REVIEW

# Plant sulfate assimilation genes: redundancy versus specialization

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**Abstract** Sulfur is an essential nutrient present in the amino acids cysteine and methionine, co-enzymes and vitamins. Plants and many microorganisms are able to utilize inorganic sulfate and assimilate it into these compounds. Sulfate assimilation in plants has been extensively studied because of the many functions of sulfur in plant metabolism and stress defense. The pathway is highly regulated in a demand-driven manner. A characteristic feature of this pathway is that most of its components are encoded by small multigene families. This may not be surprising, as several steps of sulfate assimilation occur in multiple cellular compartments, but the composition of the gene families is more complex than simply organellar versus cytosolic forms. Recently, several of these gene families have been investigated in a systematic manner utilizing Arabidopsis reverse genetics tools. In this review, we will assess how far the individual isoforms of sulfate assimilation enzymes possess specific functions and what level of genetic redundancy is retained. We will also compare the genomic organization of sulfate assimilation in the model plant *Arabidopsis thaliana* with other plant species to find common and species-specific features of the pathway.

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## Introduction

Sulfur is an essential macronutrient for all forms of life. It is present in the amino acids cysteine and methionine and thus is an important component of proteins and numerous oligopeptides. Many enzymes require sulfur-containing co-enzymes and prosthetic groups for their activity, such as iron sulfur centers, thiamine, lipoic acid, Coenzyme-A, *S*-adenosylmethionine, and many more. The sulfate group modifies proteins, oligo- and polysaccharides and lipids, modulating function of these molecules. In addition, many plant secondary metabolites responsible, e.g., for taste and smell of vegetables, contain sulfur, the best examples being glucosinolates and alliins (Leustek et al. 2000). Methionine is an essential amino acid for human and animal nutrition; therefore, sulfur-containing amino acids are important determinants of quality and suitability of plant proteins for animal feed (Wang et al. 2003).

Sulfur in nature is mostly available as inorganic sulfate, which is taken up, reduced to sulfide, and incorporated into bioorganic compounds in the sulfate assimilation pathway (Fig. 1; reviewed in Leustek et al. 2000; Kopriva 2006). Plants, algae, and many microorganisms are capable of sulfate reduction, while the pathway is missing in Metazoa and most microorganisms adapted to a parasitic life style (Patron et al. 2008). Sulfate is taken up into plant cells by sulfate transporters. Before reduction, it has to be activated by adenylation to adenosine 5′-phosphosulfate (APS) catalyzed by ATP sulfurylase (ATPS) (Fig. 1). In plants, APS is reduced by APS reductase (APR) to sulfite, which is further reduced by ferredoxin-dependent sulfite reductase

sulfate_out

*sulfate transporter* ↓

sulfate_in

*ATP sulfurylase* ↓        *APS kinase*

APS ——————→ PAPS ——→ glucosinolates

*APS reductase* ↓

serine          sulfite ——————→ sulfolipids

*serine acetyl-transferase* ↓        *sulfite reductase* ↓

*O*-acetylserine    sulfide

*O-acetylserine (thiol)lyase* ↓

cysteine
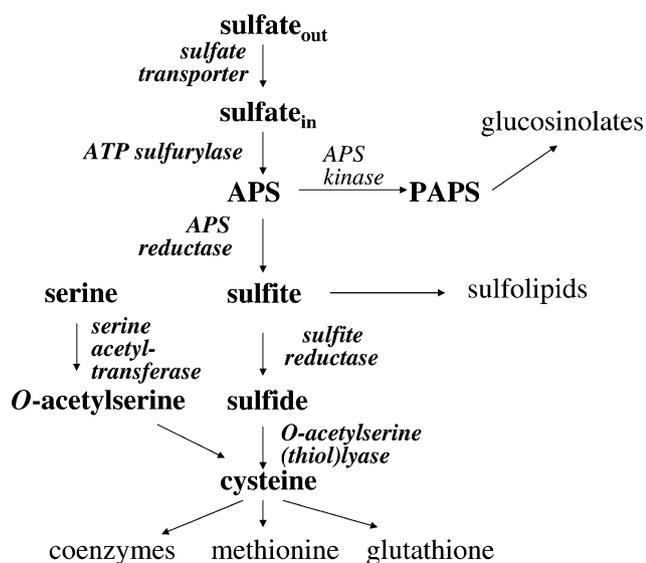
coenzymes    methionine    glutathione

**Fig. 1** Scheme of plant sulfate assimilation

(SiR) to sulfide. Sulfide is incorporated by *O*-acetylserine (thiol)lyase (OAS-TL) into the amino acid acceptor *O*-acetylserine (OAS) to form cysteine. OAS is synthesized from serine and acetyl-Coenzyme-A by serine acetyl-transferase (SAT). Cysteine is the donor of reduced sulfur for the synthesis of methionine and other S-containing metabolites (Leustek et al. 2000; Kopriva 2006). Alternatively, APS can also be phosphorylated by APS kinase (APK) to 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is the activated sulfate donor for a variety of sulfotransferases modifying proteins, saccharides and secondary metabolites. Sulfolipids, on the other hand, utilize sulfite as a source of the sulfo-group (Sanda et al. 2001). Very recently, a new enzyme has been identified in plant sulfur metabolism. Sulfite oxidase (SO) is a molybdenum cofactor possessing enzyme oxidizing sulfite to sulfate with oxygen as an electron acceptor (Eilers et al. 2001; Hänsch et al. 2006). The peroxisomal enzyme is spatially separated from sulfate reduction to prevent a futile cycle (Nowak et al. 2004). The only known function of SO so far seems to be protection of plants against access to sulfur dioxide (Lang et al. 2007, Brychkova et al. 2007), but whether this indeed is the primary function remains debatable.

