

INVITED REVIEW

Regulation of Sulfate Assimilation in *Arabidopsis* and Beyond

STANISLAV KOPRIVA*

John Innes Centre, Norwich NR4 7UH, UK

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• **Background and Aims** Sulfate assimilation is a pathway used by prokaryotes, fungi and photosynthetic organisms to convert inorganic sulfate to sulfide, which is further incorporated into carbon skeletons of amino acids to form cysteine or homocysteine. The pathway is highly regulated in a demand-driven manner; however, this regulation is not necessarily identical in various plant species. Therefore, our knowledge of the regulation of sulfate assimilation is reviewed here in detail with emphasis on different plant species.

• **Scope** Although demand-driven control plays an essential role in regulation of sulfate assimilation in all plants, the molecular mechanisms of the regulation and the effects of various treatments on the individual enzymes and metabolites are often different. This review summarizes (1) the molecular regulation of sulfate assimilation in *Arabidopsis thaliana*, especially recent data derived from platform technologies and functional genomics, (2) the co-ordination of sulfate, nitrate and carbon assimilations in *Lemna minor*, (3) the role of sulfate assimilation and glutathione in plant–*Rhizobia* symbiosis, (4) the cell-specific distribution of sulfate reduction and glutathione synthesis in C₄ plants, (5) the regulation of glutathione biosynthesis in poplar, (6) the knock-out of the adenosine 5′phosphosulfate reductase gene in *Physcomitrella patens* and identification of 3′-phosphoadenosyl 5′-phosphosulfate reductase in plants, and (7) the sulfur sensing mechanism in green algae.

• **Conclusions** As the molecular mechanisms of regulation of the sulfate assimilation pathway are not known, the role of *Arabidopsis* as a model plant will be further strengthened. However, this review demonstrates that investigations of other plant species will still be necessary to address specific questions of regulation of sulfur nutrition.

Key words: Sulfate assimilation, plant nutrition, glutathione, cysteine, C₄ photosynthesis, *Arabidopsis thaliana*, *Physcomitrella patens*, poplar, *Lemna minor*.

INTRODUCTION

Sulfur is found in nature in different oxidation states in inorganic, organic and bioorganic forms. For living organisms sulfur is an essential element with many different functions. It is found in reduced form in amino acids, peptides and proteins, in iron–sulfur clusters, lipoic acid and other co-factors, and in oxidized form as sulfonate group modifying proteins, polysaccharides and lipids. In addition, reduced sulfur compounds, such as hydrogen sulfide, serve as electron donors for chemotrophic or phototrophic growth in a large and diverse group of bacteria and archae, including purple and green sulfur bacteria (Trüper and Fischer, 1982). By contrast, oxidized sulfur compounds such as sulfate can function as terminal electron acceptor in respiration to support growth of sulfate-reducing bacteria (Postgate, 1984). Plants, yeast and most prokaryotes cover their demand for reduced sulfur by reduction of inorganic sulfate to sulfide, which is then incorporated into organic compounds. Sulfur is the least abundant of the six macronutrients required by plants, and perhaps therefore its metabolism has been least studied. In the last decade, however, significant progress in understanding the pathway of sulfate assimilation in plants has been made, and has been summarized in several reviews (Hawkesford and Wray, 2000; Leustek *et al.*, 2000; Kopriva and Koprivova, 2004; Kopriva and Rennenberg, 2004; Saito, 2004). A combination of biochemical and molecular methods have revealed that adenosine 5′phosphosulfate reductase is the

key enzyme of this pathway (Koprivova *et al.*, 2000; Suter *et al.*, 2000; Bick *et al.*, 2001; Tsakraklides *et al.*, 2002; Vauclare *et al.*, 2002). Recently, a combination of transcriptome and metabolome analyses has significantly increased our knowledge of the cellular processes affected by sulfur starvation, which is becoming a serious problem in agriculture (Hirai *et al.*, 2003, 2004, 2005; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003, 2005). Furthermore, the first reports on molecular mechanisms of regulation of sulfate uptake and assimilation have emerged very recently, opening a new era of our understanding of the pathway (Maruyama-Nakashita *et al.*, 2004, 2005). These new developments were enabled by the availability of numerous platform resources for the model plant *Arabidopsis thaliana*. However, it is also becoming increasingly apparent that not all biological questions on sulfur metabolism can be addressed in *Arabidopsis* and that not all knowledge gained on this model is transferable to other species. In this review, therefore, the newest developments in the field of plant sulfur nutrition will be summarized with special emphasis on choice of different plant species for particular experiments and on differences in regulation of the pathway in different plants.

PLANT SULFUR ASSIMILATION

The major source of sulfur for plants is inorganic sulfate, although they are also able to use reduced sulfur compounds from the atmosphere, such as sulfur dioxide or hydrogen sulfide (Leustek *et al.*, 2000). Sulfate is taken up and

* For correspondence. E-mail stanislav.kopriva@bbsrc.ac.uk

distributed within cells and the plant as a whole by sulfate transporters. Plants possess multiple sulfate transporters with different properties and functions (reviewed in Buchner *et al.*, 2004). The first plant sulfate transporters were cloned from a tropical legume, *Stylosanthes hamata*, by Smith *et al.* (1995), who utilized functional complementation screening of a yeast sulfate transporter mutant. *Arabidopsis* possess 14 genes for sulfate transporters, divided into five groups according to sequence similarity and function. All these genes have significant sequence similarities with the originally identified cDNA clone, SHST1 (Smith *et al.*, 1995). However, an exception appears with the group 5 members, which show limited sequence identity and structural similarity with the remaining sulfate transporter family members, and their transport activity has yet been unverified. High-affinity sulfate transporters, group 1, are responsible for uptake of sulfate from soil solution into the root cells (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002). Low-affinity transporters, group 2, are required for translocation of sulfate within the plant; therefore, they are localized in xylem parenchyma and phloem cells of roots and leaves (Takahashi *et al.*, 2000). Group 4 transporters are localized in tonoplast and are responsible for sulfate efflux from the vacuole (Kataoka *et al.*, 2004a). Very little is known about the function of sulfate transporters of groups 3 and 5, except a demonstrated ability of SULTR3;5 to increase the rate of root-to-shoot sulfate translocation in *Arabidopsis* (Kataoka *et al.*, 2004b). Unexpectedly, group 3 sulfate transporter was identified as essential for nitrogen fixation in legume nodules (Krusell *et al.*, 2005; see also below). The identity of the plastidic sulfate uptake system remains one of the greatest challenges for the sulfate transport community.

Intracellular sulfate is further metabolized into a large variety of primary and secondary metabolites. For assimilation into cysteine, sulfate has to be transported into plastids and activated by adenylation to adenosine 5' phosphosulfate (APS) in a reaction catalysed by ATP sulfurylase (ATPS; EC 2.7.7.4). APS is reduced to sulfite by APS reductase (APR; EC 1.8.4.9); the electrons are derived from glutathione. 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 (OASTL; 2.5.1.47) into the amino acid skeleton of *O*-acetylserine (OAS) to form cysteine. OAS is synthesized by acetylation of serine with acetyl-CoenzymeA catalysed by serine acetyltransferase (SAT; EC 2.3.1.30) (Leustek *et al.*, 2000; Suter *et al.*, 2000; Kopriva and Koprivova, 2003; Fig. 1). SAT and OASTL form a multi-enzyme complex of cysteine synthase (Hell *et al.*, 2002). Cysteine can be directly incorporated into proteins or peptides, such as glutathione (GSH), the most abundant low-molecular-weight thiol with a plethora of functions in plant stress defence, redox regulation, and sulfur storage and transport. Alternatively, cysteine is further metabolized and serves as a donor of reduced sulfur for synthesis of methionine, iron-sulfur centres, and various coenzymes and secondary metabolites. An intermediate of the sulfate assimilation pathway, sulfite, is metabolized into sulfolipids, essential components of chloroplast membranes (Sanda *et al.*, 2001). Whereas

