2003).

Sulfate Uptake and Transport: Dissecting Fundamental Steps with "Omics" Technologies

The major S form available to plants is sulfate. Uptake of sulfate by plants is considered to be the fundamental step of the S cycle in the nature.

Sulfate is taken up to plant cells by sulfate transporters (Sultr). Recently, the first transcription factors responsible for regulation of sulfate uptake and assimilation have been identified (Maruyama-Nakashita et al. 2006; Hirai et al. 2007). Once sulfate is taken up from the soil solution by dedicated root membrane transport systems, it is either transitorily accumulated in the vacuoles of roots or shoots, or it enters the complex sulfate reductive metabolic pathway (Kopriva 2006). In between the step of sulfate uptake into root cells and its reduction in the far-away leaf chloroplasts, several cell-to-cell inter- and intracellular transports through numerous transmembrane and plasmodesmata exist. Coordination of such short- and long-distance sulfate transport requires the timely regulations of gene expressions encoding proteins involved in sulfate uptake and transport. In order to understand how components of the network interact, it is necessary to analyze the temporal and spatial transcription behavior of all (or most) of the genes in the genome (the transcriptome) simultaneously (Kopriva 2006).

Nascent seedlings and generative tissues have a constitutive demand for S. The uptake and subsequent distribution of sulfate is regulated in response to demand and environmental factors. Many studies have shown that the changes in the capacity of plant S transport were paralleled by changes in the steady-state contents of mRNAs and protein of the group 1 sulfate transporters (Smith et al. 1997; Takahashi et al. 2000; Hawkesford and Wray 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002, 2003; Howarth et al. 2003). The derepression/repression of gene expression seems to be a major factor in the regulation of sulfate uptake in plants. Differential expression of transcripts for S-metabolic enzymes was systematically validated by reverse transcription quantitative PCR in different plants. Since the first reported cloning of a plant sulfate transporter in *Stylosanthes hamata* (Smith et al. 1997), it has gradually become clear that sulfate transport in plants is carried out by a complex system of transporters governed by multigene family. In recent years, many genes controlling expressions of sulfate transporters from diverse plant taxa have been isolated and characterized (reviewed Buchner et al. 2004a). Sulfate transport consists of both constitutive and S nutrition-dependent regulated transport. A decreased intracellular content of sulfate, Cys, and GSH is concomitant with increasing transporter activity (Smith et al. 1997). Gene and protein expression studies have confirmed that regulation occurs predominantly at the level of the mRNA (Smith et al. 1997; Takahashi et al. 2000; Hawkesford and Wray 2000; Yoshimoto et al. 2002; Hawkesford 2003; Kopriva 2006; Takahashi et al. 2011; Talukdar and Talukdar 2013d). Transcriptomic analysis of the sulfate transporter gene family reflects a complex pattern of regulation: (1) cell-specific expression of some groups 1, 2, and 4 transporters under adequate S nutrition which is upregulated by inadequate S nutrition (AtSultr1;2, 1;3, 2;1, 4;1); (2) expression of the group 3 transporters with tissue/organ specificity but no regulation by S nutrition; and (3) cell-/tissue-specific S deficiency-related derepression of some group 1 and 2 transporters (AtSultr1;1, 1;2, 2;1, 2;2) (Buchner et al. 2004a). The process of so-called long-distance sulfate translocation may require several types of transporters responsible for cell-to-cell movement of sulfate across the plasma membrane. Loading of sulfate into the vascular tissues in roots and unloading of sulfate into the leaf cells are assumed to be the two important steps in this process. These two events are controlled by the same sulfate transporter gene, *AST68*, in *A. thaliana* (Takahashi et al. 1997). Using an array hybridization/transcript profiling method in *Arabidopsis* plants subjected to 6, 10, and 13 days of constitutive and induced S starvation, Nikiforova et al. (2003) revealed induction of sulfate transporter *AST68* (Sultr2;1) and Sultr4;1. Analysis of the *Arabidopsis* and rice genome sequences (The Arabidopsis Genome Initiative 2000) has till date enabled the identification of 14 putative sulfate transporter genes in each genome (Vidmar et al. 2000; Buchner et al. 2004a). Alignment and phylogenetic analysis of the 14 *Arabidopsis* and rice proteins subdivides the plant sulfate transporter family into four closely related groups, all with 12 membrane-spanning domains and a STAS

(sulfate transporter and anti-sigma antagonist) domain at their carboxy-terminus (Aravind and Koonin 2000), and a fifth more diverse, but clearly related, group with two smaller proteins lacking the STAS domain (Hawkesford 2003). However, nothing is known about plastidic sulfate transporters in higher plants. The sulfate transport in plastids is necessary for the synthesis of many sulfur-containing compounds. For example, in *Spinacia oleracea*, the lack of sulfates leads to considerable changes in the expression of Cys synthesis genes (Lyubetsky et al. 2013). Plastomes of vascular plants lack genes of the sulfate transport system except for rare instances of cyst and cysA. However, the green alga *Helicosporidium* sp. retains cysT. Plastomes of the rhodophyte *Cyanidium caldarium* and *Cyanidioschyzon merolae* and the cyanelle genome of *Cyanophora paradoxa* lack cysT homologues but possess distant homologues of cysA presumably involved in the transport of zinc or manganese (Lyubetsky et al. 2013). In some liverworts, the plastid-encoded sulfate transporter gene *cys*A has been lost up to 29 times, yet intact copies of *cys*A are evolving under selective constraints. Gene loss is more frequent in groups with an increased substitution rate in the plastid genome of liverworts (Wickett et al. 2011). None of the 14 *Arabidopsis* genes encoding putative sulfate transporters seems to be involved in such a crucial function. Plastidic sulfate transporters are, however, identified in *Chlamydomonas reinhardtii* and shown to belong to the bacterial ABC type of transporter (Lyubetsky et al. 2013). Thus, identification of plant plastidic sulfate transporter is undoubtedly one of the greatest challenges in S research (Davidian and Kopriva 2010).

Group 1 and 2 Sulfate Transporters

Group 1 and 2 sulfate transporters, which are localized at the plasma membrane, have been the subject of several studies and are the best characterized groups. Members of group 1 represent high-affinity transporters that facilitate uptake of sulfate by the root (Sultr1;1 and Sultr1;2) or translocation of sulfate from source-to-sink organs (Sultr1;3) (Takahashi et al. 2000, 2011; Shibagaki et al. 2002; Hawkesford 2003; Kopriva 2006; Talukdar and Talukdar 2013d). In wheat, the high-affinity sulfate transporter homologue to the AtSultr1;1 was detected in aleurone cells of wheat grains by immunolocalization (Kopriva 2006). Group 2 is composed of low-affinity sulfate transporters whose gene products may rather play a role in vascular tissues, facilitating the translocation of sulfate around the plant. Differences in the kinetic and expression pattern of AtSultr2;1 (Km 0.41 mM) and AtSultr2;2 (Km 1.2 mM) indicate specific functions in the process of vascular movement of sulfate. AtSultr2;1 is expressed in the xylem parenchyma and phloem cells of leaves, but in the root in xylem parenchyma and pericycle cells. By contrast, AtSultr2;2 is localized specifically in the phloem of roots and in vascular bundle sheath cells of leaves (Takahashi et al. 2000; Maruyama-Nakashita et al. 2003). The observed upregulation of AtSultr2;1 in roots during sulfate starvation, and the increase of the mRNA transcripts level of AtSultr2;1 under selenate treatment, may be an indication of this function (Takahashi et al. 2000; Maruyama-Nakashita et al. 2003). The leaf phloem expression suggests a role in phloem loading for sulfate transport to other organs. The leaf xylem parenchyma localization of AtSultr2;1 might indicate absorption of sulfate from the xylem vessels or reabsorption for further xylem transport. In leaves, however, the expression in the bundle sheath cells surrounding the vascular veins suggests the uptake of sulfate released from xylem vessels at millimolar concentrations for transfer to the primary sites of assimilation in leaf palisade and mesophyll cells. The expression pattern of both Group 2 transporters suggests that the two transporters are involved in balancing the vascular movement of sulfate in relation to the sulfate status of the different tissues (Takahashi et al. 2000; Maruyama-Nakashita et al. 2003). In addition to such transcriptional regulation, both AtSultr 1;1 and AtSultr 1;2, when expressed under control of constitutive 35S promoter, accumulated exclusively in the root and responded to sulfate deficiency in a similar manner to native

transporters (Yoshimoto et al. 2007), indicating posttranscriptional control of gene expressions. Possibly, STAS domain is involved in such regulation, as mutations in this domain interfere with correct targeting of sulfate transporters to the plasma membrane (Shibagaki and Grossman 2006). Likewise, protein–protein interactions between two transporters such as between AtSultr 2;1 and AtSultr 3;5 in *Arabidopsis* are important for optimal sulfate transport capacity (Kataoka et al. 2004). In addition to these regulations, plant microRNAs (miRNA) have recently been implicated in regulation of sulfate transport. For example, AtSultr 2;1 expression was downregulated in shoots in response to sulfate starvation and upregulated in the absence of S, which can be explained by an increase in the miRNA in both cases revealing differential roles of miRNAs in different root tissue territories (Takahashi et al. 2000; Jones-Rhoades and Bartel 2004; Kawashima et al. 2009; Davidian and Kopriva 2010). In *Arabidopsis*, a new complex regulatory interplay between Sultr1;1 and Sultr1;2 based upon an extensive comparison of a wide set of growth conditions and metabolite signaling pathways has been proposed (Rouached et al. 2009). Differential expression and alternative splicing of rice sulfate transporter family members regulate S status during plant growth, development, and stress conditions (Kumar et al. 2011a, b).

Group 3 and 4 Sulfate Transporters

Group 3 is composed of low-affinity transporters localized at the plasma membrane. Unlike groups 1 to 3, group 4 sulfate transporters have been localized to the vacuolar membrane/tonoplast. The Sultr4;1 gene is shown to be expressed in roots under S-sufficient and S-deficient conditions, where it may play a role in the efflux of sulfate from the vacuolar lumen into the cytoplasm and influence the vacuolar storage capacity for sulfate (Kataoka et al. 2004). Contrastingly, Sultr4;2 gene expression is shown to be highly inducible by S limitation in the same tissue. The Sultr4;1/Sultr4;2 double knockout mutants contained higher amounts of sulfate than did wild-type plants. Comparison of single and Sultr4;1/Sultr4;2 double knockout mutants suggested that Sultr4;1 plays a major role and Sultr4;2 has a supplementary function (Kataoka et al. 2004). In addition to the verification of the subcellular localization of this group 4 sulfate transporters in *Arabidopsis*, analysis of T-DNA mutations showed an increased accumulation of sulfate and decrease of Cys and GSH contents when plants were grown on low sulfate (Takahashi et al. 2003). The drastic reduction of root sulfate concentrations under sulfate deficiency is accompanied by an upregulation of sulfate transporter 4;1 in *Brassica* (Hawkesford 2003). Increased expression of this transporter maximizes the vacuolar efflux of stored sulfate under these conditions. Analysis of qRT-PCR expression profiles reveals that *Arabidopsis* Sultr4;1 gene is strongly expressed (10-fold higher than the Sultr4;2 gene) in developing seeds and that its disruption significantly increases seed sulfate content, suggesting that Sultr 4;1 is involved in the efflux of sulfate from vacuoles within developing seeds. Furthermore, a proteome analysis of Sultr4;1 mutant seeds reveals metabolic modulations suggesting adaptations to altered sulfate compartmentation. This event implicates Sultr4;1-mediated sulfate transport in establishment of defense mechanisms against oxidative stress during seed development (Zuber et al. 2010). This study also pointed out that Sultr4;1 is highly expressed during grain filling stage, whereas Sultr4;2 is expressed at almost constitutively from embryogenesis to the dry mature stage. Also, the qRT-PCR data revealed that the Sultr4;1 gene was more highly expressed than Sultr4;2 in most plant organs. Interestingly, expression studies of both Sultr 4 transporters in oilseed rape (*Brassica napus*) leaves revealed a differential S-dependent expression pattern: BnSultr 4;2 was more highly expressed than BnSultr 4;1 in response to sulfate depletion (Parmar et al. 2007; Dubousset et al. 2009). In the same sulfur-depletion conditions, rapeseed leaf sulfate content decreased significantly in relation to upregulation of BnSultr 4;1, confirming the involvement of both Sultr4 members in vacuolar sulfate remobilization in *Brassica* (Dubousset

et al. 2009). A transcriptional regulator, Sulfur LIMitation1 (SLIM1), regulating sulfate uptake and assimilation has been shown to induce AtSultr1;1, AtSultr1;2, and AtSultr 4;2 gene expression in response to sulfate starvation (Maruyama-Nakashita et al. 2006). Clearly, cytosolic and plastidic sulfate homeostasis is important to avoid toxification when excess sulfate is accumulated in the vacuole.

Group 5 Sulfate Transporter

Among the fifth group, the *Arabidopsis Sultr5;2* gene has recently been demonstrated to encode a high-affinity root molybdate (Mo) transporter, MOT1 (Tomatsu et al. 2007), which raises the question of the role of group 5 genes in sulfate transport. However, cotransport of Mo through plant sulfate transporter SHST1 has been reported (Fitzpatrick et al. 2008). Indeed, expression of the sulfate transporter SHST1 from *Stylosanthes hamata* in a *Saccharomyces cerevisiae* mutant defective in sulfate transport, YSD1, increased its capacity to take up Mo when grown in the presence of low Mo concentrations (Fitzpatrick et al. 2008). While sulfate did not inhibit the transport of Mo through this transporter, Mo reduced sulfate transport via SHST1 (Fitzpatrick et al. 2008). However, a complex interaction between S, Mo, and selenium in S-deficient and S-sufficient plant has recently been studied in Indian mustard, *Brassica juncea* (Schiavon et al. 2012).