The components of sulfate uptake and assimilation have been identified and the corresponding genes cloned. Interestingly, with the exception of SiR and SO, all enzymes are encoded by small multigene families in the model plant *Arabidopsis thaliana* (Leustek et al. 2000; Kopriva 2006; Patron et al. 2008). The recent progress in genomic sequencing revealed that the same is true for many basal plants and algae (Table 1; Kopriva et al. 2007a; Patron et al. 2008). The question thus arises whether this is a mere consequence of recent gene duplications with high

functional redundancy or whether the individual genes have specific functions. Gene duplications can now be readily assessed by a Plant Genome Duplication Database (PGDD, http://chibba.agtec.uga.edu/duplication/) which identifies intragenome or cross-genome syntenic relationships (Tang et al. 2008). In addition, the availability of Arabidopsis reverse genetics resources has recently allowed several studies to systematically address functions of individual members of these gene families, using T-DNA insertion lines (Barberon et al. 2008; Heeg et al. 2008; Watanabe et al. 2008a, b; Krueger et al. 2009; Mugford et al. 2009). Here, we review these and other attempts to decipher functions of individual genes encoding components of sulfate assimilation. We will not only address the question of gene redundancy in Arabidopsis but also compare the organization of the pathway in other plant and algal species with special attention to evolution of the pathway.

## The family of sulfate transporters

The first plant sulfate transporters were identified by complementation of a yeast sulfate transport mutant by a cDNA library from a tropical legume *Stylosanthes hamata* (Smith et al. 1995). Three different cDNA clones were isolated, showing immediately that multiple sulfate transporters exist in plants. Biochemical analysis of these transporters also revealed that they facilitate sulfate uptake at different sulfate concentrations, at a high affinity range and a low affinity range (Smith et al. 1995). It was thus obvious that specialization among the individual transporters indeed occurs. This was corroborated in the first studies with *A. thaliana* (Takahashi et al. 2000). With the publication of the Arabidopsis genome sequence (Arabidopsis Genome Initiative 2000), it became apparent that the family of sulfate transporters in Arabidopsis consists of 14 genes (Hawkesford 2003). These genes can be divided into five groups according to sequence similarity (Hawkesford 2003; Buchner et al. 2004). Despite this high number, the PGDD identified only two pairs of recently duplicated genes, the *SULTR1;1* and *SULTR2;2*, and the adjacent *SULTR2;2* and *SULTR1;2*. Exploration of other sequenced plant genomes confirms the presence of 7–15 genes and all five groups of sulfate transporters (Table 1). In algae, the number of sulfate transporter genes is lower than in vascular plants; however, the complexity of the gene family is extended. While all sulfate transporters in vascular plants are of the same type, the algae possess an ABC type transporter as well (Chen et al. 2003). Interestingly, this transporter or at least some of its components are present in the chloroplast genome of a few basal plant species, most notably the liverwort *Marchantia polymorpha* (Kohn and

**Table 1** Genomic organization of transporters and enzymes for sulfate assimilation in sequenced plant and algal genomes

|  | A. thaliana | O. sativa | S. bicolor | P. trichocarpa | P. patens | S. moellendorffii | C. reinhardtii | P. tricornutum | T. pseudonana | E. huxleyi |
|---|---|---|---|---|---|---|---|---|---|---|
| Sulfate transporter | 14 | 13 | 9 | 15 | 7 | 7 | 5 | 2 | 2 | 5 |
| ATPS | 4 | 2 | 2 | 4 | 2 | 1 | 2 | 2 | 2 | 2 |
| APS kinase | 4 | 3 | 3 | 3 | 4 | 4 | 1 | 1 | 2 | 2 |
| APR | 3 | 2 | 1 | 2 | 1 + 1[a] | 1 + 1[a] | 1 | 1[a] | 2[a] | 1[a] |
| SiR | 1 | 2 | 1 | 3 | 3 | 1 | 2 | 1 | 1 | 2 |
| OASTL | 9 | 9 | 6 | 10 | 3 | 4 | 3 | 3 | 3 | 11 |
| SAT | 5 | 5 | 3 | 5 | 5 | 3 | 2 | 3 | 3 | 3 |

The number of genes was determined by tBLAST against genomic sequences available in the GenBank (A. thaliana, O. sativa, P. patens, S. moellendorffii) or on the US Department of Energy Joint Genome Institute homepage (http://genome.jgi-psf.org/)

[a] APR-B type of APS reductase without FeS cluster

Schumann 1993). In the eukaryotic microalgae, the situation is more complicated. Remarkably, these organisms contain orthologues of group 5 sulfate transporters as well as one or more genes distantly related to bacterial sulfate transporters. Unfortunately, the nomenclature of the different types of sulfate transporters is very confusing, since the same name SulP (for **sul**fate **p**ermease) has been adopted for both the bacterial/eukaryotic anion exchanger (Saier et al. 1999) and the ABC type sulfate transporter from green algae (Chen et al. 2003).