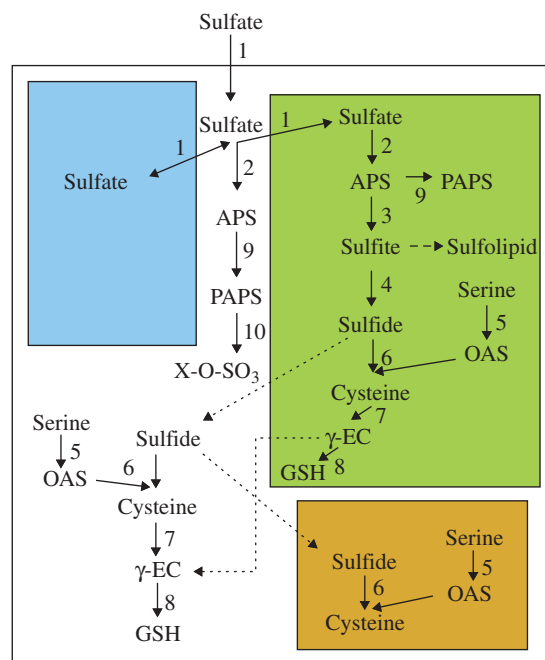


FIG. 1. Schema of plant sulfur assimilation and subcellular localization of its major steps. Green colour represents plastids, brown mitochondria and blue vacuoles. Numbers represent enzymes as follows: 1, sulfate transporter; 2, ATP sulfurylase; 3, APS reductase; 4, sulfite reductase; 5, serine acetyltransferase; 6, *O*-acetylserine thiolylase; 7, γ -glutamylcysteine synthetase; 8, glutathione synthetase; 9, APS kinase; 10, sulfotransferase. Dashed lines represent multiple reaction steps; dotted lines indicate unconfirmed transport steps.

sulfate reduction is localized in plastids only, cysteine is synthesized in all three compartments capable of protein synthesis: plastids, mitochondria and the cytosol (Leustek *et al.*, 2000).

Sulfur is, however, also present in plant metabolites in the oxidized state as a sulfo-group modifying carbohydrates, proteins and many natural products. A large proportion of the known sulfated metabolites play various roles in plant defence against biotic and abiotic stress. A well-studied example of such compounds is glucosinolates, which participate in defence against herbivores and pathogens in *Brassicales* (Mikkelsen *et al.*, 2002). They are also responsible for taste and flavour of cruciferous vegetables, and some of their degradation products, isothiocyanates, possess an anti-cancer activity (Mithen, 2001; Fahey *et al.*, 2001). Another large group of sulfated compounds important for medicine are sulfated flavonoids, present in more than 250 species of 32 families (Barron *et al.*, 1988), where they are involved in detoxification of reactive oxygen species and regulation of plant growth (Varin *et al.*, 1997). Several other sulfated compounds were shown to participate directly in plant defence against pathogens, such as a sulfated derivative of jasmonic acid identified in *Arabidopsis* (Gidda *et al.*, 2003) or sulphated β -1,3 glucan (Menard *et al.*, 2004). The transfer of the sulfo-group, i.e. sulfation, is catalysed by sulfotransferases (SOT) (Klein and Papenbrock, 2004). The SOT reaction requires 3'-phosphoadenosyl 5'-phosphosulfate (PAPS) as the sulfate donor and a compound with a free hydroxyl group as

an acceptor. Multiple SOT isoforms are found in higher eukaryotes because of the structural diversity of the biological acceptors of the sulfate group (Klein and Papenbrock, 2004; Table 1). PAPS is synthesized by phosphorylation of APS by APS kinase (Fig. 1). APS thus can be withdrawn from the primary sulfate assimilation pathway in plastids. In addition, there seems to be a sulfation-dedicated PAPS synthesis in the cytosol, as ATPS and APS kinase activity are present both in plastids and in the cytosol, in contrast to APR and SiR, which are strictly plastidic (Leustek *et al.*, 2000; Rotte and Leustek, 2000; Koprivova *et al.*, 2001).

Very recently, a further complexity was added to the sulfate assimilation pathway. First, sulfite oxidase, an enzyme oxidizing sulfite to sulfate, was detected in plants (Eilers *et al.*, 2001). This enzyme is responsible for catabolism of sulfur-containing metabolites in animal mitochondria (Kisker *et al.*, 1997). In plants it is localized in peroxisomes, i.e. spatially separated from sulfate assimilation, but its function is not yet known (Nowak *et al.*, 2004). Secondly, PAPS reductase, an enzyme reducing PAPS to sulfite in yeast, fungi and some enteric bacteria, was found in the moss *Physcomitrella patens* (Koprivova *et al.*, 2002a; see also below). The greatest progress in understanding the regulation of sulfate assimilation has been achieved in *Arabidopsis thaliana*. This recent development is therefore summarized in the next section, before the species-specific variations of sulfur metabolism are discussed.

SULFATE ASSIMILATION IN *ARABIDOPSIS THALIANA*

Arabidopsis thaliana became the model plant for studies of sulfur assimilation only recently, when the Arabidopsis sequencing project was initiated. This plant was long used predominantly for molecular cloning (Hell *et al.*, 1994; Leustek *et al.*, 1994; Brühl *et al.*, 1996; Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996; Noji *et al.*, 1998). These experiments revealed a great complexity of the sulfate assimilation pathway in *Arabidopsis*. With the exception of SiR and the enzymes of GSH synthesis, all other enzymes were encoded by small gene families (Table 1). It is not yet clear whether this is a functional redundancy or if all genes have a specific function. The sulfate transporter family is the best understood in this respect (Takahashi *et al.*, 2000; Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002, 2003; Kataoka *et al.*, 2004a, b). Each analysed isoform has a specific localization, affinity to sulfate or regulation, indicating different biological functions. Differences in subcellular localizations and in kinetic parameters were found also among the five members of the *Arabidopsis* SAT gene family (Kawashima *et al.*, 2005); however, their different functions remain to be demonstrated *in vivo*, e.g. by analysis of knock-out lines. By contrast, very little is known about the individual members of the ATPS, APR and APS kinase families. In the case of ATPS, all four genes code for proteins with putative organelle targeting peptides, so that it is not known which isoform is responsible for the cytosolic ATPS activity. This contrasts with other plant species, e.g. rice or

TABLE 1. Genomic organization of transporters and enzymes for sulfate assimilation, GSH synthesis and sulfation reactions in sequenced plant and algal genomes

	<i>Arabidopsis thaliana</i>	<i>Oryza sativa</i>	<i>Populus trichocarpa</i>	<i>Chlamydomonas reinhardtii</i>
SULTR	14	13	14	5
ATPS	4	2	4	1
APS kinase	4	3	3	1
APR	3	2	2	1
SiR	1	2	2	1
OASTL	9	9	10	4
SAT	5	5	5	2
γ -ECS	1	2	2	1
GSHS	1	3	3	1
SOT	22	27	22	0

The number of genes was determined by tblastn against genomic sequences available in GenBank (*Arabidopsis thaliana*, *Oryza sativa*) or from the DOE Joint Genome Institute website (<http://genome.jgi-psf.org/>).

ATPS, ATP sulfurylase; APS, adenosine 5' phosphosulfate; APR, APS reductase; SiR, sulfite reductase; OASTL, *O*-acetylserine (thiol)lyase; SAT, serine acetyltransferase; γ -ECS, γ -glutamylcysteine synthetase; GSHS, GSH synthetase; SOT, sulfotransferase.

potato, for which a cytosolic and plastidic ATPS were clearly identified (Klonus *et al.*, 1994; Table 1). GSH biosynthesis is also differently organized in *Arabidopsis* and other plant species. Whereas in *Arabidopsis* and possibly other *Brassicales* the first enzyme of GSH synthesis, γ -glutamylcysteine synthetase (γ -ECS), seems to be exclusively localized in plastids and the second one, GSH synthetase (GSHS), is dually targeted to plastids and the cytosol from a single gene (Wachter *et al.*, 2005), other plant species contain multiple copies of both genes (Table 1) and possess γ -ECS activity also in the cytosol (Hell and Bergmann, 1990).