Induction, Regulations, and Tissue Distributions of Sulfate Transporters

The two distinct group 1 sulfate transporters are different in their inducibilities in relation to the nutritional status of the plant. One transporter (AtSultr1;2, LeSultr1;1) mediates the uptake of sulfate under both S-replete and S-deficient conditions, and expression is relatively insensitive to external sulfate concentrations. The second transporter (AtSultr1;1, LeSultr1;2) is highly inducible under sulfate limitation but almost absent in non-S-stressed plants (Yoshimoto et al. 2002, 2003; Hawkesford 2003; Howarth et al. 2003). The higher inducibility of AtSultr1;1 compared to AtSultr1;2 can be explained by the absence of the SURE (sulfur-responsive element) element in the promoter of AtSultr1;2. This suggests that the upregulation of the root major transporter AtSultr1;2 is probably essentially controlled by SLIM1 when sulfate availability in the external medium is restricted but that an additional regulatory mechanism dependent on the SURE element drives a strong expression of AtSultr1;1 during S-deficient conditions. This dual inducible uptake system was confirmed by the identification of selenate-resistant (sel) mutants of *Arabidopsis* (Shibagaki et al. 2002), exhibiting that a lesion in the AtSultr1;2 sulfate transporter isoform restricted the uptake of both sulfate and its toxic analog, selenate. Analysis of another mutation of AtSultr1;2, (*sel1-10*) indicated that AtSultr1;2 serves as a major facilitator for the acquisition of sulfate, and despite upregulation of AtSultr1;1 expression in the *sel1-10* mutant, growth is reduced (Maruyama-Nakashita et al. 2003). Besides the use of *sel* mutant, the power of genetic approach for dissecting unexplored events in thiol-metabolisms has also been reflected by green fluorescent protein (GFP). GFP expression from the well-characterized bSR promoter fragment from conglycinin (Awazuhara et al. 2002) was used as a tool in the search for mutants with altered S deficiency response. Seeds from plants harvesting the bSR::GFP construct were mutagenized and mutants were selected with increased GFP expression at normal S supply (Ohkama-Ohtsu et al. 2004). In one of these mutants, the level of OAS was increased and, in addition to GFP expression, the mRNA levels of several other genes responsive to sulfate starvation were increased even at normal S concentration. Map-based cloning and sequence analysis identified a thiol reductase to be responsible for the elevated OAS levels (Ohkama-Ohtsu et al. 2004).

Expression pattern of sulfate transporters greatly differs in different plant species and during different growth stages and tissues of the same plant. Spatial expression analysis of AtSultr1;3 in *Arabidopsis*, and of group 1 high-

affinity sulfate transporters in other plant species, indicated that sulfate transport in vascular tissues is not restricted to low-affinity transport. In sulfate-deficient barley roots, HvSultr1;1 was expressed within the stele (Rae and Smith 2002). This was not observed for the homologous *Arabidopsis* AtSultr1;1 and 1;2. Presumably, in barley, a single transporter is responsible for functions carried out by more than one transporter in *Arabidopsis* (Rae and Smith 2002). In the rice genome, two group 2 transporters are present, but pattern of their spatial expression is unknown. In tomato, LeSultr1;1 is expressed under sulfate-deprived conditions in the pericycle (Howarth et al. 2003). This indicates that under sulfate stress, plants are able to induce additional high-affinity sulfate transport to maintain vascular movement of sulfate under low-sulfate concentrations. In *Arabidopsis*, AtSultr1;3 seems to mediate the interorgan transport of sulfate as high-affinity transporter by specific expression exclusively in the phloem of all *Arabidopsis* organs analyzed (Yoshimoto et al. 2003). Analysis of a AtSultr1;3 T-DNA insertion mutant provided direct evidence for this function by restricting movement of labeled sulfate from the cotyledon to the other organs, indicating importance of AtSultr1;3 for source-to-sink transport of sulfate (Yoshimoto et al. 2003). The specific expression of lower high-affinity transporters in the root tip, as well as in axillary buds, indicates the importance of an adequate sulfate supply to fast-growing tissues (Takahashi et al. 1997, 2000; Rae and Smith 2002). In addition, in root tips, high levels of expression are likely to be of functional value to facilitate “foraging” (Buchner et al. 2004a). Developing leaves are strong S-sinks. The upregulation of *Arabidopsis* AtSultr2;2 and 1;3 in leaves under sulfate starvation revealed participation of both transporters in the vascular allocation of sulfate from leaves to other tissues (Takahashi et al. 2000; Yoshimoto et al. 2003). In *Arabidopsis*, AtSultr1;1 and AtSultr 1;2 expression is upregulated in leaves subjected to S starvation. AtSultr1;2 promoter activity was found in the guard cells under normal growth conditions (Yoshimoto et al. 2002). The upregulation of the *Arabidopsis* group 4 transporter, AtSultr4;1, under S deficiency in roots as well as in leaves (Takahashi et al. 2000) indicated the importance of vacuolar efflux of sulfate regulated by the S demand. In tomato, high-affinity sulfate transporter, LeSultr1;2, was overexpressed in the stem and in the leaves due to S-stress (Howarth et al. 2003). LeSultr1;2 expression was also observed induced in the vascular tissue of the *Verticillium*-resistant tomato line, GCR 218, after infection by *Verticillium dahlia*. This suggested prominent roles played by the sulfate transporter in the mechanism of *Verticillium* resistance involving elemental sulfur formation.

Among prominent food legumes, transcript levels of two sulfate transporters, Sultr1;2 and Sultr3;3, were elevated in the active phase of storage protein accumulation in common beans, *Phaseolus vulgaris* L. (Liao et al. 2012). Transcriptomic profiling identifies predominant expression of Sultr3 members in *P. vulgaris*, whereas expression of Sultr5 members was predominant in *Vigna mungo* (Liao et al. 2013). Understanding the role of S in food legumes growth is important from the point of view that the deficiency of the S-containing amino acids Cys, cystine, and Met may limit the nutritional value of food and feed (Khan and Mazid 2011; Liao et al. 2012). The increased levels of Sultr1;2 and Sultr 3;3 transcripts in common beans are consistent with *Arabidopsis* studies implicating the high-affinity sulfate transporter Sultr1;3 in phloem transport of sulfate (Yoshimoto et al. 2003) and low-affinity group 3 sulfate transporters in the transport of sulfate from the seed coat to the embryo (Zuber et al. 2010). In chickpeas, expression of the low-affinity AtSultr2;1 homologue was not restricted to vegetative tissues but was also found in pods and in the testa of developing embryos (Tabe et al. 2003). The group 3 sulfate transporter homologue of AtSultr3.1 is also expressed in the pods, in the testa, and, additionally, in developing chickpea embryos. Expression of the sulfate transporter homologue of AtSultr3;3 was detectable in almost all organs with high abundance in the developing chickpea embryo, suggesting participation in sulfate transport in many cell types (Tabe et al. 2003).

Unexpectedly, group 3 sulfate transporter was identified as essential for nitrogen fixation in legume nodules (Krusell et al. 2005). In lentil (*Lens culinaris* Medik.) seedlings, two putative sulfate transporter genes, LcSultr1;1 and LcSultr1;2, were upregulated in roots of L 414 genotype in response to arsenate (Talukdar and Talukdar 2013d). Temporal expression pattern revealed initial downregulation of both LcSultr1;1 and LcSultr1;2 in L 414, but their significant elevations were observed during later stages of metalloid exposures. Transcripts of LcSultr2;1 and LcSultr2;2 initially changed nonsignificantly in L 414, followed by their downregulations. Expression levels of two group 1 transporters were initially low in lentil genotype DPL 59 and further downregulated during prolonged arsenate exposure. Transcripts of both LcSultr2 ;1 and LcSultr2 ;2 in DPL 59 were initially unchanged but increased after 24 h of metalloid exposures (Talukdar and Talukdar 2013d). This observation indicates genotypic differences in regulation of gene expression of sulfate transporters in lentil crop. A detailed discussion of the current status of sulfate transporter regulation in *Arabidopsis* and crop plants can be found in Takahashi et al. (2011).

Sulfate Assimilation and Cys Biosynthesis-Cellular Regulations, Homeostasis, and Functional Interplay

Plant S-assimilation is the effective delivery of sulfate to the plastid, the major site of the assimilatory reductive pathway. For assimilation into Cys, sulfate is activated by adenylation to adenosine 5′ phosphosulfate (APS) in a reaction catalyzed by ATP sulfurylase (ATPS; EC 2.7.7.4). APS is reduced to sulfite by APS reductase (APR; EC 1.8.4.9) with electrons derived from GSH. Sulfite is further reduced by a ferredoxin-dependent sulfite reductase (SiR; EC 1.8.7.1) to sulfide, which is incorporated by O-acetylserine (thiol)lyase (OAS-TL; 2.5.1.47) into the amino acid skeleton of O-acetylserine (OAS) to form Cys. OAS is synthesized by acetylation of serine with acetyl-Coenzyme A catalyzed by serine acetyltransferase (SAT; EC 2.3.1.30) (Leustek et al. 2000; Suter et al. 2000; Kopriva et al. 2001, 2009) (Fig. 1). Transcript levels of APR in soybean decrease significantly in the absence of N and increase under S-deprivation conditions. Likewise, expression of both ATPS and APR undergoes concomitant changes in both gene expressions and enzyme activity across developmental stages of soybean (Phartiyal et al. 2006, 2008). In vast majority of reports, changes in APR activity correlated well with changes in mRNA and protein accumulation, indicating a simple transcriptional regulation of the corresponding genes, with a rare occurrence of additional level of posttranscriptional redox regulation (Koprivova et al. 2008). In contrast to ATPS and APR genes, the SiR gene shows little transcriptional regulation. Nevertheless, the SiR single copy gene At5g04590 is not only essential for survival; the encoded SiR activity can become limiting for the flux through the reduction pathway (Khan et al. 2010). OAS and sulfide are the substrates for the Cys synthesis and thus are fundamental for the homeostasis of reduced S in the plant. OAS has now been considered as the best signal, if not sulfate itself, of sulfate starvation in plants. Feeding of OAS to *Arabidopsis* induced low S-responsive genes such as sulfate transporters and APR (Koprivova et al. 2000; Hesse et al. 1999, 2004; Hirai et al. 2003). Evidence that OAS functions as a putative signaling molecule has come from computational analysis of time-series experiments and on studies of transgenic plants, conditionally displaying increased OAS levels (Hubberten et al. 2012). Transcripts whose levels correlated with the transient and specific increase in OAS levels observed in leaves of *Arabidopsis thaliana* plants 5–10 min after transfer to darkness and with diurnal oscillation of the OAS content, showing a characteristic peak during the night, were identified. Induction of a SAT in transgenic *A. thaliana* plants expressing the genes under the control of an inducible promoter resulted in a specific time-dependent increase in OAS levels (Hubberten et al. 2012). Monitoring the transcriptome response at time points at which no changes in

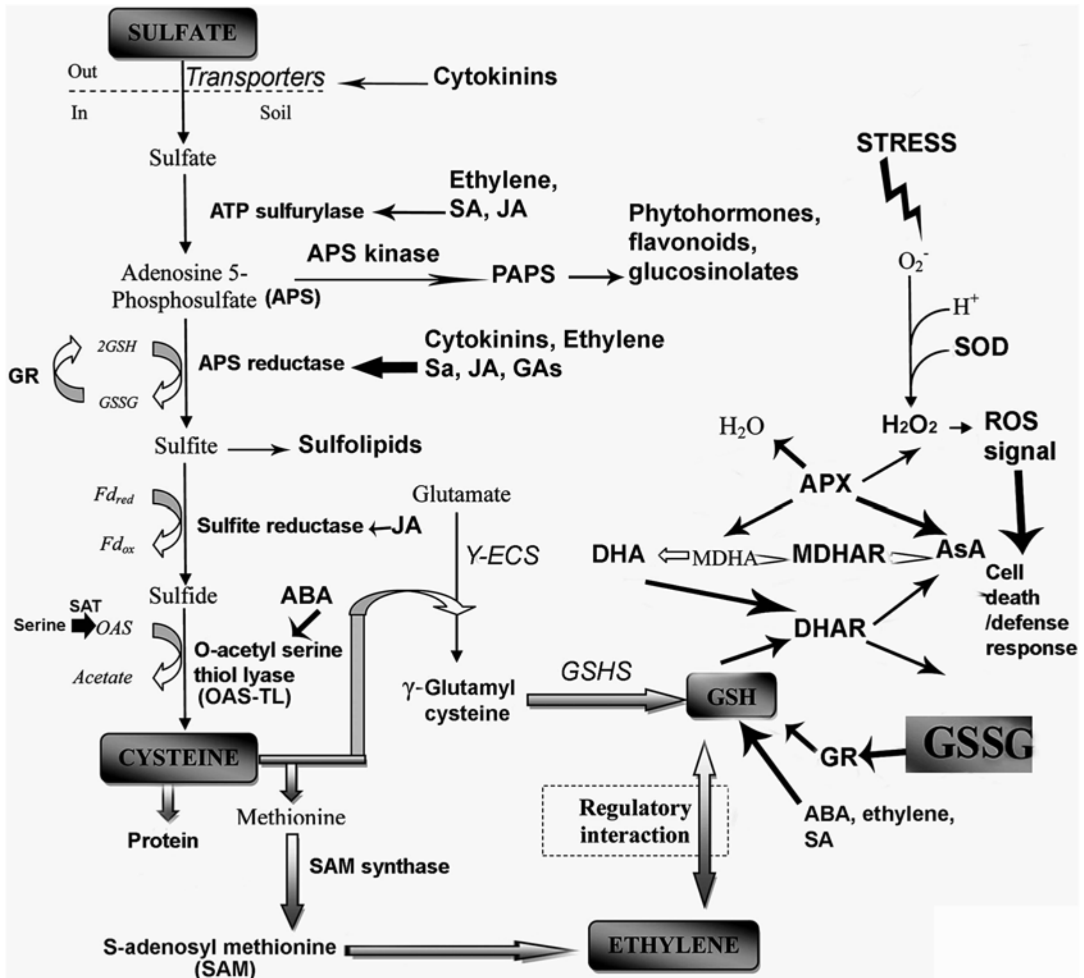

Fig. 1 Overview of thiol-cascade and integration with stress response and hormonal regulations; *JA* jasmonate, *SA* salicylic acid, *ABA* abscisic acid, *SOD* superoxide dismutase, *APX* ascorbate (AsA) peroxidase, *DHA* dehydroascorbate, *MDHAR* monodehydroascorbate reductase, *DHAR* DHA reductase, *GR* GSH reductase, *GSH* reduced glutathione, *OAS* O-acetyl serine, *PAPS* 3′-phosphate-5′-adenosine phosphosulfate, *SAT* serine acetyl transferase, *γ-ECS* γ-glutamyl cysteine synthetase, *GSHS* GSH synthase