The clear distinction of five groups of plant sulfate transporters and the fact that both high affinity and low affinity transporters were isolated (Smith et al. 1995; Takahashi et al. 2000) indicated specific biochemical functions of the different groups with a possible further functional differentiation of the individual isoforms due to tissue specificity. Indeed, the functionally characterized high affinity transporters from S. hamata, Arabidopsis, barley, or tomato (Smith et al. 1995, 1997; Takahashi et al. 2000; Yoshimoto et al. 2002; Howarth et al. 2003) all belong to group 1, while the low affinity transporters (Smith et al. 1995; Takahashi et al. 2000) form group 2 (Hawkesford 2003). Transporters of group 4 also have their function assigned, as in Arabidopsis they were localized to the tonoplast and shown to be responsible for efflux of sulfate from the vacuoles (Kataoka et al. 2004a). Much less is known about the function of group 3 and group 5 transporters. Until now, only one group 3 transporter has been demonstrated to actually facilitate sulfate uptake into the cell (Krusell et al. 2005). Another group 3 transporter, SULTR3;5 (for AGI numbers of the isoforms, see Supplemental Table 1) from A. thaliana was not capable of sulfate uptake on its own, but in co-expression assays highly enhanced the capacity of the low affinity transporters for sulfate uptake (Kataoka et al. 2004b). Accordingly, in Arabidopsis, the SULTR3;5 co-localizes with the low affinity SULTR2;1 and its disruption reduces root-to-shoot

transport during conditions of sulfur deficiency (Kataoka et al. 2004b). Interestingly, a group 3 transporter, SST1, has been shown to be specifically localized in the symbiosome membrane in Lotus japonicus nodules and to be essential for symbiotic nitrogen fixation in these nodules (Krusell et al. 2005). The group 5 transporters are the most diverse in sequence from the other groups and their function in sulfate transport has not yet been confirmed. On the contrary, Arabidopsis SULTR5;2 and its orthologue from Chlamydomonas reinhardtii have been characterized as molybdate transporters, and SULTR5;2 was not capable of sulfate transport when expressed in yeast (Tomatsu et al. 2007; Tejada-Jiménez et al. 2007; Baxter et al. 2008). Therefore, the nature of the substrate for SULTR5;1 and its function still needs to be determined, before it is possible to reconsider whether group 5 transporters indeed belong to the sulfate transporter family.

While it appears that sulfate transporters of different groups have specific functions, the relation of the individual isoforms within the groups is much less understood, especially in plant species other than Arabidopsis. The issue of redundancy versus special function has been thoroughly investigated and discussed for the high affinity transporters of group 1 (Yoshimoto et al. 2002; Rouached et al. 2008; Barberon et al. 2008). It seems that in Arabidopsis the SULTR1;1 and -1;2 transporters are involved in sulfate uptake from soil solution while the SULTR1;3 has a specific function in phloem distribution of sulfate between organs (Yoshimoto et al. 2002, 2003). SULTR1;1 and -1;2 seem to have overlapping functions but are differently regulated and do not contribute equally to total sulfate uptake (Yoshimoto et al. 2007; Rouached et al. 2008; Barberon et al. 2008). Nevertheless, Arabidopsis mutants disrupted in both SULTR1;1 and 1;2 are still capable of sulfate uptake, albeit at much reduced rates (Yoshimoto et al. 2007; Barberon et al. 2008). The two Arabidopsis low affinity transporters also differ in their

tissue-specific expression and thus, presumably, function (Takahashi et al. 2000). Generally, it seems that the individual sulfate transporters have specific functions in specific tissue, which can however be taken over by another member of the family.

How far is the described specialization in function general for other plant species? While all sequenced higher plant genomes possess all five groups of sulfate transporters, the transporters of the model basal plants *Physcomitrella patens* and *Selaginella moellendorffii* cannot be assigned unequivocally to all the groups (Kopriva et al. 2007a). These two species possess transporters of group 4 and group 5, but the other four isoforms form a cluster positioned between the main branches separating group 1 and group 2. It is thus not known whether these species also possess distinct high affinity and low affinity sulfate uptake systems. Detailed characterization of sulfate uptake in these species thus remains an important open question as well as the nature of higher plant plastidic sulfate transporters.

## ATP sulfurylase

ATP sulfurylase catalyzes the entry point of sulfate into the sulfate assimilation pathway. Sulfate has a low reduction potential and must therefore be activated by ATP-dependent conversion to APS (Schmidt and Jäger 1992). This reaction is, however, energetically very unfavorable so that the reaction equilibrium is strongly shifted towards the reverse reaction. This is actually utilized in sulfur oxidizing bacteria to generate ATP (Laue and Nelson 1994). The forward reaction generating APS is facilitated in plants by coupling to inorganic pyrophosphatase, which hydrolyses the second reaction product, the pyrophosphate. In bacteria, ATP sulfurylase forms a complex with GTPase (Patron et al. 2008). ATPS is a widespread enzyme present not only in sulfate assimilating plants, algae, fungi, and bacteria but also in Metazoa which do not reduce sulfate. In the latter organisms, ATPS together with APK is necessary to synthesize PAPS, the active sulfate for sulfation reactions that are essential for many aspects of animal life (Zhu et al. 2007).

In all organisms investigated so far, with the exception of *S. moellendorffii*, at least two isoforms of ATPS have been found, with Arabidopsis and *Populus trichocarpa* possessing four-member gene families (Table 1). This seems to be physiologically relevant, since ATPS activity is present in the plastids as well as in the cytosol (Lunn et al. 1990; Rotte and Leustek 2000). Indeed, spinach (*Spinacia oleracea*) and potato (*Solanum tuberosum*) contain two ATPS isoforms, specific to the cytosol and plastids (Lunn et al. 1990; Renosto et al. 1993; Klonus et al. 1994).

In spinach, ∼80% of total ATPS activity was found in the chloroplast fraction, suggesting this is the major isoform (Lunn et al. 1990). However, the existence of cytosolic and plastidic ATPS isoforms does not seem to be universal. In other plant species including rice, poplar, and Arabidopsis, all ATPS genes encode proteins with putative plastid targeting peptides (Leustek et al. 1994; Murillo and Leustek 1995; Hatzfeld et al. 2000a). The identity of the corresponding cytosolic and plastidic isoforms is thus not known, and the role of cytosolic ATPS activity is also unclear. As APS reduction occurs exclusively in plastids, cytosolic ATPS may be linked to cytosolic APS kinase and play a role in provision of PAPS for secondary metabolism (Rotte and Leustek 2000). The latest data show that Arabidopsis lacking cytosolic APS kinase is perfectly viable and does not show any phenotypic alterations (Mugford et al. 2009), thus indicating that the real function of cytosolic ATPS still awaits elucidation.