The availability of cDNA sequences allowed using *Arabidopsis* for investigation of regulation of sulfate metabolism at the molecular level. Many reports primarily addressed changes in accumulation of mRNA of sulfate assimilation genes; for example, Takahashi *et al.* (1997) showed that a low-affinity sulfate transporter, SULTR2;1, and one isoform of APR and SAT were strongly induced by 2 d of sulfate starvation, and Harada *et al.* (2000) demonstrated regulation of sulfate assimilation by jasmonate. However, it was also possible to conduct more complex studies of the level of enzyme activities, mRNA and protein accumulation, metabolites (Cys and GSH), and even fluxes through the pathway. The first among such studies was the investigation of the control of sulfate assimilation by light (Kopriva *et al.*, 1999). APR activity was shown to undergo a diurnal rhythm with maximum activity 4 h after light onset and minimum activity at the beginning of the night. Furthermore, sucrose was able to mimic the effect of light, revealing an interaction of sulfate assimilation with carbon metabolism (Kopriva *et al.*, 1999). Sulfate assimilation is also strongly interconnected with the assimilation of nitrogen (Reuveny *et al.*, 1980; Brunold and Suter, 1984). In *A. thaliana* withdrawal of nitrogen from nutrition for 3 d led to a specific decrease of APR activity, whereas OASTL

and thiol contents were not affected (Koprivova *et al.*, 2000). In all these experiments, the changes in APR activity corresponded to changes in mRNA levels of all three APR isoforms and APR protein accumulation, showing that APR is primarily regulated on the level of transcription. In addition, however, Bick *et al.* (2001) revealed a further, post-translational level of APR regulation by redox processes. These and other experiments concentrated on APR, as this enzyme was long known to be strongly regulated by various environmental factors, nutrient availability and stress (Brunold, 1990). The finding that treatment of *Arabidopsis* root cultures with thiols reduced solely APR, but not ATPS, SiR or OASTL, enabled use of feeding experiments with $^{35}\text{SO}_4^{2-}$ for control flux analysis to quantify the contribution of APR in the control of sulfate assimilation (Vauclare *et al.*, 2002). Starting from internal sulfate the flux control coefficient of APR was calculated to be between 0.7 and 0.9 (equivalent to 70 and 90% of the total control), indicating strong control of the pathway by APR. However, because sulfate uptake was even more inhibited by GSH than APS reduction, APR shares control of the flux with the sulfate uptake system. APR is thus indeed a key enzyme of the sulfate reduction pathway (Vauclare *et al.*, 2002). This conclusion was further corroborated by Tsakraklides *et al.* (2002), who demonstrated that expression of bacterial APR in *Arabidopsis* results in deregulation of the pathway and accumulation of thiols and other reduced sulfur compounds, such as thiosulfate.

In the search for molecular mechanisms of regulation of sulfate assimilation, several potential signals were identified (Fig. 2). The most prominent signal is the precursor of cysteine, OAS. OAS has been identified as a limiting factor for cysteine synthesis (Rennenberg, 1983) and was shown to induce APR activity and rate of thiol synthesis in *Lemna minor* (Neuenschwander *et al.*, 1991). In *Arabidopsis*, OAS induced mRNA accumulation of all genes of sulfate assimilation and dramatically increased flux through sulfate assimilation (Koprivova *et al.*, 2000). OAS also strongly affects the cysteine synthase complex: even a less than two-fold increase in OAS concentration results in dissociation of the complex and inactivation of SAT (Berkowitz *et al.*, 2002). Because OAS accumulates during sulfur deficiency, and because of its effects on cysteine synthase and expression of sulfate assimilation genes, OAS was proposed to act as a mediator of plant sulfur status (Hell *et al.*, 2002). Accordingly, the activity of a β_{SR} S-responsive region of the β -subunit of soybean conglycinin (Awazuhara *et al.*, 2002) was up-regulated by OAS (Ohkama-Ohtsu *et al.*, 2005). In the search for mutants with altered sulfur deficiency response using the β_{SR} -driven green fluorescent protein (GFP) expression as a tool, a mutant accumulating OAS was identified, again indicating OAS as a signal of sulfur deficiency (Ohkama-Ohtsu *et al.*, 2005). This conclusion was further strengthened by a transcriptome analysis suggesting the role of OAS as general regulator of gene expression (Hirai *et al.*, 2003). The mRNA levels of more than 850 genes were affected by treatment of *Arabidopsis* with 1 mM OAS for 48 h. However, the correlation of changes in expression levels after sulfur deficiency and OAS treatment was significant only in the leaves. Further

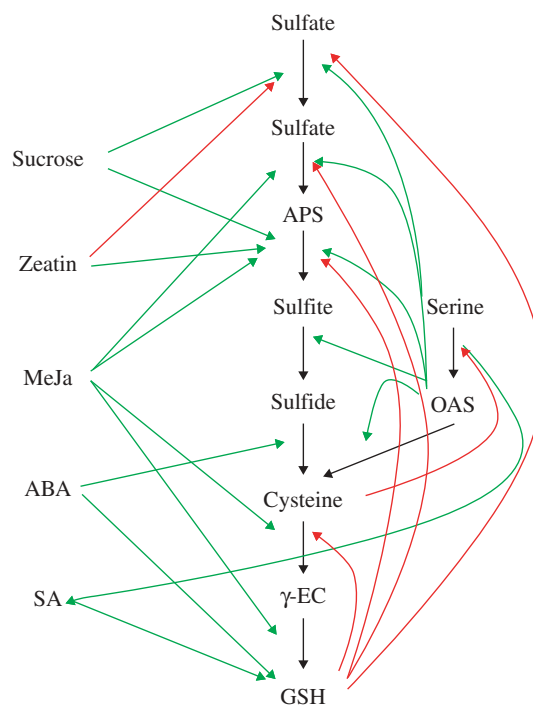


FIG. 2. Schematic representation of putative signals involved in regulation of sulfate assimilation and GSH synthesis. Red arrows represent repression of the metabolic step; green arrows indicate an increase in metabolite accumulation (pointed towards metabolite) or induction of the reaction.

analysis of metabolite-to-gene networks revealed that expression of several genes of sulfate assimilation, such as ATPS3, APR2 and 3, SULTR1;1, 1;2 and 2;1, was indeed tightly linked to OAS level (Hirai *et al.*, 2005). Transcript levels of other genes also regulated by sulfur deficiency, such as SiR, APS kinase or group 3 sulfate transporters, by contrast, were not correlated with OAS, showing that OAS cannot be the sole sensor of sulfur deficiency (Hirai *et al.*, 2005). It is thus evident that OAS is an important player in regulation of plant sulfur homeostasis but its exact molecular function and the other components of the regulatory circuit are unresolved.

The general role of OAS as signal in the sulfur deficiency response was further questioned in experiments with potato, in which induction of sulfate uptake and mRNA levels of sulfate transporter and APR by sulfur starvation preceded accumulation of OAS (Hopkins *et al.*, 2005). Whether this is due to different regulatory circuits in *Arabidopsis* and potato remains to be elucidated. This explanation is possible, as it is not the first major variation to be described in the regulation of sulfur metabolism between the two species. The cystathionine γ -synthase (CgS), first step in synthesis of methionine from cysteine and O-phosphohomoserine, is strongly down-regulated by Met. In *Arabidopsis* this is achieved by S-adenosylmethionine-dependent degradation of CgS mRNA (Chiba *et al.*, 2003). The CgS protein plays an important role in this autoregulation, as *mto1* mutants accumulating Met and CgS transcript have alterations in amino acid sequence of the N-terminal part of the protein (Chiba *et al.*, 1999).

Met levels in *Arabidopsis* can be modulated by changes in CgS mRNA level using a transgenic approach (Hesse and Hoefgen, 2003). However, in potato, neither overexpression nor antisense downregulation of CgS affected Met levels (Kreft *et al.*, 2003). In addition, potato CgS mRNA expressed in *Arabidopsis* does not undergo the same degradation as the endogenous transcript (Kreft *et al.*, 2003). It is thus possible that different molecular mechanisms of regulation of sulfate assimilation exist in *Arabidopsis* and potato (and potentially in other plants) so that the knowledge obtained from the model species may not always be simply transferred to other, for example, crop species.

Another compound possibly regulating sulfate assimilation directly is glutathione (Fig. 2). Reduced forms of sulfur, such as H₂S, cysteine or GSH, trigger a strong decrease in sulfate uptake and assimilation (Brunold and Schmidt, 1978; Lappartient *et al.*, 1999; Westerman *et al.*, 2001). In *A. thaliana* root cultures APR activity and transcript levels were decreased by feeding either cysteine or GSH (Vauclare *et al.*, 2002). Because external GSH supply also increases the accumulation of cysteine, both Cys and GSH might be responsible for the control of SO₄²⁻ uptake and assimilation. Blocking GSH synthesis by L-buthionine [S, R] sulfoximine, an inhibitor of γ -ECS, relieved the repression APR, indicating that GSH is most probably the acting molecular signal (Vauclare *et al.*, 2002).

Carbohydrates induce APR mRNA accumulation and activity in the dark (Kopriva *et al.*, 1999, 2002a; Hesse *et al.*, 2003); the mechanism involved is, however, distinct from the carbohydrate regulation of the ADP-glucose pyrophosphorylase subunit ApL3 gene used to decipher sugar sensing in *Arabidopsis* (Rook *et al.*, 2001; S. Kopriva, unpubl. res.).

