Sulfate Assimilation in Basal Land Plants – What Does Genomic Sequencing Tell Us?

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Abstract: Sulfate assimilation is a pathway providing reduced sulfur for the synthesis of cysteine, methionine, co-enzymes such as iron-sulfur centres, thiamine, lipoic acid, or Coenzyme A, and many secondary metabolites, e.g., glucosinolates or alliins. The pathway is relatively well understood in flowering plants, but very little information exists on sulfate assimilation in basal land plants. Since the finding of a putative 3′-phosphoadenosine 5′-phosphosulfate reductase in *Physcomitrella patens,* an enigmatic enzyme thought to exist in fungi and some bacteria only, it has been evident that sulfur metabolism in lower plants may substantially differ from seed plant models. The genomic sequencing of two basal plant species, the Bryophyte *Physcomitrella patens,* and the Lycophyte *Selaginella moellendorffii,* opens up the possibility to search for differences between lower and higher plants at the genomic level. Here we describe the similarities and differences in the organisation of the sulfate assimilation pathway between basal and advanced land plants derived from genome comparisons of these two species with *Arabidopsis thaliana* and *Oryza sativa,* two seed plants with sequenced genomes. We found differences in the number of genes encoding sulfate transporters, adenosine 5′-phosphosulfate reductase, and sulfite reductase between the lower and higher plants. The consequences for regulation of the pathway and evolution of sulfate assimilation in plants are discussed.

Key words: Sulfate assimilation, cysteine synthesis, *Physcomitrella patens, Selaginella moellendorffi,* genomics.

Abbreviations:

APS:	adenosine 5′-phosphosulfate
APR:	adenosine 5′-phosphosulfate reductase
ATPS:	ATP sulfurylase
γ-ECS:	γ-glutamylcysteine synthetase
GSH:	glutathione
GSHS:	glutathione synthetase
OAS:	*O*-acetylserine
OASTL:	*O*-acetylserine (thiol)lyase
PAPR:	PAPS reductase
PAPS:	3′-phosphoadenosine 5′-phosphosulfate
PCS:	phytochelatine synthase
SAT:	serine acetyltransferase
SiR:	sulfite reductase
SOT:	sulfotransferase
SULTR:	sulfate transporter

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Genomics of Basal Land Plants

Unlike algae, where multiple genomic and EST projects have been initiated with the aim of exploring the great diversity in form and function, and to facilitate phylogenetic and evolutionary studies (Grossman, 2005; http://megasun.bch.umontreal.ca/pepdb/pep_main.html), little attention has been paid to genomic studies of basal land plants. Whereas genome sequencing of the green alga *Chlamydomonas reinhardtii* and diatom *Thalassiosira pseudonana* has been successfully accomplished (Grossman, 2005; Armbrust et al., 2004), with several other species in the assembly with released trace files (the green algae *Ostreococcus tauri* and *Volvox carteri,* the red alga *Cyanidioschyzon merolae,* the haptophyte *Emiliania huxleyi,* and the diatom *Phaeodactylum tricornutum*), only two species of basal land plants are in the process of being sequenced with available trace files. Certainly this is partly due to the generally larger sizes of plant genomes, although the *Selaginella moellendorffii* genome, with 88 Mbp, is smaller than the genomes of *C. reinhardtii* and *E. huxleyi*. Also, in the most recent dbEST section of GenBank (release 020207) nine algal species with more than 10000 ESTs can be found, but only four basal plants *(Physcomitrella patens, Marchantia polymorpha, Adiantum capillus-veneris,* and *Mesostigma viride)*.

The moss *Physcomitrella patens* has been increasingly used as a model system to study the function of plant genes (Reski, 1998a; Schäfer, 2002; Cove et al., 2006; Kamisugi et al., 2006). The attractiveness of *Physcomitrella* is related to its ease of culture under controlled conditions, simple body plan, and dominance of the haploid gametophyte stage of its life cycle (Reski, 1998b). In addition it possesses an efficient system of homologous recombination, allowing gene targeting (Schäfer and Zrÿd, 1997; Strepp et al., 1998). Thus, *Physcomitrella* is the only plant where gene knockouts can be routinely produced using a highly efficient and straightforward approach (Hohe and Reski, 2003). *P. patens* is widely utilised for molecular, cytological, and developmental questions in plant biology, and it also represents a key link for understanding plant evolutionary

questions (Decker et al., 2006; Fattash et al. 2007; Richardt et al., 2007). Because of the strong scientific interest in *Physcomitrella,* many genomic resources are available, including EST collections (Rensing et al., 2002; Nishiyama et al., 2003; Lang et al., 2005), trace files from genome sequencing (www.cosmoss.org), and a first release of the genome assembly from 8 × coverage (http://genome.jgi-psf.org/).