Arabidopsis contains four ATPS isoforms compared to two genes in most other species, indicating some level of genetic redundancy. In contrast to several sulfate transporters or sulfotransferase genes that occupy adjacent positions in the genome, indicating their recent origin by gene duplication, the ATPS genes are located on different chromosomes. However, the analysis of the PGDD revealed that *ATPS1* and *ATPS4* are present in duplicated segments of the genome. There seems to be substantial differences in regulation of the individual genes as evident in the available microarray data (Matthewman et al. 2009). In addition, differential regulation and possibly different biological functions of ATPS isoforms, at least in Arabidopsis, have recently been revealed. ATPS is a target of a microRNA, miR395, that is inducible by sulfate deficiency and conserved in plant species from *Physcomitrella* to rice (Jones-Rhoades and Bartel 2004; Axtell and Bowman 2008; Kawashima et al. 2009). In Arabidopsis, miR395 was confirmed to target three of the four ATPS transcripts (*ATPS1*, *-3* and *-4*) causing their cleavage (Kawashima et al. 2009). This specific regulation strongly suggests differences in function between *ATPS2* and the three miR395-targeted isoforms. Another difference in regulation has been revealed by analysis of regulation of the ATPS family by MYB factors controlling glucosinolate biosynthesis (Gigolashvili et al. 2007a, b). Only *ATPS1* and *ATPS3* are targets of these transcription factors and the strength of response differs depending on whether the MYB factors are involved in regulation of aliphatic or indolic glucosinolates (Yatusevich R, Mugford S, Matthewman C, Gigolashvili T, Flügge U-I, Kopriva S, unpublished). Thus, while some level of functional redundancy can be expected, it seems that individual ATPS isoforms have distinct biological roles.

In contrast to high identity among the plant ATPS genes, the ATPS isoforms from diatoms and haptophytes are very different and have a different evolutionary origin (Kopriva et al. 2008; Patron et al. 2008). Indeed, the phylogenetic relationship of ATPS from different sources is rather unexpected. Plant ATPS genes are more closely related to ATPS genes from animals than to those in green algae (Patron et al. 2008). The diatoms and haptophytes possess both the Chlorophyte-like ATPS isoform and the plant/animal-like form. Interestingly, the latter has been subjected to frequent gene fusions. Not only is this form of ATPS in *Thalassiosira pseudonana* and *Emiliania huxleyi* fused to APS kinase as their animal counterparts, the enzymes also contain a domain similar to inorganic pyrophosphatase (Fig. 2). Although not yet proven experimentally, this fusion protein is most probably capable of a much higher APS synthesis rate since the coupling of sulfate adenylation with pyrophosphate hydrolysis is physically linked. Similarly, no kinetic data are available on the ATPS from Chlorophytes, so that the biological reason for such divergence in the form of ATPS among closely related organisms is unclear. Also interestingly, the animal and yeast ATPS is fused to APK, although the order of the two enzymes differs between these kingdoms (Fig. 2). Another way of improving ATPS efficiency might be the fusion to APR found in the dinoflagellate *Heterocapsa triquetra*.

## APS reductase

APS reductase is the key enzyme of plant sulfate assimilation. The enzyme undergoes extensive and complex regulation by multiple environmental factors and signaling compounds (Kopriva and Koprivova 2004; Koprivova et al. 2008). Many treatments specifically affect APR and sulfate uptake but not other enzymes of the sulfate



**Fig. 2** Isoforms and fusions of ATP sulfurylase in different organisms

reduction pathway. Correspondingly, as determined in Arabidopsis by control flux analysis, APR and sulfate transport have the highest control over the pathway (Vauclare et al. 2002).

In Arabidopsis and most vascular plants, except *Sorghum*, APR is encoded by a small gene family. The three Arabidopsis isoforms are similar in sequence and generally, apart from small variations in the strength and timing of a response, they are regulated in the same way (Kopriva and Koprivova 2004). This seems to be invariably true for *APR1* and *APR3*, which were co-regulated in all studies so far and which also share the highest sequence homology, which is supported by the PGDD. *APR2* was shown to respond differently than *APR1* and *APR3* to several hormone treatments (Koprivova et al. 2008). This indicates that individual APR isoforms might have specific functions. This view is corroborated by promoter analysis. Although the general expression pattern is similar and the three APR isoforms are expressed in most tissues, in a more detailed analysis, it is obvious that they do have distinct tissue-specific expression patterns (Fig. 3).

While these differences may be important at specific growth conditions, Arabidopsis plants lacking functional *APR1* or *APR2* are perfectly viable without any obvious developmental effects or growth penalties (Loudet et al. 2007; own unpublished work). Inactivation of *APR1* reduces APR activity by ca. 20% (Koprivova A and Kopriva S, unpublished). Disruption of *APR2* has, however, a large influence on the performance of sulfate assimilation. *APR2* was identified in a QTL analysis as being responsible for a difference in sulfate accumulation between two Arabidopsis ecotypes Bay-0 and Shahdara (Loudet et al. 2007). A single nucleotide polymorphism between the ecotypes resulted in an amino acid exchange in the Shahdara allele of *APR2*. This substitution of glutamine for alanine in the vicinity of the thioredoxin active site (Kopriva and Koprivova 2004) resulted in a large increase in $K_M$ for GSH and decrease in $V_{max}$ of the Shahdara protein. The total APR activity was significantly reduced which resulted in the accumulation of sulfate. Similarly, a T-DNA insertion line in *APR2* in Col-0 background also accumulated sulfate. As the total APR activity in the T-DNA line was reduced by ca. 80% (Loudet et al. 2007), *APR2* seems to be the major APR isoform in Arabidopsis.