Little is known about the role of phytohormones in the control of sulfur assimilation, but recent developments indicate that this group of compounds is very important for regulation of S nutrition (Ohkama *et al.*, 2002; Maruyama-Nakashita *et al.*, 2004, 2005). Ohkama *et al.* (2002) used transgenic *Arabidopsis* plants, expressing GFP under control of a chimeric promoter containing a sulfur responsive element of β -conglycinin (Awazuhara *et al.*, 2002), to test the influence of phytohormones on the sulfur deficiency response. Whereas abscisic acid (ABA), indole-3-acetic acid (IAA), aminocyclopropane carboxylic acid (ACC, precursor of ethylene), gibberellic acid (GA₃) and jasmonic acid (JA) were not able to induce expression of GFP derived from the sulfur responsive element and, thus, mimic the sulfur starvation response, *trans*-zeatin caused an increase in GFP synthesis both in sulfur-sufficient and in sulfur-deficient 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 repressed the expression of high-affinity sulfate transporters and sulfate uptake capacity of *Arabidopsis* roots. The CRE1/WOL/AHK4 cytokinin receptor was found to be important for regulation given that the effect of cytokinins was attenuated in *cre1-1* mutants (Maruyama-Nakashita *et al.*, 2004). Cytokinins are known to be closely related to the nitrogen

status of the plant and to be involved in regulation of nitrogen assimilation (Samuelson *et al.*, 1995; Takei *et al.*, 2001; Collier *et al.*, 2003). Recently, they were shown to be involved also in regulation of phosphate uptake (Martin *et al.*, 2000). It therefore seems likely that cytokinins may play a more general role in co-ordination of uptake and assimilation of nutrients, including sulfur.

Although auxin was not able to induce expression of GFP from the sulfur responsive element, its role in regulation of sulfur assimilation cannot be excluded. A *NIT3* nitrilase, involved in synthesis of IAA, belongs to genes strongly induced by sulfur deficiency (Kutz *et al.*, 2002). In addition, the *cis*-acting element conferring sulfur starvation response recently identified in *Arabidopsis* SULTR1;2 promoter contains an auxin response factor (ARF) binding sequence (Maruyama-Nakashita *et al.*, 2005). In contrast to typical ARF sites the motif was found as a monomer in the sulfur responsive element and the promoter activity was not affected by naphthalene acetic acid (Maruyama-Nakashita *et al.*, 2005). However, more information is needed to evaluate comprehensively the regulatory interactions of auxin with sulfur metabolism.

JA did not affect the expression of the sulfur responsive promoter element (Ohkama *et al.*, 2002), but is nevertheless involved in regulation of sulfate assimilation. Treatment of *Arabidopsis* with methyljasmonate resulted in a fast but transient increase in mRNA levels of many genes involved in sulfate assimilation and GSH synthesis, but without affecting sulfur metabolite levels (Xiang and Oliver, 1998; Harada *et al.*, 2000; Jost *et al.*, 2005). The mRNA for sulfate transporters was not affected, confirming that JA does not participate in the regulation by sulfur nutrition although genes of jasmonate biosynthesis are among those induced by sulfur starvation (Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). The induction of sulfate assimilation by JA is not surprising as JA is known to participate in the transduction of stress responses (Reymond and Farmer, 1998) and sulfur compounds often play an important role in plant stress defence (Foyer and Rennenberg, 2000).

The interaction of sulfate assimilation and GSH synthesis with stress defence is further corroborated by the finding that the level of GSH increased in plants treated with abscisic acid (Jiang and Zhang, 2001) and salicylic acid (SA) (Fodor *et al.*, 1997). ABA plays an important role in adaptive responses to environmental stresses (Chandler and Robertson, 1994) and leads to increased production of reactive oxygen species (Guan *et al.*, 2000). It is therefore not clear whether GSH synthesis is regulated by ABA itself or by the oxidative stress resulting from ABA treatment. Because ABA induces mRNA accumulation of cytosolic OASTL (Barroso *et al.*, 1999) it seems that this compound may have a more profound effect on control of sulfur metabolism. SA plays a central role in plant defence against pathogens. SA accumulates upon pathogen attack, induces expression of pathogenesis-related genes and is a necessary component of systemic acquired resistance (Kunkel and Brooks, 2002). Treatment of tobacco leaves with SA as well as infection with tobacco mosaic virus resulted in an increase in GSH content in inoculated but not in systemic

leaves (Fodor *et al.*, 1997). In addition, treatment with the biologically active SA analogue 2,6-dichloroisonicotinic acid increased the GSH level leading to a reduction of NPR1, a regulator of systemic acquired resistance, 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* resulted in an increase in SAT activity and GSH content and, consequently, in increased tolerance to Ni (Freeman *et al.*, 2005). Whether SA regulates the expression of γ ECS and *GSHS*, if and how it affects SAT, or if it utilizes another mechanism to increase GSH synthesis remain to be elucidated.

Arabidopsis is the plant model of choice for global investigations of transcriptome and metabolome. As the major physiological problem connected with sulfur metabolism is sulfur deficiency, the extent to which this condition affects plant metabolism was 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.*, 2005). More than 2700 genes were found to be affected by sulfur starvation. As expected, the genes induced by sulfur deficiency included those coding for sulfate transporters and APR; other genes of sulfate assimilation were not significantly and/or consistently affected. However, the genes involved in jasmonate and auxin biosynthesis were induced irrespective from the experimental set-up, as well as a gene for NADPH oxidoreductase, which is involved in oxidative stress defence. Several genes were strongly regulated in one experiment but not others. Examples of such genes are thioglucosidase, which was strongly induced in the roots after 24 h of sulfur deficiency (Maruyama-Nakashita *et al.*, 2003) and was suggested to be involved in providing additional sulfur via degradation of glucosinolates (Wittstock and Halkier, 2002), or phenylalanine ammonia-lyase, strongly reduced after 6 and 10 d of sulfur starvation (Nikiforova *et al.*, 2003). Metabolome analysis revealed that from approx. 6000 analysed metabolites, 11.5% were significantly affected by 13 d of sulfur starvation (Nikiforova *et al.*, 2005). Examples are tryptophan, concentration of which increased up to 28-fold, other amino acids and flavonoids, whereas levels of thiols, lipids and chlorophyll decreased (Nikiforova *et al.*, 2005). 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. This led, for example, to prediction of function of three sulfotransferase genes in glucosinolate metabolism that were confirmed after biochemical analysis of corresponding recombinant proteins. Altogether, transcriptome and metabolome analyses revealed the complexity of the interactions between S, N and C metabolism and opened new perspectives for dissecting the molecular mechanisms of regulation of sulfate assimilation. In this context, the finding of 49 transcription factor genes that specifically responded to sulfur deficiency is of greatest importance (Nikiforova *et al.*, 2003).

Compared with the number for other metabolic pathways, only few recent reports have described the use of genetic approaches for studies of sulfate assimilation (Shibagaki *et al.*, 2002; Ohkama-Ohtsu *et al.*, 2005). Analysis of selenate-resistant mutants resulted in functional characterization of SULTR1;2 and its identification as the major uptake system for sulfate into the roots (Shibagaki *et al.*, 2002). GFP expression from the well-characterized β_{SR} promoter fragment from conglycinin (Awazuhara *et al.*, 2002) was used as a tool in the search for mutants with altered sulfur deficiency response. Seeds from plants harvesting the $\beta_{SR}::GFP$ construct were mutagenized and mutants were selected with increased GFP expression at normal sulfur supply (Ohkama-Ohtsu *et al.*, 2005). 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 sulfur concentration. Map-based cloning and sequence analysis identified a thiol reductase to be responsible for the elevated OAS levels (Ohkama-Ohtsu *et al.*, 2005). This result, although not easy to interpret, indicates the power of the genetic approach, which allows identification of unexpected connections in plant metabolism. Surely, this approach, together with the use of other genetic resources, such as T-DNA or transposon mutants, will deliver the much needed identity of molecular signals and transcription factors regulating sulfur metabolism. *Arabidopsis thaliana* will certainly be the major player in this process.