The spike-moss *Selaginella moellendorffii,* belonging to the *Lycopodiophyta,* has the smallest genome size (*ca.* 88 Mbp) among land plant species. The *Lycopodiophyta* are an ancient lineage of vascular plants that arose about 400 million years ago, which lack true leaves and roots and thus represent the key node of the plant evolutionary tree. Because of its position between bryophytes and seed plants, *Selaginella* is important for understanding the evolution of flowering plants. Therefore, an EST library has also been produced for this species (Weng et al., 2005) and the genome has been sequenced at the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/sequencing/cspseqplans.html), with trace files released into the GenBank.

Sulfur metabolism

Sulfur is found in the amino acids cysteine and methionine, often responsible for protein structure and enzymatic activity; in coenzymes, such as iron-sulfur centres, thiamine, lipoic acid, or Coenzyme A; and in many secondary compounds, e.g., glucosinolates, alliins, etc. Not surprisingly, therefore, sulfur is an essential macronutrient for all living organisms. In most bioorganic metabolites sulfur is found in the reduced form as S^{II-}, whereas the majority of sulfur in nature is in the oxidised form of sulfate (S^{VI+}). The inorganic sulfate thus has to be reduced and incorporated into carbon skeletons. The sulfate assimilation pathway is present in plants, fungi, and many bacteria (Fig. **1**; reviewed in Leustek et al., 2000; Kopriva, 2006). However, metazoans and cellular parasites lack the ability to assimilate sulfate and are dependent on sulfur-containing amino acids from their diet or host. Sulfate assimilation clearly differs from the dissimilatory sulfate reduction used for energy conversion in a number of sulfate-reducing bacteria and Archaea. In the pathway of assimilatory sulfate reduction, sulfate is taken up into the cells by sulfate transporters. Because sulfate is chemically very stable, before reduction it has to be activated via adenylation to adenosine 5′-phosphosulfate (APS), catalysed by ATP sulfurylase (ATPS). APS can be reduced to sulfite by APS reductase (APR) in plants, algae, and the majority of bacteria. However, fungi, some cyanobacteria, and some γ-proteobacteria require a second activation step: phosphorylation of APS to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) is needed to enable reduction to sulfite by thioredoxin-dependent PAPS reductase (PAPR). In the second reductive step, sulfite is reduced to sulfide by sulfite reductase (SiR). Sulfide is then incorporated into the amino acid skeleton of *O*-acetylserine (OAS) to form cysteine in a reaction catalysed by OAS (thiol)lyase (OASTL). In bacteria and plants, OASTL forms a multienzyme complex with serine acetyltransferase (SAT), which catalyses the synthesis of OAS from serine and acetyl-coenzyme A, called cysteine synthase (Leustek et al., 2000; Kopriva, 2006). In yeast and fungi, the acceptor of sulfide is not OAS but *O*-acetylhomoserine. Therefore, the final product of sulfate assimilation in these organisms is homocysteine, which can be converted to cysteine by the transsulfuration pathway.

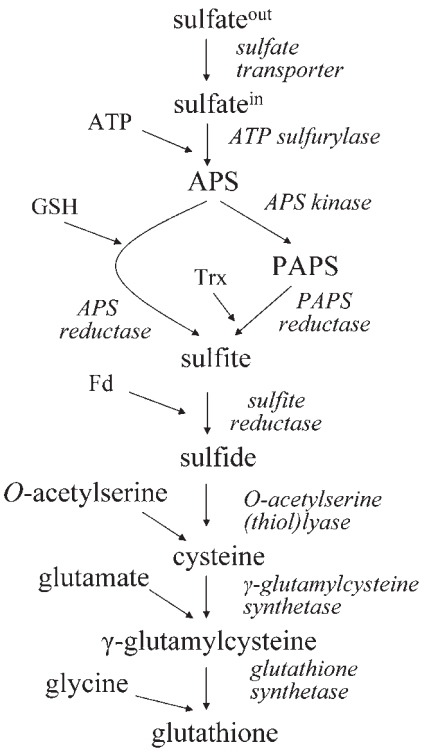

Fig. 1 Scheme of sulfate assimilation and GSH synthesis.