Nothing is known about differences between individual APR isoforms in other higher plants. However, the moss *P. patens* possesses two very different APS reductases. One isoform is an orthologue of APR from other plant species, binding the FeS cluster essential for APR activity and including the C-terminal thioredoxin-like domain (Kopriva et al. 2001, 2007b). On the other hand, the recently discovered APR-B shares moderate sequence identity and the same active center but does not need the FeS center for
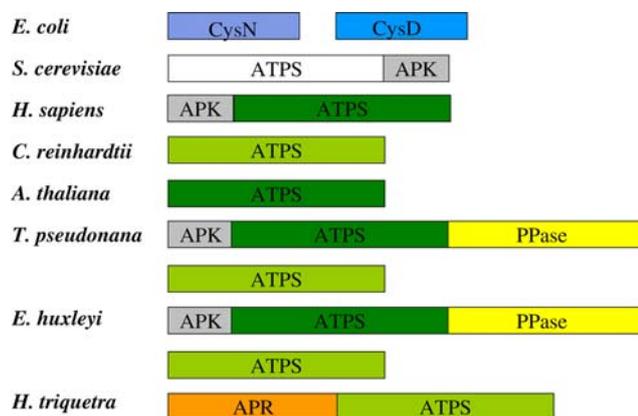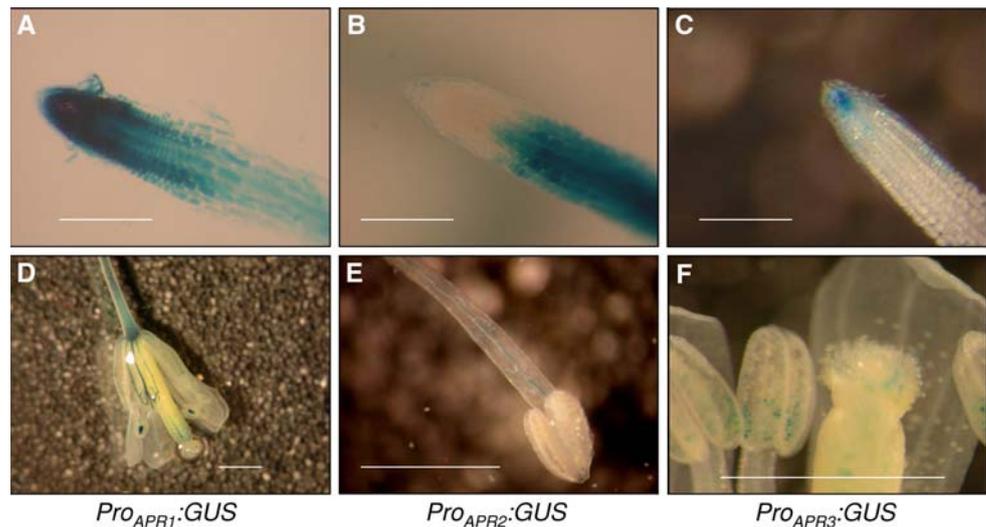
**Fig. 3** Histochemical GUS staining of root tips (**a–c**) and flowers (**d–f**) of plants expressing GUS under control of APR1 promoter (**a, d**), APR2 promoter (**b, e**), and APR3 promoter (**c, f**), *bar* 1 mm



catalytic activity. In addition, similar to bacterial APS and PAPS reductases, APR-B lacks the thioredoxin-like domain and is dependent on free thioredoxin or glutaredoxin (Kopriva et al. 2007b). Interestingly, APR-B-like genes are found in two species of *Sellaginella* and the liverwort *M. polymorpha*, so they must have been part of the genome of the first plants colonizing the Earth's surface (Kopriva et al. 2007a; Patron et al. 2008). APR-B, but not higher plant-like APR, was identified in EST collections of *S. lepidophylla* and *M. polymorpha*. The sequenced genome of *S. moellendorffii* possesses both isoforms, but the product of the gene encoding APR is most probably nonfunctional, because of the substitution of an invariant cysteine present in the active center of all APR, APR-B, and PAPS reductase-related proteins (Kopriva et al. 2007a). Thus, in these basal plants, it might be APR-B which is the major enzyme reducing activated sulfate. In *P. patens*, both APR and APR-B on their own are able to support normal rates of sulfate assimilation, but under conditions where there is a high demand for reduced sulfur, due to exposure to the heavy metal cadmium, both genes are necessary for optimal growth (Koprivova et al. 2002; Wiedemann et al. 2007). In vitro, the FeS-containing APR is more efficient at catalyzing APS reduction than APR-B (Kopriva et al. 2007b). APR-B, however, is more stable and does not require the cofactor, so it may confer a specific advantage during iron and/or sulfur limiting conditions. Although only a little is known about the regulation of APR and APR-B in *P. patens*, it seems that the regulation is different from higher plants. Treatments that strongly regulate APR activity in Arabidopsis and other flowering plants did not affect APR or APR-B in *P. patens* (Wiedemann et al. 2007). It is possible that because of the more complex APS reductase family in this species the control of the pathway has moved to another component.