SULFATE ASSIMILATION IN *LEMNA MINOR*

Traditionally, the water plant *Lemna minor* was used as a model to study the regulation of sulfate assimilation in higher plants. The advantages of *Lemna* compared with other plant systems, such as tobacco cell cultures or spinach chloroplasts, were (1) the use of an intact plant, (2) direct contact of the fronds with nutrient solutions, (3) rapid increase in biomass and (4) the possibility of growth in aseptic liquid cultures under steady-state conditions. *L. minor* was used predominantly to decipher the regulation of sulfate assimilation by sulfur compounds (Brunold and Schmidt, 1978; Brunold *et al.*, 1987) and the co-ordination of sulfate reduction with the assimilation of nitrogen (Brunold and Suter, 1984; Suter *et al.*, 1986). Sulfur deficiency induced activity of APR; other enzymes of the sulfate reduction pathway were much less influenced (Brunold *et al.*, 1987). An increase in sulfate concentration in the nutrition or addition of cysteine or H₂S led to decreased APR activity, but activity could be quickly re-established on a return to control conditions (Brunold and Schmidt, 1978; Brunold *et al.*, 1987). The activities of ATPS and APR decreased under nitrogen-deficient conditions (Brunold and Suter, 1984). At the same time, addition of nitrate or ammonia to the medium quickly restored the activity of these two enzymes. By contrast, addition of ammonia or amino acids (Arg, Asn, Gln) to the nutrient solution caused a 50–110% increase in extractable APR activity in *Lemna* (Brunold and Suter, 1984; Suter *et al.*, 1986). Addition of ammonia increased the flux through the

sulfate assimilation pathway, measured as incorporation of ^{35}S in proteins after feeding [^{35}S]sulfate (Brunold and Suter, 1984). Finally, APR was shown to be repressed by dark, a process attenuated by OAS (Neuenschwander *et al.*, 1991). Addition of OAS also led to an increase in thiol concentration both in light and in dark and to an increased flux through the sulfate assimilation pathway. From these results, Neuenschwander *et al.* (1991) for the first time suggested a role for OAS in regulation of APR and sulfate assimilation.

Lemna proved to be useful also in molecular studies. The purification of plant APR was successful only from *L. minor*, which together with cloning the corresponding mRNA led to the conclusion that APS sulfotransferase and APS reductase are identical enzymes (Suter *et al.*, 2000). In order to study the interconnection of sulfate, nitrogen and carbon assimilation, *L. minor* was used to analyse the effects of CO_2 omission from the atmosphere and simultaneous application of alternative carbon sources on APR and nitrate reductase (NR) (Kopriva *et al.*, 2002a). Incubation in a CO_2 -free atmosphere led to a severe decrease in APR and NR activities and mRNA levels, but Rubisco was not affected to any great degree. Simultaneous addition of sucrose prevented the reduction in enzyme activities but not in mRNA levels. OAS could also attenuate the effect of missing CO_2 on APR, but did not affect NR. $^{35}\text{SO}_4^{2-}$ feeding showed that withdrawal of CO_2 severely inhibited sulfate uptake and the flux through the sulfate assimilation pathway. After re-supply of normal air or addition of sucrose, incorporation of ^{35}S into proteins and glutathione greatly increased. OAS treatment resulted in high-level labelling of cysteine; incorporation of ^{35}S in proteins and glutathione was increased to a much lower extent (Kopriva *et al.*, 2002a). These results corroborated the close link between sulfate, nitrate and carbon assimilation and indicated that OAS might signal the plant N status towards S assimilation, but most probably not *vice versa*.

SULFATE ASSIMILATION IN LEGUMES

Legumes are unique among plants because they are able to form symbiosis with rhizobial bacteria. These bacteria are able to reduce atmospheric nitrogen to ammonium, which is incorporated into carbohydrate skeletons provided by the plant to form amino acids (Udvardi and Day, 1997). Infection of plants with Rhizobia causes development of nodules, where the bacteria in the cytoplasm of plant cells differentiate into bacteroids (Van Rhijn and Vanderleyden, 1995). Nodulation is very important for plant nitrogen nutrition, especially in soils with low nitrogen content. Sulfur and sulfur compounds were shown to play an important role in establishing the symbiosis and its function (Zhao *et al.*, 1999; Frendo *et al.*, 2005; Harrison *et al.*, 2005; Krusell *et al.*, 2005). Growth and nitrogen fixation of pea were greatly enhanced by providing additional sulfur to the soil (Zhao *et al.*, 1999). Legumes are the only plant family with significant amounts of a GSH analogue homoglutathione, which contains a β -alanine instead of glycine

(Klapheck, 1988). Homoglutathione (hGSH) is synthesized by an hGSH synthase, which is distinct from GSHS. The presence of the two genes in legumes is the result of relatively recent gene duplication after divergence of the Fabales (Frendo *et al.*, 2001). No functional differences between GSH and hGSH are apparent as GSH to hGSH ratios vary largely between different legume species (Matamoros *et al.*, 1999). Nodules are organs with the highest GSH and/or hGSH content in legumes because of their role in defence of the nitrogenase against reactive oxygen species (Matamoros *et al.*, 2003). However, GSH and hGSH are important also for establishing the symbiosis. Reducing the tripeptide contents either by antisense expression of the corresponding biosynthetic genes or by inhibition of the GSH/hGSH synthetase with buthionine sulfoximine dramatically inhibited the formation of nodules (Frendo *et al.*, 2005). In addition, the bacteria have a requirement for GSH. *Sinorhizobium* mutants in γ -ECS were not able to nodulate plant host whereas disruption of GSHS led to slow growth, delayed and deformed development of nodules, and a reduced capacity to fix nitrogen (Harrison *et al.*, 2005). By contrast, *Rhizobium* mutants unable to reduce sulfate and nitrate due to disruption of siroheme synthase and thus with non-functional sulfite and nitrite reductase were unable to grow in the soil but produced functional nodules (Tate *et al.*, 1997). It therefore seems that Rhizobia are able to take up and utilize cysteine but not GSH.

The importance of sulfur for nodule function was further corroborated by analysis of *Lotus sym* mutants with non-functional nodules (Krusell *et al.*, 2005). They cloned the gene responsible for the *sym13* and *sym81* mutations, which display nitrogen deficiency syndromes under symbiotic but not non-symbiotic growth conditions and form smaller nodules with reduced nitrogenase content and nitrogen fixing capacity. In both cases, an SST1 sulfate transporter was defective. SST1 is a group 3 sulfate transporter expressed in a nodule-specific manner and located in the symbiosome membrane (Krusell *et al.*, 2005). Thus, interestingly, the first sulfate transporter with clearly a unique function is essential not for sulfur nutrition but for metabolism of nitrogen. However, the strong *sym* phenotype of the *sst1* mutants is surprising as at least one additional sulfate transporter is expressed specifically in *Lotus* nodules (Colebatch *et al.*, 2002). In addition, the reduction of total sulfur content of 20–25 % in the mutants compared with wild-type nodules can hardly explain the severe disruption in nitrogen fixation as plants can reduce sulfur content by up to 70 % without phenotypic changes (Nikiforova *et al.*, 2003). The mechanism by which the loss of SST1 aborts nitrogen fixation thus needs to be addressed in more detail.

SULFATE ASSIMILATION IN C_4 PLANTS

An interesting variation in sulfate assimilation was observed in plants with C_4 photosynthesis (reviewed in Kopriva and Koprivova, 2005). C_4 photosynthesis is characterized by spatial separation of a primary CO_2 fixation step into C_4 acids from their decarboxylation and refixation of the

released CO₂ by Rubisco. This separation of the photosynthetic reactions is usually linked with occurrence of two distinct cell types: the bundle sheath cells (BSCs) and mesophyll cells (MCs) arranged around vascular tissue in a radial pattern known as Kranz anatomy (Laetsch, 1974). CO₂ is initially fixed by phosphoenolpyruvate carboxylase (PEPCase) in the MCs to form a C₄ compound oxaloacetate, which is subsequently converted to malate and/or aspartate. These C₄ acids diffuse to BSCs, where they become decarboxylated and the released CO₂ is refixed by Rubisco in the Calvin cycle, as in C₃ plants (Edwards and Huber, 1981; Hatch, 1987). The CO₂ concentration is therefore increased at the site of its photosynthetic fixation and thus eliminates the oxygenation reaction of Rubisco and photorespiration. A characteristic feature of C₄ plants is a cell-specific localization of many enzymes of primary metabolism in BSCs or MCs. Clearly, the enzymes involved in the primary CO₂ fixation and malate and/or aspartate synthesis, such as cytosolic carbonic anhydrase, PEPCase, pyruvate phosphate dikinase and NADP-malate dehydrogenase, are localized predominantly or exclusively in the MCs, whereas NAD(P)-malic enzyme, Rubisco, Rubisco activase and some enzymes of the Calvin cycle are found exclusively in BSCs (reviewed in Sheen, 1999; Edwards *et al.*, 2001). In addition, glycine decarboxylase, a key enzyme of photorespiration, is localized exclusively in BSCs of C₄ and C₃-C₄ intermediate plants (Hylton *et al.*, 1988). C₃-C₄ intermediate plants were originally identified by having a CO₂ compensation point intermediate between that of C₃ and C₄ species and can be considered as evolutionary intermediates in the path from C₃ to C₄ photosynthesis (Monson and Moore, 1989; Kopriva *et al.*, 1996). Interestingly, enzymes participating in the assimilation of nitrogen are also localized in a cell-specific manner in various C₄ plants; nitrate reductase and nitrite reductase are specifically localized in MCs, whereas glutamine synthetase is equally distributed between MCs and BSCs, and glutamate synthetase and glutamate dehydrogenase are predominantly but not exclusively localized within BSCs (Rathnam and Edwards, 1976; Moore and Black, 1979).