Sulfur compounds are especially important for plant responses to stress. The tripeptide glutathione (GSH) occupies the central position in plant defence against abiotic stress. GSH is synthesised in two ATP-dependent steps. First, γ-glutamylcysteine synthetase (γ-ECS) links glutamate and cysteine in γ-glutamylcysteine, which is subsequently joined with glycine in a reaction catalysed by GSH synthetase (GSHS) (May et al., 1998; Noctor et al., 1998). Apart from its role in oxidative stress, due to its function in the regeneration of the primary reactive oxygen species scavenger, ascorbate, in the Halliwell-Asada pathway (Noctor and Foyer, 1998), GSH is important for plant responses to xenobiotics and heavy metals. Many toxic compounds are dealt with by conjugation to GSH and transport of the conjugate into the vacuole (Edwards et al., 2000). Heavy metals are either chelated directly by GSH or, much more frequently, by small cysteine-rich peptides phytochelatins. Phytochelatins are synthesised enzymatically from GSH by phytochelatine synthase (PCS) (Grill et al., 1989), resulting in a higher demand for GSH during metal exposure.

A well-studied example of compounds involved in plant defence against biotic stress are glucosinolates, which participate in defence against herbivores and pathogens in *Brassicales* (Halkier and Gershenzon, 2006). Glucosinolates are sulfated thioglucosides, derived from amino acids, which, after enzymatic reaction of myrosinase, produce toxic volatile isothiocyanates and nitrils. The last step in their synthesis is sulfation by sulfotransferase (SOT), which is essential for their function (Halkier and Gershenzon, 2006). Plants possess many other sulfated compounds, such as sulfoflavonoids and sulfated oligosaccharides, with different functions in defence against plant stress (Varin et al., 1997; Menard et al., 2004). In mammals, sulfation is a major contributor to the homeostasis

Table 1 Genomic organisation of transporters and enzymes for sulfate assimilation, GSH and phytochelatin synthesis, and sulfation reactions in sequenced plant and algal genomes. 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. thaliana	*O. sativa*	*P. patens*	*S. moellendorffii*	*C. reinhardtii*
Sulfate transporter	14	13	7	7	5
ATPS	4	2	2	1	2
APS kinase	4	3	4	4	1
APR	3	2	1	1	1
(P)APS reductase	0	0	1	1	0
SiR	1	2	3	1	2
OASTL	9	9	4	4	3
SAT	5	5	4	3	2
γ-ECS	1	2	3	2	1
GSHS	1	3	1	1	1
PCS	2	2	0	1	1
SOT	18	26	0	0	0

and regulation of numerous biologically potent endogenous chemicals, such as catecholamines, steroids, and iodothyronines, as well as contributing to the detoxification of xenobiotics (Coughtrie et al., 1998). In bacteria, sulfation is essential for the signalling of rhizobial nod factors to the plant (Truchet et al., 1991).

For a long time, carrier-bound sulfite was believed to be the reaction intermediate in plant sulfate assimilation (Schmidt, 1972; Schmidt and Jäger, 1992; Kopriva and Koprivova, 2004). However, detailed biochemical studies of plant APS reductase revealed the identity of its reaction product to be free sulfite and the presence of an FeS cofactor bound to the enzyme, confirmed a reductase reaction mechanism (Suter et al., 2000; Kopriva et al., 2001). APR is highly regulated and represents the major regulatory step of the pathway (Brunold, 1990; Vauclare et al., 2002; Kopriva and Koprivova, 2004). Although the organisation and regulation of sulfate assimilation is very similar among various plants, subtle differences have been demonstrated (Kopriva, 2006). Little is known about the pathway in basal land plants, however, identification of putative PAPS reductase in *P. patens* (Koprivova et al., 2002; see details below) indicates that there might be major differences. In this review, we summarise our findings on sulfate assimilation in *P. patens* and explore very recently available information from the genomic sequencing of *P. patens* and *S. moellendorffii* to learn about the organisation and evolution of sulfate assimilation in basal plants.