Interestingly, the genomes of several microalgae, the diatoms, haptophytes, and dinoflagellates include APR-B-like genes. The APS reductase activity in some of these organisms is, however, at least 50-fold higher than in plants (Gao et al. 2000; Kopriva S, unpublished). In contrast to APR-B from *P. patens*, however, the deduced structures of these proteins are more complicated. Two APR-B-like enzymes from the diatom *T. pseudonana* contain C-terminal extensions that might have similar function to the thioredoxin-like domain of higher plant APR. The APR-B from *H. triquetra* is fused to ATPS in a unique combination of two enzyme activities rapidly reducing sulfate to sulfite (Patron et al. 2008). The function of these proteins and their biochemical characteristics, however, remain to be elucidated. The general presence of the APR-B-like form of APS reductase in marine organisms thus supports the hypothesis that this isoform is beneficial during low Fe supply.

## APS kinase

APS kinase phosphorylates APS to form PAPS, the sulfate donor for sulfotransferases. These enzymes catalyze sulfation of free hydroxyl groups of suitable acceptors to form sulfated secondary metabolites, including glucosinolates, and sulfated peptides. In yeast, fungi, and some $\gamma$-proteobacteria and cyanobacteria, APK is an essential component of sulfate assimilation, since these organisms reduce PAPS instead of APS (Kopriva and Koprivova 2004). Higher plants do not possess PAPS reductase, so in these organisms APK can be considered the branch point of sulfur partitioning between primary and secondary metabolism.

Again, Arabidopsis and other plants possess multiple APK genes. In *A. thaliana*, APK exists as a four-member

family, all of them functional (Mugford et al. 2009), rice and poplar contain three isoforms, and *P. patens* four. APK is similarly as widespread as ATPS, and is present in all kinds of Eukaryotic algae, fungi, metazoan, and bacteria (Table 1). In contrast to ATPS and APR, the sequence conservation among APKs from different sources is high, which did not allow dissection of the evolutionary origin of plant APK (Kopriva et al. 2008). However, a clear separation of plastidic and cytosolic isoforms, at least in plants, is apparent as all species possess isoforms with and without putative target peptides. The presence of such peptides seems to be a good indication of the subcellular localization, as in *A. thaliana*, the three isoforms containing the peptide have been confirmed to be localized in plastids while the isoform without the N-terminal extension, *APK3*, is cytosolic (Mugford et al. 2009).

The presence of multiple APK isoforms is intriguing. In Arabidopsis and other Brassicaceae, it may not be surprising, as these species produce high levels of sulfated secondary compounds, the glucosinolates, important for defense against herbivores and other pathogens (Halkier and Gershenzon 2006; Bednarek et al. 2009). The Arabidopsis genome encodes at least 18 sulfotransferases (SOT), most of them without known substrate specificity (Klein and Papenbrock 2004), so specific APK isoforms might possibly supply PAPS for specific sets of SOTs. Creation of knock-out mutants of this family, however, did not reveal any phenotype in single APK knock-outs, showing again at least a partial redundancy in function (Mugford et al. 2009). A correlation between the number of APK isoforms and SOT genes or levels of sulfated metabolites, however, does not seem to exist, as no SOT homologues are present in *P. patens*. PAPS is also a substrate for tyrosine sulfotransferases that catalyze sulfation of proteins and peptides, many with important growth regulatory functions (Komori et al. 2009). Therefore, APK seems to be essential independently from primary sulfate metabolism.

The functional redundancy of APK isoforms in *A. thaliana* is, however, only limited. Analysis of APK double mutants revealed that while five out of six double mutant combinations did not show any phenotype, growth and sulfur metabolism are greatly perturbed in the *apk1 apk2* mutants (Mugford et al. 2009). The *apk1 apk2* plants are significantly smaller than the WT and the single knock-out parents, but otherwise develop normally. Levels of glucosinolates are dramatically reduced in *apk1 apk2* while the precursors over-accumulate 11-fold, indicating that *APK1* and *APK2* are the major isoforms of APK in Arabidopsis. The low glucosinolates seem to be compensated by increased levels of cysteine and GSH, revealing an upregulation of the primary sulfate assimilation in this mutant and an important role of APK in controlling sulfur partitioning between primary and secondary metabolism (Mugford et al. 2009). Interestingly, the single *APK3* knock-out mutants, lacking the only cytosolic APK, had a normal glucosinolate chemotype, strongly suggesting a rapid transport of PAPS from plastids to the cytosol, since the desulfo-glucosinolate SOTs are cytosolic enzymes (Klein and Papenbrock 2004). Similarly, plants with all three plastidial APKs disrupted are viable and contain low levels of sulfated glucosinolates, confirming the interchangeability of plastidial and cytosolic PAPS pools (own unpublished data). Remarkably, a combination of triple APK knock-out which left only *APK1* isoform active was indistinguishable from the wild type, exactly as was the *apk1* mutant (Mugford et al. 2009; Mugford S, unpublished). On the other hand, a combination of triple mutants leaving *APK2* as the sole APK isoform was not viable confirming that APK indeed is essential for plant viability (Mugford S et al., unpublished). These data present a strong case for both partial redundancy and functional specialization of APK isoforms. This view has been corroborated by the analysis of regulation of APK by the glucosinolate MYB factors. Similar to ATPS, two isoforms of APK, *APK1* and *APK2*, were shown to be part of the glucosinolate biosynthesis network (Yatusevich et al., unpublished).

## Sulfite reductase

Sulfite reductase is the only component of sulfate assimilation which is encoded by a single gene in Arabidopsis and most other vascular plants. The *SiR* gene, however, still has a close paralogue in plant genomes, the nitrite reductase (NiR) (Patron et al. 2008). Plant NiR and SiR are plastidic enzymes catalyzing six electron reduction of nitrite and sulfite in sulfate and nitrate assimilation pathways, respectively (Nakayama et al. 2000; Swamy et al. 2005). Both enzymes use siroheme and FeS centers as cofactors and ferredoxin as an electron donor. Indeed, they were shown to be able to catalyze reduction of both compounds, their specificity derived mainly from $K_M$ values for the respective substrates (Krueger and Siegel 1982). The two enzymes are similar enough to conclude that they result from gene duplication (Patron et al. 2008). This duplication must have preceded the initial endosymbiotic event that gave rise to plastids, since both SiR and NiR from plants and all groups of algae are closely related to cyanobacterial sequences within the respective clades (Patron et al. 2008).