S-related metabolites except OAS were observed and correlating this with the light/dark transition and diurnal experiments resulted in identification of six genes (adenosine-5′-phosphosulfate reductase 3, sulfur-deficiency-induced 1, sulfur-deficiency-induced 2, low-sulfur-induced 1, serine hydroxymethyltransferase 7, and ChaC-like protein) whose expression was highly correlated with that of OAS. These data suggest that OAS displays a signaling function leading to changes in transcript levels of a specific gene set irrespective of the S status of the plant (Hubberten et al. 2012). Multiple experimental approaches indicate that SAT catalyzes the limiting step in Cys biosynthesis (Droux 2003; Bonner et al. 2005). OAS-TL and SAT physically interact to form multimeric complex known as cysteine synthase (CS) complex (Hell and Wirtz 2011) or recently called as cysteine regulatory complex (CRC) (Yi et al. 2010). Five SAT and nine OAS-TL genes are found in the *Arabidopsis* genome (Kopriva et al. 2009), while the soybean genome contains

8 putative SAT and 15 putative β-cyanoalanine synthase (BSAS) (OAS-TL plus related enzymes) genes (Yi et al. 2010). Characterization of OAS-TL-encoding genes reveals their distinct but cooperative expression in Cys synthesis of soybean (Zhang et al. 2008). In *Vicia sativa*, a putative OAS-TL gene, designated as *Voas-tl5* (GenBank Accession No. DQ456491), was cloned and characterized (Novero et al. 2008). The mRNA transcription patterns generated from semiquantitative RT-PCR revealed that the *Voas-tl5* gene was highly transcribed in leaf, pod, and seed tissues. Phylogenetic analysis revealed that the gene belonged to the BSAS 5 subgroup (Novero et al. 2008). At the transcript level, BSAS isoforms show dynamic but partially overlapping expression pattern depending on organ types and developmental stages (Chronis and Krishnan 2004; Zhang et al. 2008). Presumably, overall rate of Cys synthesis at a certain time and location in soybean may be determined by interplay among BSAS isoforms expressed: some carry out Cys biosynthesis while the others are more involved for β-cyanoalanine synthesis and desulfuration using Cys as substrate. A discrepancy in OAS-TL activity and mRNA expression level of a BSAS isoform in wild soybean (*Glycine soja*) may be related to differential expression patterns of multiple isoforms and/or difference in preferred biochemical activity among BSAS isoforms (Zhang et al. 2008). Although the mRNA expression level of BSAS isoforms studied so far largely corresponds to the total OAS-TL activity during seed development, it is not yet clear exactly how many BSAS isoforms are expressed during seed development and what is the in vivo function of each enzyme expressed (Chronis and Krishnan 2004; Zhang et al. 2008). The relevance of compartmentation of Cys biosynthesis in phototrophic organisms has been studied in vascular model plant *Arabidopsis*, unicellular green alga *Chlamydomonas reinhardtii*, and the colonizer moss, *Physcomitrella patens* (Birke et al. 2012). In *Arabidopsis thaliana*, synthesis of Cys and its precursors OAS and sulfide is compartmentalized between the cytosol, chloroplasts, and mitochondria, contributing regulation of Cys synthesis. The predominant cytosolic isoforms are SAT2, SAT4, SAT5, and OAS-TL A, whereas SAT1 and OAS-TL B are targeted to the plastids and SAT3 and OAS-TL C localize to mitochondria. Mitochondrial SAT3 contributes to approximately 80 % of total SAT activity in the *Arabidopsis* leaf cell, suggesting prominent role of mitochondria for total OAS production (Birke et al. 2012; Wirtz et al. 2004, 2012). Conversely, contribution of mitochondrial OAS-TL C to total OAS-TL activity in leaves is very low (<5 %). Cytosolic and plastidic isoforms of OAS-TL in *Arabidopsis* account both for more than 45 % of total OAS-TL activity; however only loss of a cytosolic isoform leads to decrease in total Cys production demonstrating its predominant role in the synthesis of Cys (Watanabe et al. 2008a, b; Krueger et al. 2009). In contrast, Cys synthesis is exclusively restricted to chloroplasts in the unicellular green alga *Chlamydomonas reinhardtii*. The moss *Physcomitrella patens* colonizes land but is still characterized by a simple morphology compared to vascular plants. Native OAS-TL proteins demonstrated the presence of five OAS-TL protein species encoded by two genes in *Physcomitrella*. At least one of the gene products is dual targeted to plastids and cytosol, as shown by combination of GFP fusion localization studies, purification of chloroplasts, and identification of N termini from native proteins (Birke et al. 2012). The bulk of OAS-TL protein is targeted to plastids, whereas there is no evidence for a mitochondrial OAS-TL isoform and only a minor part of OAS-TL protein is localized in the cytosol. This demonstrates that subcellular diversification of Cys synthesis is already initialized in *Physcomitrella* but appears to gain relevance later during evolution of vascular plants. Multiple lines of evidence suggest a critical role for formation of the CS complex in different subcellular compartments in plants. The evidence that mitochondrial CS complex regulates OAS biosynthesis in plants has come from biochemical analyses of recombinant plant SAT and OAS-TL. This indicates that the reversible association of the proteins in the CS complex controls cellular S homeostasis (Wirtz et al. 2010, 2012). In this study, NMR spectroscopy of isolated mitochondria from wild type, *serat (SAT)2;2*, and *oastl-C*

mutant plants exhibited SAT-dependent export of OAS. The presence of Cys resulted in reduced OAS export in mitochondria of *oastl-C* mutants but not in wild-type mitochondria. This is in agreement with the stronger in vitro feedback inhibition of free SAT by Cys compared with CS complex-bound SAT and explains the high OAS export rate of wild-type mitochondria in the presence of Cys. The predominant role of mitochondrial OAS synthesis was validated in planta by feeding [^{3}H]serine to the wild-type and loss-of-function mutants for OAS-TLs in the cytosol, plastids, and mitochondria (Wirtz et al. 2012). Computational modeling coupled with transgenomic and transcriptomic analysis provided ample proof of more complex interaction of both enzymes underlying the mechanism of their reciprocal regulation (Wawrzyńska et al. 2013). At the mRNA level, SAT and OAS-TL are constitutively expressed, although expressions of some isoforms increase/decrease under nutritional and environmental stress conditions (Yamaguchi et al. 1999; Domínguez-Solís et al. 2004; Kawashima et al. 2005; Yi et al. 2010; Talukdar and Talukdar 2013d). The fact that SAT and OAS-TL expression does not compensate at either the RNA or protein level in mutants, lacking one or two isoforms, further supports that transcriptional control plays a limited role in regulating expression of Cys synthesis (Haas et al. 2008; Heeg et al. 2008; Watanabe et al. 2008a, b). Alternatively, interaction between SAT and OAS-TL, which is mediated by C-terminal tail of SAT and active site pocket of OAS-TL, appears to provide an effective regulatory mechanism that readily responds to cellular concentration of sulfide and OAS (Bonner et al. 2005; Francois et al. 2006; Kumaran et al. 2009). High concentrations of OAS and/or low levels of thiol compounds in the cell are likely signals that induce high-affinity sulfate transporter and restore the conditions favoring formation of the CS in *Arabidopsis* and potato (Hirai et al. 2003; Hopkins et al. 2005). The importance of OAS in plant growth and development has been elucidated in *Arabidopsis* point mutations impaired in cytosolic OAS-TL expressions, leading to early leaf death (old 3–1). The early leaf death phenotype is temperature dependent and is associated with increased expression of defense-response and oxidative stress marker genes. Independent of the presence of the *odd-ler* gene, *OAS-A1* is involved in maintaining S and thiol levels and is required for resistance against (Cd) stress (Shirzadian-Khorramabad et al. 2010). In addition to increasing SAT activity, CS formation can alleviate the inhibitory effect of Cys on SAT activity (Kumaran et al. 2009). Transient expression of soybean GmSerat (SAT) 2;1 fused with GFP revealed its dual targeting to cytosol and plastid (Liu et al. 2006). ATSAT 2;1 is also found in both cytosol and plastid in the later developmental stage but is exclusively targeted to the plastid in the earlier stage of *Arabidopsis* (Noji et al. 2001). Transcriptomic profiling revealed that expression of the cytosolic SAT1;1 and SAT1;2 was approximately fourfold higher in *Phaseolus vulgaris* while expression of the plastidic SAT2;1 was twofold higher in *Vigna mungo* (Liao et al. 2013). Among *BSAS* family members, BSAS4;1, encoding a cytosolic cysteine desulfhydrase, and BSAS1;1, encoding a cytosolic OAS-TL, were most highly expressed in both species. This was followed by BSAS3;1 encoding a plastidic BSAS which was more highly expressed by 10-fold in *P. vulgaris*. The data identify BSAS3;1 as a candidate enzyme for the biosynthesis of *S*-methylcysteine through the use of methanethiol as substrate instead of cyanide. Expression of GLC1 would provide a complete sequence leading to the biosynthesis of γ-Glutamyl-*S*-methylcysteine in plastids. The detection of *S*-methylhomoglutathione in *P. vulgaris* suggested that homoglutathione synthetase may accept, to some extent, γ-Glutamyl-*S-methylcysteine* as substrate, which might lead to the formation of *S*-methylated phytochelatins (Liao et al. 2013). Using unique quadruple knockout mutants of SAT that retained only one functional isoform in *Arabidopsis*, Watanabe et al. (2010) nicely compared metabolite and transcriptome data from these mutants with N-, P-, K-, and S-depleted plants. The study revealed many similarities with general nutrient-depletion-induced senescence (NuDIS), indicating the recruitment of existing regulatory programs for nutrient-starvation responses. Several candidate

genes that could be involved in these processes were identified, including transcription factors and other regulatory proteins, as well as the functional categories of their target genes. These results outline components of the regulatory network controlling plant development under sulfate stress. For example, increased gene expression of Sultr1;2, Sultr1;3, Sultr4, and APR in the group 2 SAT mutant indicates that these genes, which have been assumed to be regulated by OAS or thiols, are also regulated by OAS/thiol-independent factors, as OAS and thiol contents remain unchanged in the mutants (Watanabe et al. 2010).

The transcriptional and metabolic responses of plants to S supply limitation have been investigated by several groups (Nikiforova et al. 2003, 2005a, b, 2006; Maruyama-Nakashita et al. 2003, 2004; Maruyama-Nakashita and Takahashi 2005; Hirai et al. 2003, 2005). Using array hybridization experiments, Nikiforova et al. (2003) examined transcript levels of 16 128 *Arabidopsis* EST clones representing approximately 7,200 individual genes, corresponding to about 30 % of the total *Arabidopsis* genome, and 18 genes linked to S-related pathways were sorted out. Besides Sultr 2;1 and 4;1, the other prominent members are GSH-dependent dehydroascorbate reductase (DHAR), cytosolic isoform of glutathione reductase (GR), glutathione peroxidase (GPX), SAT-1, OAS-TLC, S-adenosylmethionine synthetase 2, and γ-ECS (Nikiforova et al. 2003). Further, transcriptome data were overlaid with >100 nonredundant compounds of known chemical structure (Nikiforova et al. 2005a). Integration of these data sets allowed the first multifactorial correlation network to be created, revealing potential relationships among genes and metabolites under S limitation (Nikiforova et al. 2005b). Transgenic poplars overexpressing γ-ECS offered an opportunity to address the effects of increased GSH synthesis on the sulfate assimilation pathway because Cys availability is most critical for the rate of GSH synthesis (Noctor et al. 2012). Although leaf GSH levels were three- to fourfold higher in transgenic poplars overexpressing γ-ECS in the cytosol, foliar activities of enzymes of sulfate assimilation, ATPS, APR, SiR, SAT, and OAS-TL, and their mRNA levels were not different from those of wild-type poplars (Hartmann et al. 2004). This indicates the fact that sulfate reduction in poplar is sufficient to provide the additional Cys necessary to accommodate the enhanced GSH synthesis. Remarkably enough, the increased GSH level in transgenic poplars did not downregulate ATPS and APR as commonly observed in several herbaceous plant species and crops (Vauclare et al. 2002; Talukdar and Talukdar 2013d). The lack of regulation of APR and ATPS in the transgenic poplar lines must be caused by a second signal that positively influences APR mRNA accumulation and activity and overrides the negative signal of GSH.

Glutathione: The Center of Thiol-Cascade

In plants, the thiol-containing tripeptide GSH (in reduced form) is a major regulator of cellular redox state as well as an essential contributor to processes such as the detoxification of xenobiotics, regeneration of ascorbate in AsA–GSH cycle, thiol–disulfide exchange reactions through GRXs (glutaredoxins), protein *S*-glutathionylation or thiolation by forming stable mixed disulfide bonds with protein Cys residues, the sequestration of heavy metals, the storage of excess S in the form of Cys, weed-induced phytotoxicity, hormone signaling, as an electron donor for APR in sulfur assimilation, and numerous other metabolic and cellular processes involved in plant growth and development (Mullineaux and Rausch 2005; Meyer and Rausch 2008; Rouhier et al. 2008; Noctor et al. 2012; Talukdar 2013c, e, f). The role of GSH in cell cycle progression during promotion of root growth has earlier been demonstrated in *Arabidopsis rml 1* (*root meristem less 1*) mutant (Vernoux et al. 2000) and in an ascorbate-deficient mutant line *asfL-1* of hardy legume grass pea (Talukdar 2012a). Versatility of this thiol peptide as efficient buffer has also been demonstrated in two catalase-deficient mutants of lentil where failure of defense cross talk between thiol-linked enzymes led to onset of oxidative stress and cell-division

anomalies (Talukdar and Talukdar 2013a). Besides pea and beans, grass pea and lentil have drawn increasing attention due to their low-input requirement, availability of robust cytogenetic and biochemical mutant stocks, and escalating demand for legume food and forage (Vaz Patto et al. 2006; Kumar et al. 2011a, b; Talukdar et al. 2002; Talukdar and Biswas 2005, 2007a, b; Talukdar 2008, 2009a, b, 2010a, b, c, 2011a, d, 2012b, c, d, 2013g). Very recently, the role of GSH redox in leaf photosynthesis and mitigating oxidative metabolisms in different mating types of a critically endangered legume tree, *Gymnocladus assamicus*, has been elucidated (Talukdar and Talukdar 2014). Legumes are the only plant family with significant amounts of a GSH analog homoglutathione, which contains a β-alanine instead of glycine. Nodules are organs with the highest GSH and/or hGSH content in legumes because of their role in defense of the nitrogenase against reactive oxygen species (ROS) (Matamoros et al. 2003). However, GSH and hGSH are important also for establishing the symbiosis. Our understanding of the synthesis and function of GSH is primarily based on the Brassicaceae, in particular *Arabidopsis* and Indian Mustard (*Brassica juncea*), although additional studies completed in legumes, such as soybean, grass pea, and lentil, expand the role of this peptide to species-specific analogs. GSH synthesis requires the activities of two dedicated ATP-dependent enzymes: γ-glutamyl cysteine synthase (γ-ECS) and glutathione synthetase (GS). The first enzyme, γ-ECS, utilizes L-glutamate and L-Cys to generate γ-glutamyl cysteine and is a rate-limiting enzyme (Hell and Bergmann 1990; Jez et al. 2004). From this dipeptide and glycine, GSHS, the second enzyme in the pathway, then synthesizes GSH (Jez et al. 2004; Noctor et al. 2012). Transcript analysis and activity assays in common bean (*Phaseolus vulgaris*) nodules revealed localization of γ-ECS to only chloroplasts and GSHS to both plastids and the cytoplasm as also occurs in *Arabidopsis* (Wachter et al. 2005) but differs with cowpea (*Vigna unguiculata*) nodules where γ-ECS is localized to both plastids and cytosol (Moran et al. 2000). In Brassicaceae, differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiations in different cellular compartments (Wachter et al. 2005).