Organisation of sulfur metabolism in basal land plants

All genes of the sulfate assimilation pathway of higher plants can be found in the genomes of *P. patens* and *S. moellendorffii*. The number of genes for individual steps, however, differs substantially (Table **1**). The largest difference can be found in the sulfate transporter family. Whereas flowering plants possess 13 – 14 genes (Buchner et al., 2004), only seven are present in the genomes of the two basal plants and five in the green alga *C. reinhardtii*. This reflects the complexity of seed plants, where tissue differentiation requires multiple transport steps that are unnecessary for simple organisms like mosses and algae. In addition to APR, both lower plants possess a gene with high similarity to bacterial and fungal PAPR that is otherwise not found in seed plants and green algae (see below). Unexpectedly, *P. patens* possesses three genes for sulfite reductase, which is the only gene in seed plants that is often found in a single isoform. Also, the first enzyme of GSH synthesis, γ-ECS, is encoded by more genes in *P. patens* than in any other plant. In plants and algae, sulfate assimilation is localised to plastids, it is therefore significant that for each enzyme at least one isoform with a putative targeting peptide can be identified from computer predictions with programs such as Psort or TargetP. On the other hand, the gene families for enzymes of cysteine synthesis are smaller in basal land plants.

Large differences exist, however, in the occurrence of genes involved in other aspects of sulfur metabolism. Basal land plants and *C. reinhardtii* do not possess any SOT genes, whereas multiple SOT isoforms are found in flowering plants and other eukaryotes because of the structural diversity of the biological acceptors of the sulfate group. The SOT family in *A. thaliana* consists of 18 members whereas 26 SOT genes were found in the rice genome (Table **1**; Klein and Papenbrock, 2004). The absence of SOT in the genomes of *P. patens, S. moellendorffii,* and *C. reinhardtii* could imply that these plants and algae are not able to produce sulfated compounds, which are, however, ubiquitous. In addition, all these species possess genes for APS kinase, an enzyme producing PAPS as an active form of sulfate for the sulfation reactions. Thus, it is possible that enzymes catalyzing sulfation in mosses and spike-mosses are unrelated to SOTs from higher plants and animals.

It has long been known that mosses do not synthesise phytochelatins upon exposure to heavy metals (Bruns et al., 2001). This finding can now be confirmed, since no gene for PCS is present in the *P. patens* genome. There is, however, a PCS in *S. moellendorffii* and in *C. reinhardtii,* which shows that the PCS is not a recent invention of higher plants and was present in plant genomes before the separation of chlorophytes but subsequently lost in the bryophyte lineage.

Sulfate Transporters of Basal Plants

Plant sulfate transporters can be divided into five groups according to sequence similarity and function (reviewed in Buchner et al., 2004). Group 1 represents high-affinity transporters that are responsible for the uptake of sulfate into the roots (Shibagaki et al., 2002; Yoshimoto et al., 2002). Group 2 transporters have lower affinity for sulfate. They are localised in xylem parenchyma and phloem cells of both roots and leaves and function in long-distance translocation of sulfate within the plant (Takahashi et al., 2000). Transporters of group 4 are localised in the tonoplast and are responsible for sulfate efflux from the vacuole (Kataoka et al., 2004a). Little is known about the function of group 3 and 5 sulfate transporters, apart from a demonstrated ability of AtSULTR3;5 to increase the rate of root to shoot sulfate translocation in *A. thaliana* (Kataoka et al., 2004b) and the essential contribution of a *Lotus japonicus* group 3 transporter for functional nodules (Krusell et al., 2005). In green algae, an ABC type of transporter, SulP, is localised in plastid membranes (Chen et al., 2003). This transporter is directly related to cyanobacteria but is encoded in the nucleus of *C. reinhardtii*. No homologues of components of this transporter have been found in seed plants, where the identity of the plastidial sulfate transporter is still not known. However, in line with *SulP* originating from cyanobacteria, a homologue of the *SulP* gene has been identified in the chloroplast genome of a liverwort, *Marchantia polymorpha* (Melis and Chen, 2005).