Given the number of cellular processes spatially distributed in C₄ plants, it was not a great surprise that also enzymes of sulfate assimilation were found to be differentially localized (Gerwick and Black, 1979; Gerwick *et al.*, 1980; Passera and Ghisi, 1982; Schmutz and Brunold, 1984). Several groups reported that 75–100% of total leaf ATP sulfurylase activity in maize was confined to BSCs (Gerwick and Black, 1979; Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1984). These findings were extended to 17 other C₄ species, in which 95–100% of total leaf ATPS activity was found in chloroplasts of BSCs (Gerwick *et al.*, 1980). In addition, APR activity was found almost exclusively in BSCs of maize (Schmutz and Brunold, 1984; Burgener *et al.*, 1998), whereas activities of SiR and OASTL were found in MCs and BSCs at comparable levels (Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1985). The mRNA for APR, ATPS and SiR accumulated in BSCs only, whereas OASTL transcript was detected in both MCs and BSCs (Kopriva *et al.*, 2001).

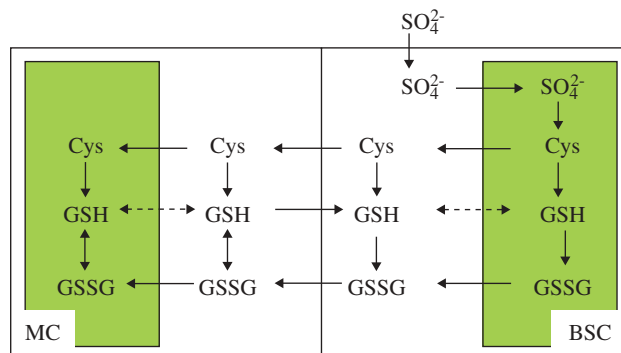


FIG. 3. Schematic representation of the distribution of the major steps in sulfate assimilation and GSH synthesis and reduction between mesophyll (MC) and bundle sheath (BSC) of maize. Dashed lines represent unconfirmed transport steps.

Not only sulfate assimilation but also the synthesis and reduction of glutathione seem to be differently localized in C₄ plants than in, for example, *Arabidopsis*. GSH is particularly important in maize and other low-temperature-sensitive C₄ plants, because it protects against chilling stress by detoxification of H₂O₂. The oxidized glutathione (GSSG) formed in this reaction is subsequently reduced by NADPH-dependent glutathione reductase (GR). At low temperature, GSH content and reduction state are higher in chilling-tolerant genotypes of maize than in chilling-sensitive genotypes (Kocsy *et al.*, 1996). When GSH content was increased in chilling-sensitive maize by treatment with herbicide safeners, chilling-induced injury was significantly reduced (Kocsy *et al.*, 2001), whereas reduction of GSH by inhibiting its synthesis in a chilling-tolerant genotype resulted in increased leaf injury at low temperature (Kocsy *et al.*, 2000). Consequently, in maize chilling induces foliar thiol levels and activities of APR, γ -ECS and GSHS (Brunner *et al.*, 1995), 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 (Kocsy *et al.*, 1997). Although GSH is essential for protection against chilling injury and other stress, it is not equally distributed between MCs and BSCs in maize. GSHS activity is greater in MCs than in BSCs, leading to GSH synthesis predominantly in the MCs (Burgener *et al.*, 1998) and higher GSH levels in this cell type (Doullis *et al.*, 1997; Burgener *et al.*, 1998; Kopriva *et al.*, 2001). Cysteine was shown to be exported from BSC protoplast as a transport form of reduced sulfur to the MCs (Burgener *et al.*, 1998; Fig. 3). The enzymes of GSH synthesis and corresponding mRNAs were, however, found to be localized in both MCs and BSCs by immunohistochemistry (Gómez *et al.*, 2004). Both enzymes were detected in chloroplasts and in the cytosol (Gómez *et al.*, 2004); this contrasts with results for the Brassicaceae, where γ -ECS is localized in plastids and GSHS is prevalently cytosolic (Wachter *et al.*, 2005). Probably as a result of the low capacity for NADPH formation in BSCs, GR was found exclusively in MCs of maize (Doullis *et al.*, 1997; Pastori *et al.*, 2000). These results thus demonstrate that there are species-specific differences

in the intercellular localization of GSH biosynthetic enzymes, which are dependent on increased capacity for transport of various sulfur compounds and possibly result in different regulatory mechanisms for sulfur assimilation in C_4 plants.

Most results on the regulation of sulfate assimilation obtained with maize fitted well to the general hypothesis of demand-driven control of the pathway (Lappartient and Touraine, 1996). A co-ordinate 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.*, 2004) and the ATPS mRNA level was repressed in the presence of reduced sulfur compounds (Bolchi *et al.*, 1999). Accordingly, ATPS and APR activities were increased upon treatments of maize with cadmium or chilling, which resulted in higher demand for reduced sulfur (Nussbaum *et al.*, 1988; Brunner *et al.*, 1995). In these and other reports the regulation of sulfate assimilation in maize was not distinguishable from that in other plants. Bolchi *et al.* (1999), however, described an interesting result which differentiates maize from other plant species analysed to date. Whereas in Brassicaceae GSH exerts the feedback repression of sulfate assimilation (Lappartient and Touraine, 1996; Vauclare *et al.*, 2002), in maize cysteine is acting directly without conversion to GSH (Bolchi *et al.*, 1999). This variation might well be a consequence of the BSC localization of sulfate assimilation (discussed in Kopriva and Koprivova, 2005).

Because the biological significance of the cell-specific distribution of sulfate assimilation and GSH synthesis is not known, the evolution of this trait is of particular interest. To address the question of whether the BSC localization of sulfate assimilation was a prerequisite or a consequence of C_4 photosynthesis, the distribution of APR was studied in *Flaveria* species with different types of photosynthesis (Koprivova *et al.*, 2001). The dicot genus *Flaveria* (Flaveriinae—Asteraceae) is an excellent model to study the evolution of C_4 photosynthesis because, in addition to C_3 and C_4 species, a relatively large number of C_3 – C_4 intermediates occur in this genus (Ku *et al.*, 1991) and a continuous gradation both in the physiology and the biochemistry of photosynthesis exists among *Flaveria* species (Monson and Moore, 1989). APR activity, and cysteine and glutathione levels were significantly higher in C_4 -like and C_4 species than in C_3 and C_3 – C_4 species. However, surprisingly, by northern analysis of cell-specific RNA and *in situ* hybridization ATPS and APR mRNA were present at comparable levels in both MCs and BSCs of the C_4 species *Flaveria trinervia*. In addition, immunogold electron microscopy confirmed the presence of APR protein in chloroplasts of both cell types (Koprivova *et al.*, 2001). Apparently, the localization of assimilatory sulfate reduction in the BSCs is not ubiquitous among C_4 plants but occurs most probably only in C_4 monocots and is, therefore, neither a prerequisite nor a consequence of C_4 photosynthesis. The finding of different compartmentation of ATPS and APR in maize and *Flaveria* clearly revealed the dangers involved in generalization, and that species-specific variations in regulation and compartmentation might be far greater than are usually assumed.