GSH-redox pool in cellular environment is maintained by delicate balance between its production and catabolism. GSH supply in cell is generally maintained by its synthesis and regeneration by the FAD-linked action of GR enzymes within AsA–GSH cycle (Noctor et al. 2012; Talukdar 2012a). In a magnificent study, Mhamdi et al. (2010) addressed the role of GR1 isoforms in H_2O_2 responses through a combined genetic, transcriptomic, and redox profiling approach. To identify the potential role of changes in GSH status in H_2O_2 signaling, gr1 mutants, which show a constitutive increase in oxidized glutathione (GSSG), were compared with a catalase-deficient background (cat2), in which GSSG accumulation is conditionally driven by H_2O_2. Parallel transcriptomics analysis of gr1 and cat2 identified overlapping gene expression profiles that in both lines were dependent on growth day length. Overlapping genes included phytohormone-associated genes, in particular implicating GSH oxidation state in the regulation of jasmonic acid (JA) signaling. GSH synthesis is regulated by the supply of the constituent amino acids and by feedback inhibition of γ-ECS by GSH (Noctor et al. 2012). To address this regulation in more detail, a poplar hybrid *Populus tremula* X *P. alba* (INRA clone no. 717-1-B4, Versailles, France) was transformed to express bacterial γ-ECS or GSHS either in the cytosol or in the chloroplast (Noctor et al. 2012). Overexpression of γ-ECS, but not of GSHS, increased foliar and root GSH concentration (Noctor et al. 2012), thus confirming the major role of γ-ECS in the control of GSH synthesis. Experiments with poplar leaf disks revealed that feeding of γ-EC dramatically enhanced GSH synthesis compared with feeding of Cys and Glu, this effect being more profound in the GSHS-overexpressing plants, whereas Cys was more effective in the γ-ECS-overexpressing poplar (Noctor et al. 2012). Interestingly, overexpression of γ-ECS either in the cytosol or in the chloroplast did not decrease Cys and Met concentrations (Herschbach et al. 2010). Presumably, sulfate reduction and Cys formation are adjusted

to the higher demand for GSH synthesis in γ-ECS transgenic trees, the detail mechanism of which is still unknown. Changes in the redox state of γ-ECS provide a posttranslational mechanism for regulation of activity (Jez et al. 2004; Hicks et al. 2007; Noctor et al. 2012). The soybean genome contains two full-length copies of the GSHS gene that are 91 % identical. Localization tag analysis suggests that both of these transcripts are targeted multiple locations (Yi et al. 2010). Several studies describe transcriptional regulation of the pathway and report increased expression of the genes encoding γ-ECS and GSHS under different stress conditions (Xiang and Oliver 1998). Interestingly, in *Arabidopsis* suspension cells, transcriptional upregulation of γ-ECS in response to various oxidative stresses was not observed, even though both GSHS activity and cellular GSH levels increased and new concepts on post-transcriptional regulations and diversity in synthesis pathway have emerged (Galant et al. 2011). GSH is efficient in repressing sulfate uptake after a period of sulfate starvation but also during normal S nutrition (Lappartient et al. 1999; Vauclare et al. 2002). S is important for chelating heavy metals through metallothioneins, i.e., Cys-rich proteins and phytochelatins (PCs), small polypeptides with repeating γ-EC units. PCs are synthesized from GSH by phytochelatin synthase (PCS). The sulfhydryl groups of Cys residues bind the heavy metal ions, and the resulting complexes are excreted to the vacuole. GSH is also important for resistance against herbicides. Differences in herbicide toxicity are often based on the capacity of the plants to detoxify the herbicide, e.g., through the glutathione S-transferase (GST) reaction and subsequent excretion of the conjugate into the vacuole (Edwards and Dixon 2005). In plants, GSTs are encoded by a large gene family with approximately 50 members in *Arabidopsis* and rice (Edwards and Dixon 2005), highlighting the importance of GSH conjugate formation for the metabolism of endogenous compounds and the detoxification of noxious compounds such as herbicides. GSH conjugates are predominantly generated in the cytosol, with minor GST activities in the nucleus, chloroplast, and mitochondrion (Dixon and Edwards 2009). Glutathionylation of compounds is an important reaction in the detoxification of electrophilic xenobiotics and in the biosynthesis of endogenous molecules. The GSH conjugates are further processed by peptidic cleavage reactions. In animals and plants, γ-glutamyl transpeptidases initiate the turnover by removal of the glutamate residue from the conjugate. Plants have a second route leading to the formation of γ-glutamylcysteinyl (γ-GluCys) conjugates. PCS is well known to mediate the synthesis of heavy metal-binding PCs. In addition, the enzyme is also able to catabolize GSH conjugates to the γ-GluCys derivative. Cellular compartmentalization of PCS and its role in the plant-specific γ-GluCys conjugate pathway have been studied in *Arabidopsis thaliana* (Blum et al. 2010; Noctor et al. 2012). Localization studies of both *Arabidopsis* PCS revealed a ubiquitous presence of AtPCS1 in *Arabidopsis* seedlings, while AtPCS2 was only detected in the root tip. A functional AtPCS1:eGFP (enhanced green fluorescent protein) fusion protein was localized to the cytosolic compartment (Blum et al. 2010).

Defining Cross Talk with Plant Metabolisms: Hormonal Response, Photosynthesis, Carbohydrate and Lipid Metabolisms, Kinase Cascades, and Other Metabolisms

Thiol-Cascade and Plant Hormone Metabolisms

Recent developments indicate that plant hormones play pivotal roles in regulating S-metabolisms (Ohkama et al. 2002; Maruyama-Nakashita et al. 2004, 2006), a brief outline of which is presented in Table 1 and Fig. 1. Whereas abscisic acid (ABA), indole-3-acetic acid (IAA), 1-aminocyclopropane 1-carboxylic acid (ACC, precursor of ethylene), gibberellic acid (GA_3), and jasmonic acid (JA) were not able to induce expression of GFP derived from the S-responsive element and, thus, mimic the S starvation response, trans-zeatin caused an increase in GFP synthesis both in S-sufficient and in S-deficient

Table 1 Phytohormone-mediated regulations of thiol-cascade gene expressions in *Arabidopsis*

Hormone	Gene(s) expressed	Functions in thiol-cascade	References
Auxins	*SULTR1;2,1;4* *SULTR4;1*	Sulfur (S) uptake, transport through root, and vacuole overexpressed	Nikiforova et al. (2003)
	At2g44460 (thioglucosidase gene), *APR2*	Repressed expressions, impeded S-reductive assimilation	Nikiforova et al. (2003)
Gibberellins	*APR1, APR2*	Differential regulations	Koprivova et al. (2008)
Cytokinins	*APR1, SULTR2;2*	Induced during vascular transport of S and during S-assimilation through an increase in sucrose concentrations	Ohkama et al. (2002)
	SULTR1; 1, SULTR1; 2	Repressed expressions in roots through involvement of CRE/WOL/AHK4-mediated signal	Maruyama-Nakashita et al. (2003)
Abscisic acid (ABA)	*AtGSTUI7, OsMCSU*	Repressed GSH pool in *Arabidopsis*. The molybdenum (Mo) cofactor sulfurase (MCSU) in rice transfers the sulfur ligand to aldehyde oxidase-bound MoCo	Maruyama-Nakashita et al. (2003)
Ethylene	*APR1, APR3*	Overexpressed	Koprivova et al. (2008)
Jasmonate	*APR 1, APR 2, SIR, SAT3, γ-ECS, APS2, APR3, ATPS*	Methyl jasmonate upregulates the expressions	Jost et al. (2000)
Salicylic acid	*APR1, APR2, APR3, γ-ECS, GSHS*	Overexpressed	Kopriva (2006)

conditions. In addition, zeatin treatment resulted in an increased accumulation of mRNA for APR and a low-affinity sulfate transporter (Ohkama et al. 2002). By contrast, cytokinins repress the expression of high-affinity sulfate transporters and sulfate uptake capacity of *Arabidopsis* roots, since feeding with zeatin downregulated transcript levels of AtSultr 1;1 and AtSultr 1;2 and also root sulfate acquisition (Maruyama-Nakashita et al. 2004). Cytokinin acts through the cytokinin response receptor (CRE1) to regulate sulfate uptake and transporter expression. In the *cre1-1* mutant, application of cytokinin only partly reduces sulfate uptake, suggesting redundancy as noted for the case of phosphate deprivation. The effect of zeatin is dependent on the CRE1/WOL/AHK4 cytokinin receptor (Maruyama-Nakashita et al. 2004).

The physiological responses to auxin involve changes in gene expressions. A NIT3 nitrilase, involved in synthesis of IAA, belongs to genes strongly induced by S deficiency (Kutz et al. 2002). In addition, the cis-acting element conferring S starvation response recently identified in *Arabidopsis* Sultr1;2 promoter contains an auxin response factor (ARF) binding sequence (Maruyama-Nakashita et al. 2006). Auxin regulates the cell-specific transcription of target genes via two types of transcription factors, ARFs and Aux/IAA proteins. In *Arabidopsis thaliana*, the Aux/IAA and ARF gene families are represented by 29 and 23 loci, respectively. Several transcription factors of both families, including IAA13, IAA28, and ARF2, are moderately upregulated during S depletion (Nikiforova et al. 2005b, 2006). IAA28 exhibits a high degree of connectivity and so was identified as a hub of the transcript/metabolite co-response network responding to S starvation (Nikiforova et al. 2005b). A gain-of-function *Arabidopsis* mutant of IAA28 has been reported to exhibit suppressed lateral root formation (Rogg et al. 2001) by repressing transcription, perhaps of genes that promote lateral root initiation in response to auxin signals. ARF2 has been identified as a transcription factor binding to AuxRE in promoters of auxin response genes, thus activating flowering, senescence, and abscission. It also functions as a light-indepen-

dent repressor of cell growth and of differential hypocotyl growth during seedling hook formation (Li et al. 2004a, b; Ellis et al. 2005; Okushima et al. 2005a, b). ARF2, a pleiotropic developmental regulator, knockout mutants develop a phenotype with increased leaf size, enhanced flower formation, and increased seed size (Ellis et al. 2005). Overexpression of ARF2 is likely to be lethal as it was impossible to retrieve ARF2 overexpressing lines, while co-suppression lines could be isolated. Alterations were observed in the contents of thiol-metabolites in response to the manipulation of Aux/IAA and ARF transcription factor expression, especially of the key thiol-metabolites Cys and GSH (Falkenberg et al. 2008). These changes were interpreted as broad spectrum shifts in amino acid metabolism and carbon/nitrogen balance caused by changes in the expression of AUX/IAA and ARF target genes, rather than a direct effect of auxin on sulfate metabolism (Falkenberg et al. 2008). Combined transcriptomic data and metabolic profiling indicated, among other potential links, a relationship between serine metabolism and tryptophan (trp)–glucosinolate–auxin metabolism. Based on these data, it was hypothesized that auxin might be involved in communicating the nutrient status of the shoot to the root, inducing lateral root formation when S is limiting (Nikiforova et al. 2003, 2005b).

JA did not affect the expression of the S-responsive promotor element (Ohkama et al. 2002) but is nevertheless involved in regulation of sulfate assimilation. Transcriptomic analysis revealed fast but transient increase in mRNA levels of many genes involved in sulfate assimilation and GSH synthesis without affecting sulfur metabolite levels in *Arabidopsis* treated with methyl jasmonate (Jost et al. 2000; Harada et al. 2001). The fact that the mRNA for sulfate transporters was unaffected confirmed that JA may not be involved in the regulation by sulfur nutrition although genes of JA biosynthesis are induced by S starvation (Hirai et al. 2003, 2004; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003). The increase of level of GSH in plants treated with ABA and salicylic acid (SA) indicates a complex interaction of sulfate assimilation, GSH synthesis, and hormone metabolisms. ABA induces mRNA accumulation of cytosolic OAS-TL (Barroso et al. 1999). Thus, it seems that this compound may have a more profound effect on the control of S-metabolism. On the other hand, SA plays a central role in plant defense against pathogens. Treatment with the biologically active SA analog 2,6-dichloroisonicotinic acid increased the GSH level leading to a reduction of NPR1, a regulator of systemic acquired resistance (SAR), and expression of the *PR1* gene for a pathogenesis-related protein (Mou et al. 2003). SA was also implicated in the mechanism of nickel tolerance in hyperaccumulator *Thlaspi* species. Elevated SA levels engineered in *Arabidopsis* led to an increase in SAT activity and GSH content and, subsequently, increased tolerance to Ni (Noctor et al. 2012). Whether SA regulates the expression of γ-ECS and GS, if and how it affects SAT, or if it utilizes another mechanism to increase GSH synthesis remains to be elucidated.