In lower plants, surprisingly, the complexity of SiR is greater than in vascular plants. *P. patens* possess three SiR genes that are 80–90% identical. All three gene products are, however, targeted to plastids (Wiedemann G, Reski R,

Kopriva S, unpublished). The three isoforms may have specific functions in sulfate assimilation, but also outside the pathway. Indeed, in vascular plants, SiR has been identified as a DNA packaging protein in plastidic nucleoli (Chi-Ham et al. 2002; Sekine et al. 2007). Such a function, however, has not been confirmed for the *P. patens* SiR so that the possible specialization of the three moss SiR isoforms is still an open question.

## Serine acetyltransferase

Serine acetyltransferase catalyzes the synthesis of the cysteine precursor, OAS, from serine and acetyl-Coenzyme-A. OAS is the acceptor of sulfide, forming cysteine in a reaction catalyzed by OAS-TL (Leustek et al. 2000; Kopriva 2006). SAT and OAS-TL form a multienzyme complex cysteine synthase (Bogdanova and Hell 1997). The complex does not simply facilitate cysteine synthesis by substrate channeling, but the assembly of the subunits strongly affects their enzymatic activity (Droux et al. 1998). Whereas SAT is activated in the complex with OAS-TL, the latter enzyme is active free from bound SAT (Droux et al. 1998). The interaction of the two enzymes is strongly affected by concentration of OAS and sulfide, thus representing another level in regulation of sulfate assimilation (Droux et al. 1998; Berkowitz et al. 2002). SAT is, however, also subjected to feedback inhibition by cysteine (Saito et al. 1995).

Originally, SAT was detected almost exclusively in mitochondria of *Phaseolus vulgaris* (Smith 1972), in chloroplasts of spinach leaves (Brunold and Suter 1982), and cytosol of watermelon (Saito et al. 1995). However, in pea, SAT activity could be measured in all three compartments (Ruffet et al. 1995). In a first systematic survey, Noji et al. (1998) cloned three SAT isoforms from Arabidopsis, revealed that they are indeed targeted into the three compartments cytosol, plastids, and mitochondria, and identified the cytosolic form as susceptible to inhibition by cysteine. With the completion of Arabidopsis genome sequence, two more cytosolic SAT isoforms were identified (Kawashima et al. 2005). The individual SAT isoforms differ very significantly in their enzymatic properties and sensitivity to cysteine. In Arabidopsis, e.g., the cytosolic SAT is inhibited by cysteine, while in pea it is the plastidic form (Noji et al. 1998; Droux 2003). While the "traditional" SAT isoforms denoted SERAT1;1 (cytosolic), SERAT2;1 (plastidic) and SERAT2;2 (mitochondrial) have a $K_M$ for serine in the physiological range of 1–3 mM, the novel SERAT3;1 and -3;2 are characterized by $K_M$ 120 and 40 mM, respectively (Noji et al. 1998; Kawashima et al. 2005). Similar differences were observed in $K_M$ values for acetyl-CoA. Tissue-specific expression

patterns of the individual genes and the responses of corresponding mRNA levels to sulfur starvation and cadmium exposure also revealed significant differences between the isoforms (Kawashima et al. 2005).

Given the localization in multiple compartments and different biochemical properties of the individual isoforms, the questions of their contribution to total SAT activity and to the degree of redundancy were obvious. Activity measurements indicated that the majority of SAT is localized in the mitochondria of pea (Ruffet et al. 1995) and Arabidopsis (Heeg et al. 2008). This view has been strengthened by the observation that specific silencing of the mitochondrial SERAT2;2 results in a strong growth reduction (Haas et al. 2008). The growth phenotype correlated with reduced OAS and cysteine levels in these plants. The conclusion that mitochondria are the major site of SAT activity, at least in Arabidopsis, was corroborated using mutants in plastidic and cytosolic SAT isoforms and nonaqueous fractionation (Krueger et al. 2009). A major advance has been achieved by Watanabe et al. (2008a, b) who analyzed T-DNA insertion mutants in each of the five *SAT* genes, and also succeeded in isolating all five quadruple mutant combinations. The comparison of growth phenotypes of the single insertion lines and wild-type plants did not reveal any growth alterations under normal or sulfur-deficient conditions. This indicates redundancy in function of the individual SAT isoenzymes and a free exchange of OAS (and Cys) between the cellular compartments. However, these results are in sharp contrast to Haas et al. (2008) who observed a strong correlation between levels of mRNA for mitochondrial SAT and growth. The discrepancy might be caused by differences in growth conditions between the two reports, especially the use of sucrose, which has a strong effect on the demand-driven regulation of APR (own unpublished results) and perhaps on the whole sulfate assimilation pathway.

Comparison of plants possessing single SAT isoforms revealed, however, that while OAS production in a single compartment is sufficient, the redundancy of the isoforms is only partial (Watanabe et al. 2008a). SERAT2;1, -3;1, and -3;2 could support completion of the life cycle of the corresponding lines, but with a large growth penalty. On the other hand, the major cytosolic form SERAT1;1 and mitochondrial SERAT2;2 on their own are able to produce sufficient amounts of OAS to support normal growth of plants (Watanabe et al. 2008a). This again shows that OAS and/or Cys are freely inter-changeable among cellular compartments. Indeed, overexpression of SAT either in cytosol or in plastids resulted in increased cysteine and GSH synthesis (Blaszczyk et al. 1999). The benefit of active OAS and Cys synthesis in the three organelles is not simple to explain. Perhaps the active SAT and OAS-TL in each compartment allow a rapid response to small

alterations in sulfide and cysteine concentration and thus participate in keeping a tight homeostasis of these metabolites.