SULFATE ASSIMILATION IN TREES

In trees the regulation of sulfate assimilation is necessarily more complex than in herbaceous plants because of their long life cycles connected with seasonal processes. Trees possess several specific physiological characteristics such as wood production, periods of dormancy combined with storage and mobilization processes that require transport between the shoot and the roots over extremely long distances. In conifers GSH accumulates in winter in the needles as storage of reduced sulfur (Schupp and Rennenberg, 1992). Deciduous trees also seem to store reduced sulfur in the form of GSH; the storage organ is the bark (Siller-Cepeda *et al.*, 1991; Herschbach and Rennenberg, 2001). Under ambient conditions, sulfate is a major sulfur source. It is taken up by the roots, loaded to xylem and transported to the leaves where its reduction takes place. Uptake of sulfate in trees is facilitated by association with mycorrhiza. Although the association with ectomycorrhiza has no impact on the rate of sulfate uptake in trees, it does affect sulfate loading into the xylem (Seegmüller *et al.*, 1996; Kreuzwieser and Rennenberg, 1998). In addition, the de-repression of sulfate transport by sulfur deficiency observed in non-mycorrhizal beech roots is attenuated in ectomycorrhizal roots, indicating a significant contribution of the fungal partner to the regulation of sulfate uptake (Kreuzwieser *et al.*, 1996; Kreuzwieser and Rennenberg, 1998). From these and other studies (Herschbach *et al.*, 2000) it is apparent that sulfate uptake and xylem loading are regulated differently. However, only little is known about the molecular mechanisms of regulation of sulfate reduction in trees. Poplar has proved to be an excellent model plant for molecular studies, as demonstrated by the fact that it was the third plant species for which the genome was sequenced (Tuskan *et al.*, 2004). The genomic organization of the pathway in poplar is similar to that in herbaceous plants; ATPS and APR are present in several isoforms, SiR and γ -ECS are single copy genes (Table 1; Kopriva *et al.*, 2004). In a first attempt to characterize the regulation of sulfur nutrition in poplar, the effects of sulfur and nitrogen deficiency were monitored (Kopriva *et al.*, 2004). Although distinct differences were found, such as no effect of sulfate deficiency on APR in leaves, the regulation of sulfate assimilation by nutrient availability observed in poplar showed strong similarities to the regulation described for herbaceous plants (Kopriva *et al.*, 2004).

In sulfur research, poplars over-expressing bacterial genes for GSH biosynthesis in the cytosol or in the chloroplast are the best characterized plants with manipulated GSH content. Indeed, our current understanding of the regulation of GSH synthesis in plants is mostly derived from experiments with transgenic poplars. GSH synthesis is regulated by the supply of the constituent amino acids and by feedback inhibition of γ -ECS by GSH (Hell and Bergmann, 1990; Noctor *et al.*, 1998b). To address this regulation in more detail a poplar hybrid *Populus tremula* \times *P. alba* (INRA clone no. 717-1-B4, Versailles, France) was transformed to express bacterial γ -ECS or GSHS either in the cytosol (Noctor *et al.*, 1996; Arisi *et al.*, 1997) or

in the chloroplast (Noctor *et al.*, 1998a). Over-expression of γ -ECS, but not of GSHS, increased foliar and root GSH concentration (Strohm *et al.*, 1995; Noctor *et al.*, 1996, 1998a; Arisi *et al.*, 1997; Herschbach *et al.*, 2000), thus confirming the major role of γ -ECS in the control of GSH synthesis. Experiments with poplar leaf discs 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 (Strohm *et al.*, 1995; Noctor *et al.*, 1996). Interestingly, over-expression of γ -ECS either in the cytosol or in the chloroplast did not decrease cysteine and methionine concentrations (Herschbach *et al.*, 2000). Presumably, sulfate reduction and Cys formation are adjusted to the higher demand for GSH synthesis in γ -ECS transgenic trees by a still unknown mechanism. The third amino acid constituting GSH, glycine, is produced through photorespiration and restricts GSH synthesis in the dark in both the cytosol and the chloroplast (Noctor *et al.*, 1997a, b, 1999). Furthermore, over-expression of γ -ECS in the chloroplast, but not in the cytosol, was accompanied by higher foliar concentrations of valine, leucine, isoleucine, tyrosine and lysine (Noctor *et al.*, 1998a). As synthesis of these amino acids is predominantly localized in the chloroplasts, γ -ECS over-expression seems to interact with general amino acid synthesis in this compartment.

GSH concentration was increased in phloem exudates from stem bark tissues of poplars over-expressing γ -ECS in the cytosol, but not in GSHS-expressing plants (Herschbach *et al.*, 1998). The higher GSH concentration in the phloem correlated linearly with the GSH concentrations in the leaves and roots. In deciduous trees both reduced sulfur, mainly as GSH, and sulfate are transported in the phloem to the roots (Herschbach and Rennenberg, 2001). Indeed, Herschbach *et al.* (2000) concluded, based on correlation analysis, that sulfate uptake and xylem loading under both enhanced and decreased sulfur demand might be controlled by the sulfate-to-glutathione ratio. How such regulation could be achieved on molecular level is, however, not clear.

GSH is an important molecule in the protection against reactive oxygen species and, therefore, increasing its foliar concentration might be a good strategy to increase the tolerance of plants to oxidative stress. However, over-expression of γ -ECS or GSHS in transgenic poplar did not increase resistance against ozone or the herbicide paraquat, although this was enhanced in wild-type poplar upon feeding GSH (Will *et al.*, 1997). In addition, exposure to heavy metals represents a stress that can be resisted by increased production of GSH and other sulfur-containing metabolites. Sulfur is important for chelating heavy metals through metallothioneins, i.e. Cys-rich proteins, and phytochelatins (PCs), small polypeptides with repeating γ -EC units (Cobbett, 2000). PCs are synthesized from GSH by phytochelatin synthase (Grill *et al.*, 1989). The sulfhydryl groups of Cys residues bind the heavy metal ions and the resulting complexes are excreted to the vacuole. Heavy metals induce the synthesis of PCs via activation of phytochelatin synthase and induction of

γ -ECS (Cobbett, 2000). Apparently, GSH synthesis is the rate-limiting step in PC synthesis as exposure of the γ -ECS-overexpressing poplars to Cd enhanced PC production (Rennenberg and Will, 2000). Although enhanced PC concentration by over-expression of genes for GSH synthesis increased Cd tolerance in *Brassica juncea* (Zhu *et al.*, 1999a, b), the same effect was not observed in poplar (Arisi *et al.*, 2000; Rennenberg and Will, 2000; Koprivova *et al.*, 2002b). Cd accumulation correlated with PC levels; poplars over-expressing γ -ECS accumulated more Cd, but without increase in Cd tolerance (Koprivova *et al.*, 2002b). 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 *et al.*, 2000). Consequently, growth of transgenic poplars over-expressing γ -ECS in the cytosol or in the chloroplast was less reduced upon treatment with chloroacetanilide herbicides than that of the wild-type (Gullner *et al.*, 2001). It seems, however, that a simple increase in cellular GSH level *per se* is not able significantly to increase plant tolerance to oxidative stress, but a more precise fine-tuning, e.g. by tissue- or compartment-specific manipulation, might be necessary.

The poplars over-expressing γ -ECS offered an opportunity to address the effects of increased GSH synthesis on the sulfate assimilation pathway because cysteine availability is most critical for the rate of GSH synthesis (Strohm *et al.*, 1995). Although foliar GSH levels were three- to four-fold higher in transgenic poplars over-expressing γ -ECS in the cytosol, foliar activities of enzymes of sulfate assimilation, ATPS, APR, SiR, SAT and OASTL, and their mRNA levels were not different from those of wild-type poplars (Hartmann *et al.*, 2004). The results show that sulfate reduction in poplar is sufficient to provide the additional cysteine necessary to accommodate the enhanced GSH synthesis. By contrast, the increased GSH level in transgenic poplars did not down-regulate ATPS and APR as described for several herbaceous plant species (Lappartient *et al.*, 1999; Vauclare *et al.*, 2002). Exogenous feeding of GSH to the roots, however, caused the APR activity and mRNA accumulation to decrease (Hartmann *et al.*, 2004). The poplars thus reacted differently to increased GSH levels due to feeding and endogenous synthesis. 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. Note that γ -ECS was over-expressed also in other plant species. However, the effects of the over-expression were either detrimental in tobacco, which ironically suffered from a highly increased oxidative stress (Creissen *et al.*, 1999), or too moderate in *Arabidopsis*, where GSH levels were increased only by approx. 50% (Xiang *et al.*, 2001). The physiology of poplar was thus especially suitable for this kind of manipulation. Poplar might therefore serve as an alternative model for genetic engineering if the more generally used herbaceous plants are affected too severely as a result of the manipulations.