The interaction between ethylene and S has been shown to control the regulation of plant processes and abiotic stress tolerance. The main pathway for ethylene biosynthesis comes from Met. Met is a fundamental metabolite in plant cells because it controls the level of several key metabolites, such as ethylene, polyamines, and biotin, through its first metabolite, s-adenosylmethionine (SAM). It is first converted to SAM, then ACC (1-amino cyclopropane 1-carboxylic acid), and finally ethylene in three consecutive reactions catalyzed by the enzymes of SAM synthetase, ACS, and ACO, respectively (Iqbal et al. 2013). Bürstenbinder et al. (2007) using an *mtk* mutant, that has a disruption of the Yang cycle, reported that the Yang cycle contributes to SAM homeostasis, especially when de novo SAM synthesis is limited, such as at S starvation. S availability and ethylene have been shown to regulate GSH synthesis and stress tolerance to ozone (Yoshida et al. 2009) and Cd stress (Masood et al. 2012). Ethylene plays important roles in selenite resistance in *Arabidopsis*. A comprehensive gene expression analysis showed that transcripts regulating ethylene synthesis (ACS6) and signaling (ERF) were upregulated by

selenate treatment, and plants overexpressing ERF1 exhibited an increase in selenium (Se) resistance (Van Hoewyk et al. 2008). These results indicate that Se resistance achieved through ethylene signaling is not mediated by S starvation resulting from the Se treatment but is a Se-specific response. The resistance mechanism may involve ethylene-enhanced S uptake and assimilation, as observed in *Arabidopsis thaliana* accessions, Columbia (Col)-0 (Iqbal et al. 2013). Koprivova et al. (2008) reported that the application of 0.2 mM ACC, which stimulates ethylene production, increased accumulation of APR activity. Recently, it has been shown that ethylene action in mustard is dependent on S availability (Masood et al. 2012). There is an indication that APR activity is increased in salt stress if ethylene signaling is disturbed, but GSH will not accumulate suggesting that components of GSH biosynthesis are under the control of ethylene (Koprivova et al. 2008).

The other level of interaction of nutrient and hormone may be visualized at the level of ROS production. The regulation and interaction between ROS and AsA–GSH cycle impact the synthesis of plant hormones such as SA, GA, ABA, and ethylene, which may signal plant response to nutrient deficiency. The involvement of ROS in S signaling may be more complex than that of K deprivation because the AsA–GSH cycle, i.e., downstream of sulfate assimilation, is involved in the removal of H_2O_2. Direct analysis of H_2O_2–GSH interactions in cat2 gr1 double mutants of *Arabidopsis* established that GR1-dependent GSH status is required for multiple responses to increased H_2O_2 availability, including limitation of lesion formation, accumulation of SA, induction of pathogenesis-related genes, and signaling through JA pathways (Mhamdi et al. 2010).

Thiol-Cascade and Photosynthesis

Photosynthesis is known to be sensitive to sulfate because sulfate is a competitive inhibitor of ribulose-1,5-biphosphate carboxylase and inhibits photophosphorylation (Dietz and Pfannschmidt 2011). Sulfate uptake is well coordinated with the uptake and assimilation of carbon. Redox proteomics is an emerging technology aimed at defining the redox protein inventory of the cells and cell compartments and analyzing the redox state of target proteins on a broad scale. Both gel- and chromatography-based redox protein screening systems have been applied to plant and chloroplast protein fractions and resulted in lists of thylakoid lumenal, stromal, and chloroplast membrane-bound candidate redox proteins that undergo thiol modifications, most commonly dithiol–disulfide transitions (Mullineaux and Rausch 2005; Rouhier et al. 2008). Novel regulators including components of thiol-cascade such as Cys, GSH, and thioredoxins in photosynthetic redox control of plant metabolism (starch biosynthesis, lipid synthesis) and gene expressions have recently been explored (Dietz and Pfannschmidt 2011).

Thiol-Cascade and Anthocyanin Biosynthesis

Like N- and P-nutrient starvation, S starvation is tightly linked to anthocyanin biosynthesis (Lillo et al. 2008). The transcription factors PAP1 (At1g56650) and PAP2 (At1g66390) are positive regulators of the anthocyanin pathway (Borevitz et al. 2000) and are upregulated in the dwarfed quadruple mutants of *Arabidopsis* (Watanabe et al. 2010). Additionally, several known transcription factors downstream of the PAP genes, TT8 (At4g09820), TTG2 (At2g37260), and EGL3 (At1g63650), are induced along with target genes within the anthocyanin biosynthetic pathway, such as anthranilate synthase (ANS; At4g22880), dihydroflavonol reductase (DFR; At5g42800), and various others. Anthocyanin accumulation is usually augmented to various stress responses and is consistent with the upregulation of ROS network genes in the dwarfed quadruple mutants in *Arabidopsis* (Watanabe et al. 2010), as has been shown, for example, in the old1/cpr5 mutant (Jing et al. 2008) and the old5 mutant (Schippers et al. 2008), which are in

a state of high-cellular oxidative stress and show early senescence phenotypes.

Thiol-Cascade and Nodulation

The importance of S for nodule function was evidenced by analysis of *Lotus* sym mutants, showing nonfunctional nodules (Krusell et al. 2005). Both the sym13 and sym81 mutations, which display N deficiency syndromes under symbiotic but not nonsymbiotic growth conditions and form smaller nodules with reduced nitrogenase content and N-fixing capacity, exhibit a defective SST1 sulfate transporter. SST1 is a group 3 sulfate transporter expressed in a nodule-specific manner and located in the symbiosome membrane (Krusell et al. 2005). However, the strong sym phenotype of the sst1 mutants is surprising as at least one additional sulfate transporter is expressed specifically in *Lotus* nodules (Krusell et al. 2005). In addition, the reduction of total S content of 20–25 % in the mutants compared with wild-type nodules can hardly explain the severe disruption in N-fixation as plants can reduce S content by up to 70 % without phenotypic changes (Nikiforova et al. 2003). The mechanism by which the loss of SST1 aborts N-fixation thus needs to be addressed in more detail. GSH and hGSH play a critical role in the nodulation process of *Medicago truncatula* (Frendo et al. 2005).

Thiol-Cascade and Plant C_4 Metabolisms

In a significant development, transcriptomic analysis revealed that in plants like *Zea mays* with C_4 metabolisms, the mRNAs for APR, ATPS, and SiR accumulated in bundle sheath cells only, whereas OAS-TL transcript was detected in both mesophyll cells (MCs) and bundle sheath cells (BSCs) (Kopriva et al. 2001, 2009). A coordinate increase in mRNA levels for sulfate transporters ATPS and APR was observed in maize roots and leaves upon sulfate starvation (Bolchi et al. 1999; Hopkins et al. 2005), and the ATPS mRNA level was repressed in the presence of reduced S compounds (Bolchi et al. 1999). Not only sulfate assimilation but also the synthesis and reduction of GSH seem to be differently localized in C_4 plants. However, GSH is not equally distributed between MCs and BSCs in maize. GSHS activity is greater in MCs than in BSCs, resulting in GSH synthesis predominantly in the MCs and higher GSH levels in this cell type (Kopriva et al. 2001). Cys is transported from BSC protoplast as reduced S to the MCs. The enzymes of GSH synthesis and corresponding mRNAs were, however, found to be localized in both MCs and BSCs (Gómez et al. 2004). Both enzymes were detected in chloroplasts and in the cytosol (Gómez et al. 2004) which is in sharp contrasts with the Brassicaceae, where γ-ECS is localized in plastids and GSHS is prevalently cytosolic (Wachter et al. 2005). Interestingly, unlike the usual observations that GSH exerts the feedback repression of sulfate assimilation as found in members of Brassicaceae (Vauclare et al. 2002) and in lentil of Fabaceae (Talukdar and Talukdar 2013d), in maize Cys acts directly without conversion to GSH probably due to BSC localization of sulfate assimilation in maize (Kopriva 2006). Presumably due to the low capacity for NADPH formation in BSCs, GR is found exclusively in MCs of maize (Kopriva et al. 2001). Thus, it seems likely that there are species-specific differences in the intercellular localization of GSH biosynthetic enzymes, which are dependent on increased capacity for transport of various thiol compounds and possibly result in different regulatory mechanisms for S-assimilation in C_4 plants.

Thiol-Cascade and Plant Kinase Cascade

A screen for soybean expressed sequence tags (EST) showing sequence similarity to known SATs identified SSAT1 (GLYMA16G03080), and an interaction screen isolated GmSerat (SAT)2;1 (GLYMA18G08910) as a substrate for a calcium-dependent protein kinase (CDPK) (Chronis and Krishnan 2004; Liu et al. 2006).

Both characterized soybean SAT isoforms are sensitive to feedback inhibition by Cys but to varying degrees (Chronis and Krishnan 2004; Liu et al. 2006). Nonetheless, GmSAT2;1 lacking the N-terminal localization sequence becomes insensitive to Cys when it is phosphorylated by CDPK at a site close to the C-terminus by CDPK (Liu et al. 2006). The finding that full-length GmSAT2;1 does not display similar phosphorylation-dependent sensitivity to Cys suggests that a combination of subcellular localization and phosphorylation determines the effect of feedback inhibition. In the case of SSAT1, which does not have a putative CDPK-phosphorylation site at its C-terminus, CS formation with OAS-TL provides a similar protection to SSAT1 against Cys (Kumaran et al. 2009). Whereas none of five *Arabidopsis* SAT isoforms contain a putative CDPK-phosphorylation site, GmSAT2;1 and four other SAT isoforms in soybean carry potential CDPK-dependent phosphorylation sites (B-X-X-S/T: where B is a basic residue lysine or arginine, X is any residue, and S/T is serine or threonine) near the C-terminus (Liu et al. 2006). Considering the fact that putative CDPK-phosphorylation sites are also found near the C-terminus in the SAT from other plants, including tobacco, sunflower, and poplar (Liu et al. 2006), it needs to be determined whether C-terminal phosphorylation of SAT affects CRC formation and whether this posttranslational modification is more widely used to modulate feedback inhibition by Cys. Integration of different signaling factors such as mitogen-activated protein kinase (MAPK), MAPK kinase kinase, and CDPK was upregulated in rice under As stress (Huang et al. 2012). Moreover, As(V) markedly increased the activity of MAPKs and CDPK-like kinases, and CDPK and NADPH oxidases were involved in As-induced MAPK activation (Huang et al. 2012).

Thiol-Cascade and Other Plant Metabolisms

Sulfate uptake is well coordinated with the uptake and assimilation of N. During N limitation, sulfate uptake is strongly reduced (Koprivova et al. 2000). This reduction of uptake corresponds to significantly lower accumulation of transcripts for AtSultr1;1 and AtSultr1;2 (Maruyama-Nakashita et al. 2004). In addition, the genes for HASulT are induced by sucrose (Maruyama-Nakashita et al. 2004). Proteomic analysis in *Arabidopsis* Sultr 4;1 mutant seed revealed strong cross talk between plant thiol-metabolisms and lipid and sterol metabolism, as well as sugar and polysaccharide metabolism. Lipid synthesis that occurs in the plastids is a strong sink for electrons. Plastid redox state affects lipid metabolism. Acetyl-CoA carboxylase (ACCase) catalyzes the committed step of malonyl-CoA production in plastid lipid synthesis. Isolated ACCase in vitro is inactive without reductant and activated after addition of DTT or reduced thioredoxins (TRXs). The chloroplast ACCase consists of four polypeptides, in which the Cys residue constitutes the important part. Biotin carboxyl carrier subunit of ACCase in *Chlamydomonas reinhardtii* is subjected to *S*-thiolation with GSH (Dietz and Pfannschmidt 2011). Biotin carboxylase is a target of glutathionylation in *Arabidopsis* cell culture. Thus, each of the subunits of ACCase is potentially controlled by redox regulation using diverse mechanisms. This fact underlines the link between thiol-linked redox state and lipid metabolism. Furthermore, it is now known that envelope-bound monogalactosyldiacylglycerol synthase (MGD) is a major lipid component of chloroplasts. Plant MGD possesses nine conserved Cys residues. Its regulation by thiol redox state is suggested to enable galactolipid synthesis along with photosynthetic activity and to foster replacement of eventually oxidized lipids under conditions that cause oxidative stress (Dietz and Pfannschmidt 2011). Several spots corresponding to enzymes involved in polysaccharide catabolism, a β-glucosidase and a β-galactosidase, increased in mutant seeds, probably as a way to sustain glycolysis and fatty-acid biosynthesis. Two proteins of amino acid metabolism were also overaccumulated in the Sultr4;1 mutant seeds that correspond to glutamine synthase and Met synthase, although level of storage proteins was not affected (Zuber et al. 2010). OAS-TL in

its unbound form catalyzes the synthesis of Cys from OAS and sulfide and becomes inactivated when bound to the complex. The CS thus constitutes a branch point where reduced S gets incorporated into a carbon backbone. At least for N and P starvation, an induction of S uptake and assimilation has been documented (Kopriva et al. 2009, 2012). Limitation of both nutrients led to an increase in APR1, APR3, Sultr4;1, Sultr1;3, and different SATs. Further confirmation regarding importance of S-metabolism pathway in controlling many other primary and secondary metabolisms in plants has come from two independent *Arabidopsis thaliana* T-DNA insertion lines deficient in SiR transcripts and enzyme activity; sir1-2 seedlings had 14 % SiR transcript levels compared with the wild type and were early seedling lethal. sir1-1 seedlings had 44 % SiR transcript levels and were viable but strongly retarded in growth (Khan et al. 2010). This unique study pointed out that disruption in SiR expression resulted in concomitant decrease in ATPS4, APR2, Sultr 2, and pathogen defense genes and increase in vegetative storage proteins (VSP1 and VSP2), NITRILASE1 (NIT1) and NIT2, and chlorophyll-degrading gene CHLOROPHYLLASE1, and steady-state levels of most of the S-related metabolites, as well as the expression of many primary metabolism genes, were changed in leaves of sir1-1. Hexose and starch contents were decreased, while free amino acids increased. Inorganic carbon, N, and S composition were also severely altered, demonstrating strong perturbations in metabolism that differed markedly from known sulfate deficiency responses (Khan et al. 2010).

Partitioning of Thiol-Cascade

In a competing pathway, APS-kinase (APSK) phosphorylates APS at the 3′-ribose position to yield 3′-phosphate-5′-adenosine phosphosulfate (PAPS) (Lee and Leustek 1998) as APS + ATP ↔ PAPS + ADP. PAPS provides an S-donor for various sulfotransferases involved in the synthesis of plant hormones, sulfolipids, flavonoids, and glucosinolates. Functional analysis reveals multiple APSK isoforms in *Arabidopsis* (Leustek et al. 2000; Mugford et al. 2009). Interestingly, T-DNA insertional knockout mutants of APSK-1 and APSK-2 in *Arabidopsis* resulted in a dwarfed phenotype and a 450 % increase in Cys content but decrease in secondary metabolites (Mugford et al. 2009). This suggests that partitioning of sulfate flux between the reductive assimilatory and APS phosphorylation pathways is important for growth and development.