Compared to the wealth of knowledge of SAT in Arabidopsis, virtually nothing is known about the enzyme from lower plants or algae. In contrast to sulfate transporters, ATPS, or APR, the SAT sequences are rather similar among different taxa and no unexpected gene fusions have yet been found. The origin of plant SAT could not be determined confidently, while it is evident that it was not acquired from cyanobacteria, both a eukaryotic and a mitochondrial origin are possible (Kopriva et al. 2008).

## *O*-Acetylserine (thiol)lyase

OAS-TL belongs to a group of $\beta$-substituted alanine synthases (BSAS) within the superfamily of pyridoxalphosphate-binding enzymes (Hatzfeld et al. 2000b). Similar to SAT, OAS-TL is present in the cytosol, plastids, and mitochondria. The OAS-TL gene family consists of nine genes in Arabidopsis, with one of them *BSAS1;2* (OASTL-A2) being regarded as a pseudogene (Jost et al. 2000; Heeg et al. 2008; Watanabe et al. 2008b). The *BSAS1;2* is indeed a result of a gene duplication of *BSAS1;1*, as revealed by analysis of the PGDD, which also identified two more recently duplicated gene pairs, *BSAS2;1* and *-2;2* as well as *BSAS4;1* and *-4;2*. The kinetic properties of recombinant major cytosolic, plastidial, and mitochondrial isoforms of Arabidopsis are, however, remarkably similar (Jost et al. 2000). Other higher plant species possess six to ten OAS-TL genes, but the gene family is much smaller in the basal species *S. moellendorffii* and *P. patens* (Table 1).

Some diversification in function of the different OAS-TL isoforms has become apparent, as several groups have identified the mitochondrial OAS-TL as $\beta$-cyanoalanine synthase (CAS), an enzyme important for cyanide detoxification (Hatzfeld et al. 2000b; Maruyama et al. 2000; Warrilow and Hawkesford 2000). However, significant species-specific differences exist in the mitochondrial OAS-TL. While Maruyama et al. (2000) and Warrilow and Hawkesford (2000) concluded that, at least in spinach and potato, the mitochondrial OAS-TL is actually CAS, both enzymes can be detected in Arabidopsis (Watanabe et al. 2008b).

The only plant species where a systematic study of OAS-TL gene family has been performed is *A. thaliana* (Jost et al. 2000; Heeg et al. 2008; Watanabe et al. 2008b). The individual genes clearly differ in their level of expression, highest steady state mRNA levels were found for the cytosolic *BSAS1;1* (OASTL-A) and *BSAS3;1* (CAS) with the plastidial *BSAS2;1* (OASTL-B) and mitochondrial *BSAS2;2* (OASTL-C) transcripts also being abundant

(Watanabe et al. 2008b). Accordingly, disruption of *BSAS1;1* and *BSAS2;1* resulted in a significant decrease in total foliar OAS-TL enzyme activity, while knocking-out *BSAS3;1* and surprisingly also *BSAS1;1* affected CAS activity specifically (Watanabe et al. 2008b). Cysteine and glutathione levels were reduced only in leaves of *BSAS1;1* plants and in roots of *BSAS1;1* and *-2;2* (Heeg et al. 2008; López-Martín et al. 2008; Watanabe et al. 2008b). Interestingly, similar to SAT, different laboratories reported different effects of disruption of individual OAS-TL genes on Arabidopsis growth. While Watanabe et al. (2008b) did not observe any phenotypic changes in any of the eight single OAS-TL knock-out lines (*BSAS1;2* was not analyzed), Heeg et al. (2008) detected a significant growth retardation of *BSAS2;2*. As the two sets of plants were grown under long and short days, respectively, the differences in phenotypes can most probably be attributed to the different growth conditions. The growth defect observed in *bsa2;2* plants is remarkable as it points to a particular importance of mitochondria for the maintenance of cellular cysteine and glutathione pools, as has recently been repeatedly reported (Zechmann et al. 2008; Dominguez-Solis et al. 2008).

Again, nothing is known about OAS-TL from basal plants and algae. Phylogenetic analysis revealed several interesting groupings of OAS-TL genes from the secondary algae in well-supported clades, but without detailed biochemical analysis it is not possible to conclude anything about possible alternative functions. Interestingly, however, some of these "exotic" OAS-TLs from a dinoflagellate *Karlodinium micrum* and a chlorarachniophyte *Bigelowiella natans* contain a glutaredoxin-like C-terminal domain that might possibly be involved in a redox regulation (Kopriva et al. 2008).

## Concluding remarks

Compared to other pathways of primary metabolism, e.g., the Calvin cycle or glycolysis, the genetic organization of sulfate assimilation in Arabidopsis is rather complex, with multiple isoforms and localizations of the individual components. In addition, under normal growth conditions cysteine synthesis requires coordination of processes localized in three compartments, sulfate reduction in plastids, OAS synthesis in mitochondria, and OAS-TL activity in the cytosol. While some of this diversity is a simple redundancy due to recent genome duplications, in many cases the individual genes have acquired specialized functions. The complexity of sulfate assimilation is not limited to Arabidopsis with its large production of sulfur-containing secondary metabolites, but seems to be a general plant feature found already in the basal species

P. patens and S. moellendorffii. Better understanding of the functions of individual isoforms of the pathway components is important for informed decisions on targets for genetic manipulations to improve specific aspects of sulfur metabolism in corresponding crops. The current progress made with Arabidopsis is a good starting point on this route.

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