SULFATE ASSIMILATION IN *PHYSCOMITRELLA PATENS*

One of the remaining questions regarding sulfate assimilation is the presence of the PAPS-dependent pathway in plants. PAPS is not only a source of activated sulfate for sulfation reactions but an intermediate of sulfate assimilation in fungi, yeast and some bacteria (Kopriva *et al.*, 2002b). In these organisms APS cannot be reduced directly but a second activation step to PAPS is necessary. The reduction is achieved in a thioredoxin-dependent reaction catalysed by PAPS reductase. As the sulfate assimilation pathway was first resolved in yeast and in PAPS-reducing enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (Jones-Mortimer, 1968; Kredich, 1971) and PAPS had been detected in plants (Schiff, 1959), the same sequence of reactions including PAPS reductase was proposed for plant sulfate assimilation (Kopriva and Koprivova, 2004). However, studies with the green alga *Chlorella* revealed that APS rather than PAPS was reduced (Tsang *et al.*, 1971; Schmidt, 1972). APS reductase activity was detected in a variety of plants and photosynthetic bacteria, and therefore it was considered to represent the major sulfate-reducing enzyme in photosynthetic organisms (Schmidt, 1975; Schmidt and Trüper, 1977). Three isoforms of APR were then cloned from *Arabidopsis* by complementation of *E. coli* mutants deficient in PAPS reductase (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996). The N-terminal part of the mature APR was homologous to *E. coli* PAPS reductase, with a C-terminal thioredoxin-like extension. Nevertheless, a PAPS-dependent pathway of plant sulfate assimilation was never excluded, in particular when the purification of PAPS reductase from spinach was reported (Schwenn 1989).

Analysis of the *Arabidopsis* genome did not identify any gene homologous to *E. coli* PAPS reductase other than APR. As PAPS reductase in plants may have a completely different structure, only analysis of plants lacking APR activity may prove or exclude the PAPS-dependent sulfate assimilation. However, no such plants have yet been described, probably because APR is encoded by small multigene families of 2–3 isoforms in most plant species analysed to date (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996; Koprivova *et al.*, 2001; Kopriva *et al.*, 2004). This problem was overcome by the use of the moss *Physcomitrella patens*, which in the last few years has become an increasingly used model system to study the function of plant genes (Schaefer, 2002). The single-copy APR gene in *P. patens* was disrupted by homologous recombination, resulting in complete loss of the correct transcript and enzymatic activity (Koprivova *et al.*, 2002a). Surprisingly, however, the knockout plants grew on sulfate as the sole sulfur source. The knockouts showed a Cd-sensitive phenotype and reduced flux through sulfate reduction, as measured by incorporation of [³⁵S]sulfate (Koprivova *et al.*, 2002a). Although PAPS reductase activity could not be measured in moss extracts, cDNA and the gene coding for this enzyme were isolated from *P. patens*. The corresponding recombinant protein possessed PAPS reductase activity, confirming the identity of the gene. The moss *P. patens* is thus the first

plant species for which PAPS reductase was confirmed at the molecular level and also the first organism for which both APS- and PAPS-dependent sulfate assimilation were found to coexist (Koprivova *et al.*, 2002a). Very recently, PAPS reductase was identified in the expressed sequence tag (EST) collection from the spike moss *Selaginella lepidophylla*, a vascular plant (Kopriva and Koprivova, 2004). PAPS reductase thus must have been present in the common ancestor of bryophytes and vascular plants. This result opens many interesting questions on the role of the two parallel sulfate-reducing systems, their coordination and regulation, and the evolution of sulfate assimilation in plants. Because of the rapid and simple method used to obtain gene knockouts, *P. patens* is an interesting alternative to *Arabidopsis* for functional genomics. Furthermore, being a lower plant makes *P. patens* an important species for evolutionary studies and a potential source of new genes.

In addition to possessing a PAPS reductase, bryophytes differ from higher plants in defence mechanisms against heavy metals. Similar to higher plants, exposure to heavy metals induces accumulation of GSH. Phytochelatinins were, however, not detected in bryophytes but the heavy metals were chelated and detoxified by GSH (Bruns *et al.*, 2001).

SULFUR METABOLISM IN ALGAE

Green algae have contributed substantially to our understanding of plant sulfur metabolism. Experiments with *Chlorella* led to the discovery of APS-dependent reduction (Tsang *et al.*, 1971; Schmidt, 1972). Most information on the biochemistry of APS reductase before its cloning from *Arabidopsis* was derived from studies of various algae, such as *Chlorella*, *Euglena* and *Porphyra* (Schmidt, 1972; Li and Schiff, 1991; Kanno *et al.*, 1996). *Euglena* is unique among all photosynthesizing organisms as its sulfate assimilation is localized in mitochondria (Brunold and Schiff, 1976). Algae are also an excellent system to study the effects of nutrient variations. For example, in the green alga *Dunaliella salina* PEPCase activity decreased 11-fold upon sulfur starvation, as did accumulation of Rubisco and light-harvesting proteins, suggesting significant reduction in photosynthetic capacity and changes in the allocation of carbon (Giordano *et al.*, 2000). In the freshwater green alga *Chlamydomonas reinhardtii* sulfur starvation resulted in changes in cell-wall composition by induction of proteins containing very low cysteine and methionine (Takahashi *et al.*, 2001). Sulfur starvation in *Chlamydomonas* results in induction of periplasmic arylsulfatase, which enables the algae to utilize alternative sources of sulfur (de Hostos *et al.*, 1988). This specific response to nutritional stress was utilized to screen for *Chlamydomonas* mutants in response to sulfur starvation (Davies *et al.*, 1994). Although some information about the signal transduction of sulfur starvation in plants has begun to appear (see above), *Chlamydomonas* is still the only organism in which this sulfur sensing is at least partially understood. Mutations in *sac* genes (for sulfur

acclimation) result in a lack of response to sulfur limitation (Davies *et al.*, 1994). The *sac1* mutants are not able to respond to sulfur starvation and induce arylsulfatase and sulfate uptake and assimilation, as well as down-regulation of photosynthesis, whereas in *sac2* and *sac3* mutants only sulfate uptake and assimilation are disturbed. The *Sac1* gene encodes a protein homologous to ion channels that seems to function as a sulfate sensor (Davies *et al.*, 1996). The *Sac3* encodes an Snf1-like kinase; its disruption leads to de-repression of the arylsulfatases even at normal sulfur concentrations but, by contrast, to the inability to induce fully the sulfate uptake system (Davies *et al.*, 1999; Ravina *et al.*, 2002). *Sac2* is involved in post-transcriptional regulation of APR, but has not yet been identified (Ravina *et al.*, 2002).

In contrast to reports on freshwater algae, there have been few studies of the basic sulfur metabolism of marine algae because, contrary to the other major inorganic nutrients nitrogen, phosphate and silicate, sulphur is assumed not to limit the growth of marine phytoplankton (Giordano *et al.*, 2005). On the other hand, the production of dimethyl sulfide (DMS) has attracted considerable interest since Lovelock *et al.* (1978) showed it to be the dominant sulfur gas in the sea. Research interest was further stimulated when Charlson *et al.* (1987) highlighted the connection between marine micro-organisms, the sulfur aerosol particles that form when DMS oxidizes in air and global climate. The major precursor for DMS is the tertiary sulfonium compound dimethylsulfonio-propionate (DMSP), which is found in various marine phytoplankton, some seaweeds and a few saltmarsh and terrestrial plants. In marine phytoplankton DMSP has some of the properties of a compatible solute in that it accumulates in response to changes in salinity and low temperature but may also represent an overflow mechanism for excess reduced compounds and energy or to counter oxidative stress (Stefels, 2000; Sunda *et al.*, 2002). The pathway of DMSP synthesis differs in higher plants and the green macroalga *Ulva intestinalis* (Hanson *et al.*, 1994; Gage *et al.*, 1997). Whereas in higher plants S-methylmethionine is metabolized to DMSP-aldehyde and then oxidized to DMSP, in the marine algae Met is first transaminated to 4-methylthio-2-oxobutyrate, reduced to methylthio hydroxybutyrate, and methylated and decarboxylated to DMSP (Gage *et al.*, 1997). However, nothing is known about the control of DMSP synthesis and its interaction with sulfate uptake and assimilation.

CONCLUSIONS

Great progress has been achieved recently in linking sulfur metabolism to a plethora of cell processes and has made sulfur deficiency one of the best characterized environmental conditions. The molecular mechanisms controlling the sulfate assimilation pathway are still far from being understood. The role of *Arabidopsis* as a model plant in deciphering these mechanisms will thus be further strengthened due to the wealth in genetic resources and available genomics data. This review, however, demonstrates that not all data from *Arabidopsis* are simply transferable to other species and that investigations of other plant species

will be necessary to address specific questions of sulfur metabolism.

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