Thiol-Metabolisms and Plant Stress Response

Plant homoeostasis is a result of complex regulatory processes that keep plant metabolic events and physiological responses in balance, despite regular, temporary, or localized changes in inputs such as light, nutrients, and water, or other environmental conditions as well as biotic stresses (Tripathi et al. 2012a, b). The system is generally thought to be able to buffer imbalances to a certain extent but may then have to shift to new homoeostatic states when severe deficiencies in nutrient supply or limitations in growth conditions occur. S-metabolism into thiol-containing compounds is critical for protecting plants from oxidative and environmental stresses (Zagorchev et al. 2013). CS overexpression in tobacco confers tolerance to S-containing environmental pollutants (Noji et al. 2001). Although stress signaling pathways are ascertained by transcriptomic analysis and promoter–reporter constructs, recently it has become apparent that posttranscriptional and posttranslational control plays an important role as well. This has been demonstrated, as, for example, in the overexpression of SOS1 under control of constitutive promoter which did not lead to accumulation of the mRNA in control plants but only under salt stress. Genes involved in proline biosynthesis are either under control of miRNA or undergo posttranscriptional regulation. Phosphorylation and ubiquitination are well-recognized processes in salt stress signaling (Kopriva et al. 2012). Therefore, the responses of genes to treatments may not always

be correlated with the responses of the encoding proteins and enzyme activities. This has been demonstrated in reports on individual genes and pathways, as well as on a much larger scale using omics technologies. Proteomics analysis of *Arabidopsis* roots treated with NaCl detected 215 differently abundant spots which resulted in the identification of 86 proteins. Among these, many known stress-related proteins were present, as well as proteins involved in metabolism, protein synthesis, and signal transduction but poorly correlated with their corresponding mRNA data. Similarly, proteome data generated upon infection with bacterial pathogen and circadian changes of 23 enzyme activities did not match greatly with corresponding variations in transcriptomics data from the same material, confirming a large contribution of posttranscriptional and posttranslational regulation (Srivastava et al. 2009). Nevertheless, transcript analysis and especially microarrays using rich repositories are still the most utilizable methods of choice for dissecting plant stress signaling.

Regulations of Thiol-Metabolisms During Salt and Drought Stresses

The S-containing group called thiol is strongly nucleophilic. This is preferably suitable for biological redox reactions and plays an important role in protection against salt and water stress-induced oxidative damage. Substantial level of study indicates that salt tolerance in plants has been associated with their capability to synthesize GSH and indicates a potential role of the S nutrition (Anjum et al. 2012; Talukdar 2011b, c, e, 2012g, 2013j, l, m; Astolfi and Zuchi 2013). Salt stress increases the activities of SAT and OAS-TL leading to higher rate of Cys biosynthesis, which results in increased accumulation of GSH for defense responses to salt stress (Astolfi and Zuchi 2013). Ruiz and Blumwald (2002) reported that S-assimilation rate and biosynthesis of Cys and GSH were greatly increased in *Brassica napus* plant exposed to saline conditions. Furthermore, changes in S-assimilation enzymes have been reported due to salt stress in *Arabidopsis* and broccoli (Lopez-Berenguera et al. 2007), respectively. Overexpression of sulfate transporters, ATP sulfurylase, Cys, OAS, and GSH resulted in increased resistance to oxidative stress (Astolfi and Zuchi 2013). The control of S partitioning and manipulating the synthesis of S-containing compounds in plants using genetic engineering may be a potential option for increasing salt tolerance. In roots of wild-type (WT) *Arabidopsis*, APR activity, protein accumulation, and mRNA levels were increased threefold after 5 h of exposure to 150 mM NaCl. Analysis of various mutants in hormone signaling revealed that the regulation was ABA insensitive; however, the response of APR activity was uncoupled from the mRNA response. However, treatment with EGTA to disrupt Ca^{2+} signaling prevented the increase in both mRNA and enzyme activity upon the salt treatment. In most of the mutants, the APR activity was not increased upon the salt treatment or even decreased, despite the increased mRNA accumulation. Assimilatory sulfate reduction may induce salt tolerance by coordinating various physiological processes and molecular mechanisms which are likely to be induced by phytohormones (Fatma et al. 2013). The involvement of phytohormones in S signaling and salt stress is a complex phenomenon as phytohormones may affect S availability and control gene expression related to S-metabolism (reviewed by Fatma et al. 2013). In a unique study, drought and salt stress tolerance of an *Arabidopsis* glutathione *S*-transferase U17 knockout mutant are attributed to the combined effect of GSH and ABA. GSH contents increased in response to NaCl stress in leaves but not in roots, the primary site of salt exposure, in gray poplar hybrid (Herschbach et al. 2010). The increasing leaf GSH concentrations correlated with stress-induced decreases in transpiration and net CO_2 assimilation rates at light saturation. Enhanced rates of photorespiration could also be involved in preventing ROS formation in chloroplasts and, thus, in protecting PS II from damage. Accumulation of glycine and serine in leaves suggested increasing rates of photorespiration. Since serine and glycine are both immediate precursors of GSH that can limit GSH synthesis, and

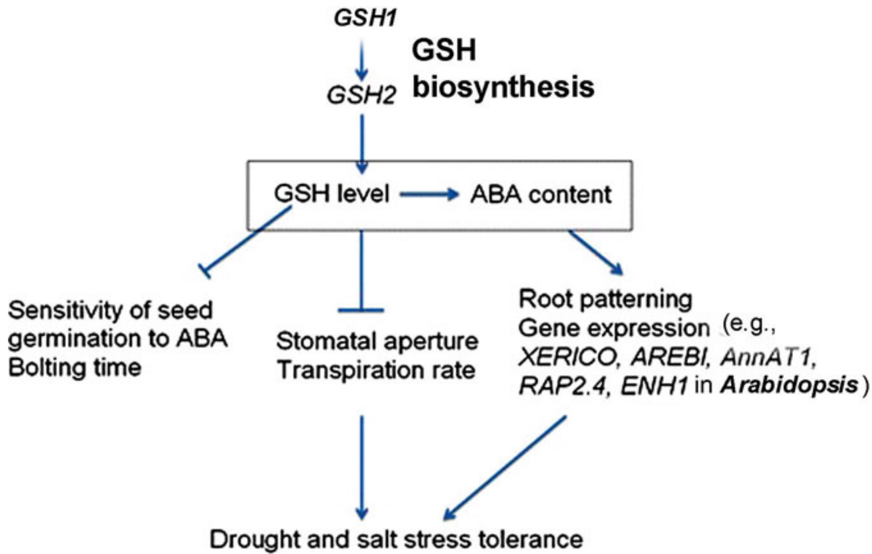

Fig. 2 A simple model for the involvement of GSH, GST, hormonal regulations, and plant phenotypes during modulation of drought and salt stress tolerance in plants

serine being involved in Cys biosynthesis, it is concluded that the salt-induced accumulation of leaf thiol-metabolites such as GSH results from enhanced photorespiration and is thus probably restricted to the cytosol (Herschbach et al. 2010). Similar roles of GSH in modulating plant growth and development have been explored in grass pea, a hardy legume, and in lentil genotypes under water stress (Talukdar 2013l). A model of involvement of GSH in drought and salt stress tolerant has been presented in Fig. 2.

Thiol-Metabolisms and Heavy Metal Stress

The pivotal roles played by thiol-cascade in conferring plants to heavy metal stress were evidenced much earlier by works of Harada et al. (2001) who engineered tobacco plants expressing a rice CS gene to make it tolerant to toxic levels of Cd, a well-known toxic heavy metal. In a significant work, Hossain and Komatsu (2012) used high-throughput comparative proteomic approaches to dissect heavy metal stress responses in soybeans and observed alteration of thiol redox homeostasis. Coordinated expression of GSH metabolic genes has been observed in *Arabidopsis* in response to heavy metal and JA (Xiang and Oliver 1998). Prominent roles of GSH-redox state, its regeneration by GR in AsA–GSH cycle, and modulation of GSH-mediated antioxidant defense have been demonstrated in grass pea mutants overproducing GSH as well as in GSH-deficient condition under Cd stress (Talukdar 2012c, d). In lentil genotypes differing in Cd tolerance, exogenous Ca significantly ameliorated Cd-induced oxidative stress by modulating antioxidant defense in which GSH, GSSG, and GSH redox played essential roles (Talukdar 2012e). These studies clearly pointed out cascading roles of GSH and GSH-derived PCs in mitigating ROS-mediated oxidative stress through induction of the entire AsA–GSH cycle components and their fine-tuned integration with defense components outside this cycle (Talukdar 2012c, d, e). In a unique study, van de Mortel et al. (2008) found expression differences for genes involved in lignin, GSH, and sulfate metabolism in response to Cd in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. Addition of 50 mM $CdCl_2$ to the irrigation medium of mature *Arabidopsis* plants induces a rapid accumulation of the mRNA for

cytosolic OAS-TL isoforms coupled with high increase in Cys content to meet growing demand for GSH and PCs throughout the leaf lamina, the root and stem cortex, and stem vascular tissues, and this suggests that increased Cys availability is responsible for Cd tolerance (Domínguez-Solís et al. 2004). Remarkably, expression of the gene encoding SAT4 responds to sulfate deprivation and to Cd exposure despite presumably contributing little SAT activity in vivo, pointing to a function in thiol-mediated stress response (Kawashima et al. 2005). Transcripts encoding SAT1 also increase 10-fold when a catalase2-deficient mutant of *Arabidopsis* is transferred from high CO_2 concentrations to ambient air, presumably to provide more Cys for GSH synthesis to be used in the detoxification of H_2O_2 by the AsA–GSH cycle (Queval et al. 2009). In the latter case the increase in SAT1 mRNA is accompanied by an increase in total levels of Cys and GSH. It seems therefore that the expression of gene encoding plastidic SAT1 is triggered by oxidative stress even though plastidic SAT activity contributes only about 10 % to the total SAT activity in nonstressed leaves and that metabolic regulation of the CS complexes in the three compartments is responsible for increased production and contents of Cys in response to environmental challenges (Hell and Wirtz 2011). Molecular responses to Cd exposure have also been identified in plants, such as *Brassica juncea*, *Arabidopsis* (Xiang and Oliver 1998), and the other plant species in which Cd exposure induced a coordinated transcriptional regulation of genes encoding γ-ECS, GSHS, and PCS (Sun et al. 2005). Combined transcript, enzymatic, and metabolic profiling in the moss *Physcomitrella patens* revealed vital involvement of sulfate assimilatory genes under Cd stress (Rother et al. 2006). Cd-induced GSH and PC synthesis have a well-established relationship with sulfate uptake, transport, and assimilation in plants in agreement with the concept of demand-driven regulation (Nocito et al. 2006). Besides GSH and hGSH in some legumes, there are other thiol-containing tripeptides in plants. Grasses and rice produce hydroxymethylglutathione, in which a serine replaces the terminal glycine, following exposure to heavy metals (Klapheck et al. 1994). Corn synthesizes an analog with a terminal glutamate, and horseradish generates a tripeptide with a glutamine in place of the glycine following Cd exposure. RNA blot analysis of transcript amounts obtained from roots and leaves of heavy metal accumulator Indian mustard, *Brassica juncea*, indicated enhanced gene expression of ATPS and APR and high level of Cys but decreased expressions of low-affinity sulfate transporters and low level of GSH (Heiss et al. 1999). Examination of steady-state mRNA levels in *Brassica napus* reveals that BnSultr2;2 transcripts were enhanced in leaves of sulfate-deficient plants and under 20–120 μM Cd exposure, but under the same conditions, the BnSultr2;2 expression in roots was severely suppressed (Sun et al. 2005). RT-PCR analysis also demonstrated that BnSultr1;1 was expressed only in roots, and its expression was upregulated by both sulfate deficiency and Cd exposure (Sun et al. 2005). Proteomic analysis in *Arabidopsis* Sultr 4;1 mutant seed revealed overaccumulation of several proteins related to stress response mechanisms (Zuber et al. 2010), of which the most important were the enzymes involved in detoxification processes, a glutathione S-transferase isoform (GST 6), an aldose reductase, a formate dehydrogenase, and a superoxide dismutase. Furthermore, spots corresponding to proteins usually upregulated during stress response and notably oxidative stress, such as glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, were increased in abundance in mutant seeds. Significantly, the mutant seeds possessed higher GSSG levels (46 % against 42 % for wild-type seeds), indicating sulfate remobilization from the vacuole to the other cell compartments is important for the seed's defense against abiotic oxidative stress during seed development and storage (Zuber et al. 2010). An upregulation of Sultr4;1 gene expression concomitantly with an oxidative stress response has also been reported in *Arabidopsis* roots and shoots under Cd stress (Herbette et al. 2006). Both constitutively elevated activity of SAT and concentration of GSH are involved in the ability of nickel-hyperaccumulator *Thlaspi goesingense*

to tolerate nickel. Further study pointed out that all three isoforms of SAT in *T. goesingense* are insensitive to feedback inhibition by Cys (Na and Salt 2011).

Thiol-Metabolisms and Metalloid Stress

Perhaps, the most breathtaking discovery in recent years regarding roles of thiol-cascade in plant stress response has come from studies on arsenic (As) tolerance of crop plants. As is a ubiquitous toxic metalloid and is highly detrimental to plant growth, development, and nutritional quality of edible parts due to formation of ROS and consequently induction of oxidative stress (Gupta et al. 2008; Srivastava et al. 2009; Talukdar 2012f, h, 2013a, h, k; Zhao et al. 2012; Sharma 2013). Functional genomic approaches are very important in analyzing plant As transport and accumulation. Thus, a new concept termed as "Arsenomics" defined as an approach dealing with transcriptome, proteome, and metabolome alterations during As exposure has emerged (Tripathi et al. 2012b). In rice, As(V) induced genes involved in abiotic stress, detoxification pathways, and secondary metabolic process. Genes involved in secondary cell wall biogenesis, cell cycle, and oligopeptide transport were mainly downregulated (Huang et al. 2012). Several lines of evidence point to the coordinated roles of thiols in As tolerance and detoxification (Chakrabarty et al. 2009; Rai et al. 2011; Talukdar 2011f; Tripathi et al. 2012a, b), and GSH plays central role of this detoxification process (Ahsan et al. 2008; Talukdar 2013b), a generalized scheme of which has been shown in Fig. 3. When plants are supplied As^{V}, typically more than 90 % of As within roots is converted to As^{III} (Finnegan and Chen 2012), and the binding of As^{III} to GSH and/or PCs is the basis for the main detoxification

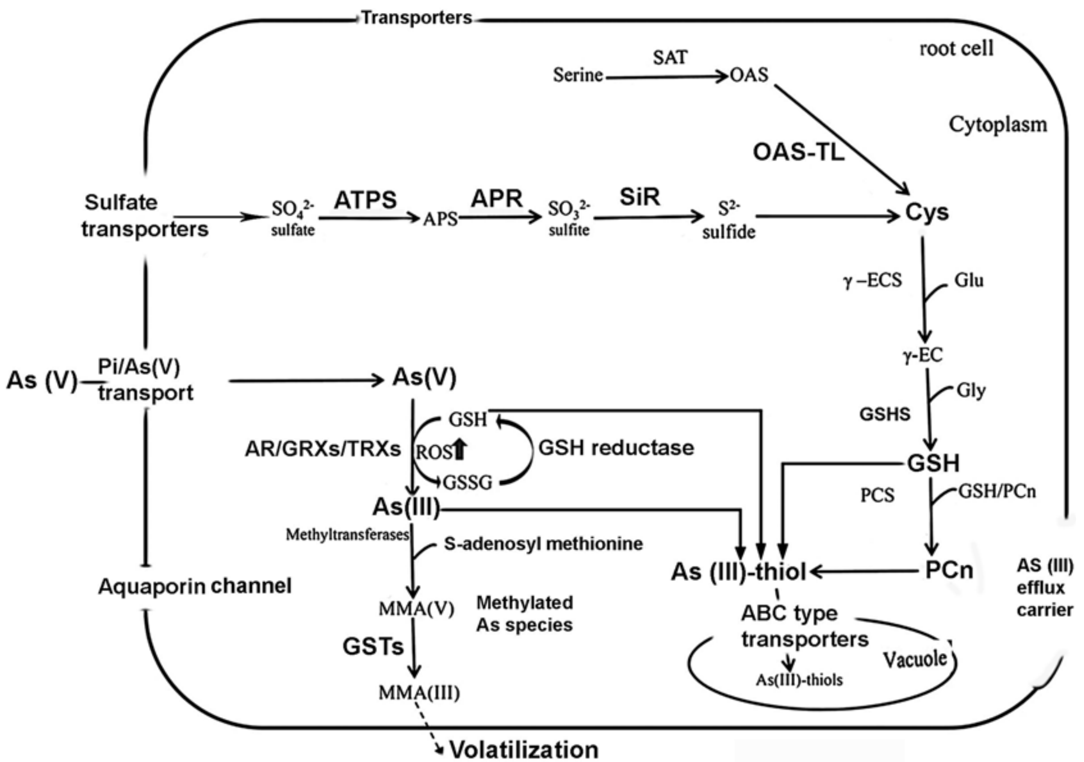

Fig. 3 A generalized scheme for involvement of components of thiol-cascade in arsenic transport and metabolisms in plant root cells

pathway for both As^V and As^{III} (Finnegan and Chen 2012; Zhao et al. 2012). In another study on rice, Tripathi et al. (2012c) observed alteration of various amino acids in rice and its synchronized role with thiolic ligand and potency for As tolerance and detoxification. Thiol biosynthesis-related enzymes were positively correlated to As accumulation-tolerant cultivar while opposite result was noticed in sensitive one (Tripathi et al. 2012b, c). Sung et al. (2009) revealed that ARS5 (arsenate reductase) is a component of the 26S proteasome complex and negatively regulates thiol biosynthesis and As tolerance in *Arabidopsis*. A recent report indicates that As exposure can significantly enhance *Fusarium* wilt infection in grain legumes (Talukdar 2013i) and is a predominant determining factor for high aggressiveness of *Leucaena leucocephala*, an invasive tree in tropical countries (Talukdar 2013d). In both cases, stimulation of GSH-mediated antioxidant defense plays vital roles in maintaining plant growth and seed yield. GSH exclusively requires Cys as one of its building blocks. Therefore, increased Cys biosynthesis to support GSH and PC production would add to the effectiveness of approaches designed to increase nonprotein thiols within plants, a process that would also require inputs from S-metabolism (Finnegan and Chen 2012). A large number of genes involved in thiol synthesis, metabolism, and transport as well as GSH-mediated antioxidant defense have recently been recorded during transcriptomic, proteomic, and metabolic analysis of rice, *Brassica*, lentil, *Pteris*, *Agrostis tenuis*, and aquatic macrophytes challenged with As stress (Norton et al. 2008; Duquesnoy et al. 2009; Srivastava et al. 2009, 2010; Bona et al. 2010; Rai et al. 2011; Tripathi et al. 2012a, b; Talukdar and Talukdar 2013d). The central role played by the GSH and PCs in the detoxification of the metalloid indicates a critical importance for S-metabolism in determining plant survival in As-contaminated soils (Ahsan et al. 2008). Transcriptomic and proteomic analysis revealed that nearly five sulfate transporter genes in rice (Norton et al. 2008; Chakrabarty et al. 2009), four in lentil (Talukdar and Talukdar 2013d), three in *Brassica* (Srivastava et al. 2009), and at least one transporter in *Arabidopsis* (Sung et al. 2009) are upregulated in roots exposed to As. In the Cys synthesizing steps, transcriptional upregulation has been observed for SAT1;1 and SAT1;2 isoforms and at least one OAS-TL each in *Arabidopsis*, rice, *Brassica*, *Hydrilla*, and lentil (Tripathi et al. 2012a, b; Talukdar and Talukdar 2013d). As^V and As^{III} exposure may cause a downregulation of OAS-TL in As-sensitive plants. OAS-TL protein disappeared from maize shoots exposed to As (Requejo and Tena 2006), while OAS-TL activity was repressed in an As-sensitive line of *B. juncea* (Srivastava et al. 2009), compromising Cys biosynthesis and, therefore, As detoxification through GSH and PC. Differential regulations of transcripts were also reported for downstream metabolites like GSH, PCs, and GSH-mediated antioxidant defense comprising different functional isoforms of AsA–GSH cycle enzymes like APX, DHAR, and GR and enzymes outside this cycle like CAT, GPXs, and GSTs in cereals, oilseeds, legumes, aquatic macrophyte *Ceratophyllum demersum*, and medicinal crops under As exposures (Li et al. 2004a, b; Mishra et al. 2008; Talukdar and Talukdar 2013b, c). An arsenate-activated GRX (PvGRX5) isolated from the As-hyperaccumulator fern *Pteris vittata* L. regulates intracellular arsenite and conferred tolerance to transgenic *Arabidopsis* by reducing As accumulation in leaves (Wei et al. 2010). Under AsV stress, the microarray experiment revealed induction of APR3, GST20 (*Tau* class), chloroplast Cu/Zn superoxide dismutase (SOD) (at2g28190), Cu/Zn SOD (at1g08830), and an SOD copper chaperone (at1g12520) in response to treatment (Abercrombie et al. 2008). Like SOD and catalase, GST, GRX and/or peroxidase transcript or protein abundance, or enzymatic activity often increases in response to As exposure (Stoeva et al. 2005; Srivastava et al. 2007; Abercrombie et al. 2008; Ahsan et al. 2008; Norton et al. 2008; Chakrabarty et al. 2009). As an example, in rice, at least 10 GST genes are upregulated in response to As^V exposure, while no more than two GST genes are downregulated (Norton et al. 2008; Chakrabarty et al. 2009). At least one γ-ECS, one PCS, three GST (Lc GSTI,

Lc GSTII, and Lc GSTIII) genes, two GR (Lc GR1 LcGR2), two Cu/Zn SOD (I, II), one each of FeSOD and MnSOD, three APXs (LcAPX1, 2, 3), and two DHAR (LcDHAR1, LcDHAR2) transcripts are differentially regulated in lentil genotypes, differing in As tolerance (Talukdar and Talukdar 2013d). Similarly, in common bean genotypes, differential expression and complementation of SOD isoforms and concomitant effect on GSH-mediated antioxidant defense were observed under As stress (Talukdar and Talukdar 2013b). In As-hyperaccumulator fern *Pteris vittata*, exogenous application of sulfate and GSH enhanced As accumulation (Wei et al. 2010). In *B. juncea* genotypes differing in As tolerance, transcriptional profiling showed an upregulation of sulfate transporters (Sultr2;1 and Sultr4;1), γ-ECS, PCS, and auxin and jasmonate biosynthesis pathway genes, whereas there was a downregulation of ethylene biosynthesis and cytokinin-responsive genes within 6 h of exposure to As^{III} (Srivastava et al. 2009). This suggested that perception of As-induced stress was presumably mediated through an integrated modulation in hormonal functioning that led to both short- and long-term adaptations to combat the stress (Srivastava et al. 2009). It seems likely that in the As-tolerant variety, there was an induction in Cys synthase activity, as well as in the activities of SAT and γ-ECS, the penultimate enzyme in GSH biosynthesis. These increases in enzyme activity were accompanied by increased levels of both Cys and GSH, indicating that increased S-metabolism may be a viable mechanism for increasing As tolerance in plants (Finnegan and Chen 2012). In order to identify the differentially expressed transcripts and the pathways involved in As metabolism and detoxification, *Crambe abyssinica*, the Ethiopian mustard plants, were subjected to arsenate stress, and a PCR-Select Suppression Subtraction Hybridization (SSH) approach was employed (Paulose et al. 2010). A total of 105 differentially expressed subtracted cDNAs were sequenced which were found to represent 38 genes. Transcripts related to thiol-cascade include GSTs which form the largest group in the subtracted cDNA library and enzymes involved in reductive assimilation of S such as APR, APS, and SiR. Most of the transcripts (12 %) of GSTs fall in the *Tau* subfamily (GST-*Tau*), while the remaining sequences (4 %) are similar to the *Phi* subfamily (Paulose et al. 2010). PCs are GSH-derived peptides synthesized in the cytosol where they form PC-metal(loid) complexes that are transported into vacuoles, thus removing these toxic elements from the cytosol. Using transcriptional responses, several vacuolar PCs and other thiol transporters including ABCC-types for long-distance transport of Cd and As have been identified in yeast, Pteris, rice, *Brassica*, and *Arabidopsis* (Indriolo et al. 2010; Song et al. 2010; Mendoza-Cózatl et al. 2011; Huang et al. 2012), and shoot-specific expression of γ-ECS, the first enzyme in GSH biosynthesis, to direct long-distance transport of thiol peptides to roots conferring Cd and As tolerance has been revealed (Li et al. 2004a, b).

Thiol-Metabolisms and Chilling Stress

Role of S-metabolisms and thiol-metabolites in conferring tolerance to chilling stress has been documented (Noctor et al. 2012; Fatma et al. 2013). In maize, a plant with C_4 metabolisms showing Kranz anatomy, chilling stress induces foliar thiol levels and activities of APR, γ-ECS, and GSHS (Kopriva et al. 2009), and total GSH content and the activities of APR and GR are increased in chilling-tolerant maize compared with a sensitive genotype even at standard growth conditions (Kopriva et al. 2009).

Response of Thiol-Metabolisms to S-Status and Selenate Stress

In order to ascertain the effect of sulfate starvation and/or selenate stress on sulfate metabolisms, transcript expression of putative sulfate/selenate transporters from one Se hyperaccumulator, *Astragalus racemosus*, and one closely related nonaccumulator species, *Astragalus drummondii*, was determined by semiquantitative RT-PCR. Transporters belonging to groups 2 and

4 and group 3 of the plant sulfate transporter family were expressed in both root and shoot tissues of *A. racemosus* and *A. drummondii*, whereas the group 1-type transporter genes, which have been shown in other species to be mainly responsible for the initial uptake (Buchner et al. 2004a), were detected only in root tissues. A 16-day time-course experiment on the same taxa revealed (a) modest increases of groups 1 and 4 transcript abundance and no influence on the expression of Sultr2 in root tissues starved for 16 days, (b) no Sultr1 transcript in shoots under sufficient S or under S starvation and a small increase in Sultr4 and no change of Sultr2 abundance in response to S starvation, and (c) that Sultr4 transcripts accumulated more in shoots of nonaccumulating *Astragalus* species compared with the Se-accumulating *Astragalus* species (Cabannes et al. 2011). Global expression profiling of S-starved *Arabidopsis* by DNA macroarray reveals the role of OAS as a general regulator of gene expression in response to S nutrition (Hirai et al. 2003). Koralewska et al. (2008) found differential regulations of gene expressions of sultr 1;1 and sultr 1;2 and APR in *Brassica pekinensis* (Chinese cabbage) by H_2S nutrition and sulfate deprivation. As mentioned earlier, a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana* (Takahashi et al. 1997), and several transporters were later identified (Takahashi et al. 2011).

Thiol-Cascade and Biosynthesis of Proteins Involved in Fatty Acids and Lipids Under Oxidative Stress

Besides proteins directly related to the oxidative stress response, an up-accumulation of proteins involved in the biosynthesis of fatty acids and lipids was revealed. Proteomic and transcriptomic analysis of *Arabidopsis* seeds provided molecular evidence for successive processing of seed proteins and its implication in the stress response to S nutrition. In *Arabidopsis* Sultr 4;1 mutant seeds, proteins such as enoyl-[acyl-carrier-protein] reductase, two isoforms of hydroxysteroid dehydrogenase 1 (HSD1), and ketoacyl carrier protein synthase I were up-accumulated (Zuber et al. 2010). An upregulation of a ketoacyl carrier protein synthase was also observed in developing *Arabidopsis* seeds under S-starved conditions. It is well known that under stress conditions, toxic oxygen derivatives are produced that inactivate enzymes and damage important cellular components, such as membranes by lipid peroxidation and fatty-acid de-esterification. It, thus, seems that the upregulation of enzymes involved in fatty acid and lipid biosynthesis may represent a mechanism to repair stress-induced membrane damage. Transgenic *Arabidopsis* lines overexpressing HSD1 have an increased tolerance to salt stress (Zuber et al. 2010).

Thiol-Cascade and Nutritional Fortifications of Crops

Substantial effort has been expended over many years to enrich the plant parts used as food and feed in essential amino acids, most notably lysine in cereal grains and Cys and methionine in legume seeds (Tabe and Droux 2002; Tabe et al. 2010). Two distinct strategies have been used: manipulation of the pathways of amino acid biosynthesis and creation of a storage sink by expression of a protein rich in the relevant amino acid (Tabe et al. 2010). In *Arabidopsis*, mutant over-accumulating OAS has been characterized (Ohkama-Ohtsu et al. 2004) and seed Met content has been enhanced by reducing the activity of HMT2, a Met biosynthetic enzyme (Lee et al. 2008). Remarkably, tissue-specific gene expressions of sulfate transporter families have been studied in relation to nutrition (Buchner et al. 2010). Overexpression of mutated forms of aspartate kinase and cystathionine γ-synthase in tobacco leaves resulted in the high accumulation of Met and threonine (Hacham et al. 2008). Protein quality in legume crops is limited by the suboptimal levels of the essential S-containing amino acids, Met and Cys. Seed development in pea has been positively modulated by increasing the phloem transport of S-methylmethionine and S as well as N metabolisms (Tan et al. 2010). In the case of the S-containing amino acids, many

different steps of the pathways of reductive S-assimilation and S amino acid biosynthesis have been manipulated in a range of plant species. Increased efficiency of wool growth and live weight gain was recorded in Merino sheep fed transgenic lupin seed containing sunflower albumin (White et al. 2007). A unique feature of *Phaseolus* and several *Vigna* species is the accumulation of a nonprotein amino acid, *S-methyl-Cys*, to a high concentration in seed, of up to 0.3 % per dry weight, mainly as a γ-Glu dipeptide (Liao et al. 2012). *S*-Methyl-Cys cannot substitute for Met or Cys in the diet (Padovese et al. 2001). Major seed proteins in common bean, the 7S globulin phaseolin and lectin phytohaemagglutinin, are poor in Met and Cys. Proteomic analysis identified several S-rich proteins whose levels are elevated in the absence of phaseolin and major lectins, including the 11S globulin legumin, albumin-2, defensin, albumin-1, and the Bowman–Birk-type proteinase inhibitor (Marsolais et al. 2010). Under these conditions, legumin becomes the dominant storage protein, accounting for at least 17 % of total protein. Integration of proteomic and functional genomic data enabled the identification and isolation of cDNAs encoding these proteins (Yin et al. 2011). These characteristics are reminiscent of the *opaque-2* mutant, which was used to develop quality protein maize (Huang et al. 2009). Till date, most approaches to improve protein quality in grain legumes have involved the transgenic expression of S-rich proteins, sometimes in combination with metabolic engineering of S amino acid pathways. In common bean, the expression of Brazil nut 2S albumin increased the Met concentration by 20 % (Aragao et al. 1999) and transcriptomic profiling identified candidate genes associated with the accumulation of distinct sulfur γ-glutamyl dipeptides in *Phaseolus vulgaris* and *Vigna mungo* seeds (Liao et al. 2013). In lupin and chickpea, expression of sunflower seed albumin (SSA) stimulated S-assimilation. S was shifted from the sulfate to the protein Met pool, elevated by 90 %, while the concentration of Cys was reduced by 10 % (Molvig et al. 1997; Tabe and Droux 2002; Chiaiese et al. 2004). Expression in developing lupin embryos of a SAT from *Arabidopsis thaliana* (AtSAT1 or AtSerat 2;1) was associated with increases of up to 5-fold in the concentrations of OAS, the immediate product of SAT, and up to 26-fold in free Cys, resulting in some of the highest in vivo concentrations of these metabolites yet reported (Tabe et al. 2010). In *Vicia narbonensis*, which accumulates little sulfate in mature seed, co-expression of Brazil nut 2S albumin with a feedback-insensitive, bacterial Asp kinase increased Met and Cys concentrations by 100 % and 20 %, respectively (Demidov et al. 2003). The increased levels of Met and Cys were accompanied by decreases in the concentration of γ-Glu-*S*-ethenyl-Cys (2-fold) and free thiols, particularly γ-GluCys and GSH. About two-third increase in Met and Cys concentration was attributed to an enhanced supply of S to the seed. In soybean, transgenic expression of Brazil nut 2S albumin increased Met concentration by 26 %, while expression of 15 kDa δ-zein increased Met and Cys concentrations by 20 % and 35 %, respectively (Dinkins et al. 2001). Constitutive overexpression of a cytosolic form of OAS-TL in transgenic soybean led to sustained enzymatic activity at the late stages of seed development and resulted in a 70 % increase in total Cys concentration in mature seed (Kim et al. 2012). This was associated with enhanced levels of the endogenous Cys-rich protein, the Bowman–Birk protease inhibitor. Two types of novel protein bodies in transitional cells situated between the vascular tissue and storage parenchyma were obtained from transgenic soybean plant overexpressing an 11 kDa Met-rich delta-zein (Kim and Krishnan 2004). High-level expression of maize γ-zein protein has been obtained (Li et al. 2005), and protease inhibitor activity has been reduced by expression of a mutant Bowman–Birk gene in soybean seed (Livingstone et al. 2007). Furthermore, S-assimilation and Cys biosynthesis has been manipulated towards engineering seed S amino acid content in food and feed (Krishnan 2008; Jez and Krishnan 2009). Although mature seed is the primary target of nutritional biofortification process, studies revealed S-metabolisms and transportations of thiol compounds in developing legume seeds. In

soybean, sulfate in pods is transformed into homoglutathione, which is mobilized into developing seed (Anderson and Fitzgerald 2001). While homoglutathione contributes Cys, *S*-methyl-Met is anticipated to be a major form of Met transported to the seed. Recent functional genomic studies have highlighted the occurrence of complete pathways of sulfate assimilation and de novo Cys and methionine biosynthesis in developing seed, both in soybean and common bean (Yi et al. 2010; Yin et al. 2011). Seed-specific transgenic expression of a chloroplastic, feedback-insensitive SAT in lupin increased the concentration of OAS and free Cys (Tabe et al. 2010). But the total concentration of Cys and Met could not be enhanced, even after co-segregation of the SSA transgene. This suggests that sulfate assimilation and Cys biosynthesis are regulated independently from Met biosynthesis, which is part of the aspartic acid-derived amino acid pathway (Galili et al. 2005). A global analysis of transcripts and free amino acids spanning the developmental period of γ-Glu-*S*-methyl-Cys accumulation in seeds of common bean revealed that (a) during seed development, phaseolin and phytohaemagglutinin transcripts are most abundant in cotyledonary stage, while phaseolin as a protein accumulates during maturation; (b) seed desiccation takes place during maturation stage. Hierarchical clustering of expression values for major seed protein transcripts that were differentially expressed between common bean genotypes of SARC1 and SMARC1N-PN1 throughout all four developmental stages were consistent (Liao et al. 2012) with previous proteomic findings (Marsolais et al. 2010); and (c) lower expression values were systematically observed for phaseolin, arcelin, lectin, and most phytohaemagglutinin contigs in SMARC1N-PN1. The microarray data were examined for differential expression of transcripts coding for S-rich proteins (Liao et al. 2012). S-rich proteins previously identified by proteomics as elevated in SMARC1N-PN1 also had increased transcript levels. They include legumin, albumin-2, defensin D1, albumin-1A and albumin-1B, and the Bowman–Birk-type proteinase inhibitor. Transcripts of additional types of S-rich proteins were identified as differentially expressed, including the basic 7S globulin, double-headed trypsin, and Kunitz trypsin protease inhibitors. Soybean basic 7S globulin, known as γ-conglutin in lupin, is a minor Cys-rich globulin with structural similarity to xyloglucan-specific endo-β-1,4-glucanase inhibitor-like protein (Scarafoni et al. 2010; Yoshizawa et al. 2011), which has insulin-binding properties and glucose-lowering nutritional effects (Hanada and Hirano 2004; Magni et al. 2004; Lovati et al. 2012). Transcripts were most elevated for albumin-1A (10-fold), albumin-1E (7-fold), and albumin-1F contigs (11-fold) and the three differentially expressed genes coding for the Bowman–Birk-type proteinase inhibitor 2 and the double-headed trypsin inhibitor/trypsin chymotrypsin inhibitor. Differential expression was observed at distinct developmental stages, also. Albumin-2, albumin-1E, the Bowman–Birk-type proteinase inhibitor 2, and the double-headed trypsin inhibitor contigs had increased transcript levels starting from cotyledonary stage. This was followed by legumin and basic 7S globulin-2 at cotyledonary stage. Defensin D1 was differentially expressed at early maturation stage and albumin-1A and albumin-1B at final maturation stage (Liao et al. 2012). Transcripts of the Kunitz trypsin protease inhibitor were elevated from early maturation stages to final maturation. In parallel with transcripts of Cys-rich proteins, those of three different chaperones involved in the formation of disulfide bridges were also elevated (Liao et al. 2012). Besides, transcripts coding for enzymes associated with several processes of S-metabolism were significantly increased; notable among these are-Sultr 1;2, Sultr 3;3, ATPS1, APSR1, SAT1;1, SAT1;2, and SAT 2;1 (Liao et al. 2012). The transcript profiling results focus on a remarkable coordination in the expression of S-metabolic genes, which includes those participating in sulfate transport and assimilation, de novo Cys, and Met biosynthesis and further confirms the fact that in vegetative tissue, transcription of S uptake and assimilation is regulated by demand, with feedback inhibition by GSH and stimulation by OAS (Liao et al. 2012). In transgenic seeds expressing SSA, upregulation of endogenous

S-rich proteins was associated with reduced levels of GSH in rice (Hagan et al. 2003) and increased levels of OAS in chickpea (Chiaiese et al. 2004). Howarth et al. (2009) identified a unique S deficiency-induced gene *sdi1*, involved in the utilization of stored sulfate pools under S-limiting conditions. This gene has potential as a diagnostic indicator of S nutritional status.

Plant Nuclear Ploidy, Sexual Reproduction, and Thiol-Metabolisms

Unique effect of the genome structure in determining the size of the sulfate transporter gene family has been studied in the Poaceae and Fabaceae. Phylogeny and expression of paralogous and orthologous sulfate transporter genes have been studied in diploid and hexaploid wheat (Buchner et al. 2004b). The partially diploidized tetraploid soybean (*Glycine max*) genome contains in total 28 sulfate transporter genes indicating gene duplications of the individual group isoforms (http://www.phytozome.net/). In contrast, in the diploid *Medicago truncatula* genome (www.medicago-hapmap.org), only 10 sulfate transporter genes have been detected. The "New World" *Astragalus* species, which include *A. crotalaria*, *A. drummondii*, *A. racemosus*, and *A. bisulcatus*, are mostly aneuploid, with a chromosome number based on $n = 11$ to 15, in contrast to the "Old World" euploid *Astragalus* ($n = 8$) species, which include *A. glycyphyllos* (Cabannes et al. 2011). Three very closely related group 1 high-affinity sulfate transporter genes (Sultr1a–Sultr1c) were identified in *A. racemosus*. Using the same RT-PCR approaches, a Sultr1b isoform from A. *crotalaria, A. bisulcatus*, and *A. drummondii* and Sultr1a and Sultr1b isoforms from *A. glycyphyllos* were isolated (Cabannes et al. 2011). No Sultr2;1 type was isolated from the *Astragalus* species, but a Sultr 2;2 isoform has been phylogenetically close to *Arabidopsis* Sultr 2;2 (Buchner et al. 2010; Cabannes et al. 2011). However, group 3 Sultr3;4 type was identified in *A. drummondii, A. racemosus*, and *A. bisulcatus*, and a group 4 type was identified from *A. racemosus* (Cabannes et al. 2011).

ROS, reactive nitrogen species (RNS), GSH and other classic buffer molecules or antioxidant proteins, and some thiol/disulfide-containing proteins belonging to the thioredoxin superfamily, like glutaredoxins (GRXs) or thioredoxins (TRXs), form a complex network of redox regulations. These components participate as critical elements not only in the switch between the mitotic to the meiotic cycle but also at further developmental stages of microsporogenesis, regulation of pollen rejection as the result of self-incompatibility, and display precise space-temporal patterns of expression and are present in specific localizations like the stigmatic papillae or the mature pollen (Traverso et al. 2013). Gene expressions of several TRXs such as TRXh1, TRXh4, TRXh5, and GRX in *Arabidopsis*, rice, *Pisum sativum*, *Crocus sativus* (saffron), and *Nicotiana* have been found to be involved in the development of stigma, style, anther, and pollen–pistil interactions and during growth of pollen tube (reviewed Traverso et al. 2013).

Origin of "Thiolomics": Progress and Future Prospects in Crop Improvement

Continuous increase in global population along with the growing urbanization and impending climate change imposes significant pressure for increasing agricultural crop productivity and nutritional quality. Crop yield is a complex trait and is found to be dependent upon three interdependent factors such as generation of photosynthetic reductant, its assimilation into the carbon product, and then translocation in different plant parts. In recent years, various transgenic-based approaches have been tested to modulate source and sink strength; however, limited success has been achieved in the terms of increased crop yield. As an alternate strategy, the concept of strengthening the plant's built in mechanism using priming mediated physiological tuning, which does not involve any genetic modification,

can be useful. Thiourea (TU), a known ROS scavenger and sulfhydryl regulator, governs source-to-sink relationship in plants through sugar dynamics, based upon the fact that most of the steps for generating photoassimilates at source and its translocation towards sink are regulated in a redox state-dependent manner (Srivastava et al. 2010; Pandey et al. 2013). This non-transgenic-based priming method has recently been successfully utilized to enhance crop productivity and nutritional biofortifications and to impart stress tolerance in crops such as mustard, wheat, mung bean, salt grass, potato, and maize (Pandey et al. 2013). High-throughput omics techniques are extensively being exploited in recent times to dissect plant molecular strategies of thiol-cascade in regulating plant growth, development, metabolisms, and stress tolerance. All these events are neatly interwoven with each other, and plant S-metabolisms, thiolic potency, its dynamisms, and regulations in each and every events either directly or indirectly focus the facts that thiol-cascade is in the central of plant growth. The components of this cascade can be effectively manipulated in favor of crop yield and nutritional quality using power of omics technology specifically employed in plant thiol-metabolisms through "thiolomics."

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