A neighbour-joining tree of amino acid sequences of sulfate transporters from *A. thaliana, O. sativa, P. patens, S. moellendorffii,* and *C. reinhardtii* clearly separates the five groups into well-supported branches (Fig. **2**). However, not all species possess transporters of all five groups. The two basal land plant species possess two genes for group 5 transporters, similar to the sequenced flowering plant models *A. thaliana, O. sativa,* and *P. trichocarpa*. Only one group 5 transporter is present in the *C. reinhardtii* genome. The only other two plant-like sulfate transporters of *Chlamydomonas* belong to group 4. Also, *P. patens* and *S. moellendorffii* contain a group 4 transporter, while all remaining transporters from these species seem to cluster within group 1. The separation of the subcluster comprising SmSULTR1;2, 1;3, and 1;4 is not well supported by bootstrap analysis, so it is still possible that all basal plant transporters could cluster together. Whether these genes encode only high-affinity transporters awaits biochemical analysis. However, the lack of group 2 and 3 transporters corroborates a high specialisation of the gene family, since no long-distance sulfate translocation is needed in the two lower plant species.

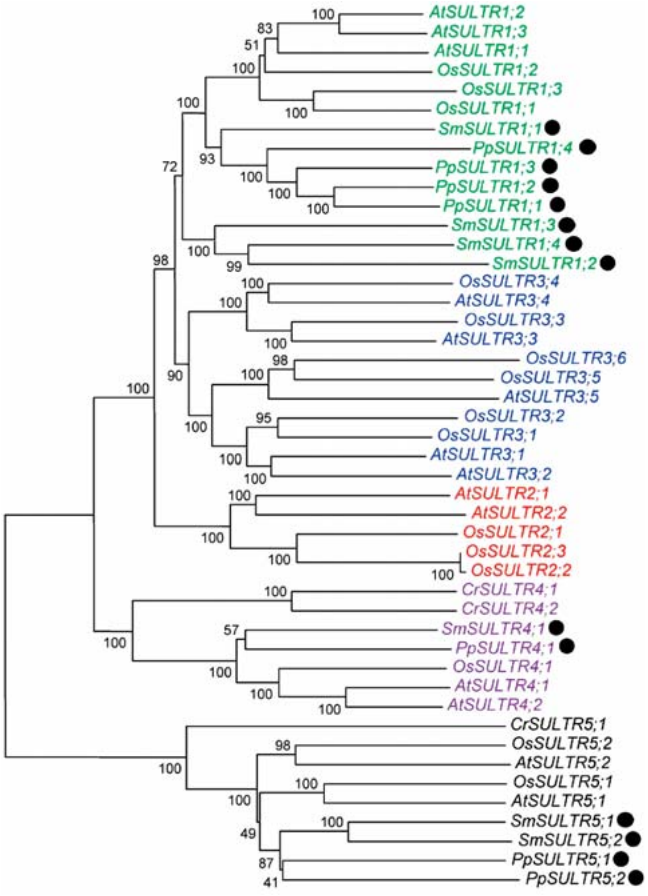

Fig. 2 Neighbor-joining tree of amino acid sequences of sulfate transporters from *A. thaliana, O. sativa, S. moellendorffii, P. patens,* and *C. reinhardtii*. The sequences were retrieved by analysis of genomic sequences from GenBank or US Department of Energy Joint Genome Institute, and aligned by CLUSTALW. The calculation of distances and tree construction were performed with the software Mega3.1 (Kumar et al., 2004). Numbers indicate scores of bootstrap analysis. The sequences of lower plants are marked by complete circles. Group 1 transporters are presented in green, group 2 in red, group 3 and group 4 in blue and magenta, respectively, and group 5 transporters are printed in black.

Sulfate Activation

In seed plants, sulfate is adenylated to APS in both the plastids and in the cytosol (Rotte and Leustek, 2000). Since APR is present exclusively in plastids (Koprivova et al., 2001), the cytosolic APS production must be utilised for processes other than sulfate assimilation. This is corroborated by the different response of cytosolic and plastidic ATPS activity to leaf development. Whereas plastidic activity is highest during earlier stages, where there is a high demand for cysteine and methionine for protein synthesis which then decreases, cytosolic activity increases with leaf age (Rotte and Leustek, 2000). The cytosolic APS is most probably phosphorylated to PAPS and utilised for synthesis of sulfated secondary compounds by SOT. Indeed, APS kinase activity is also present in plastids and cytosol. Therefore, seed plants possess multiple ATPS isoforms (Table **1**). In some species there are distinct cytosolic and plastidic isoforms, in *A. thaliana,* on the other hand, all four genes seem to encode proteins with targeting peptides. Which ATPS isoform in *A. thaliana* is responsible for cytosolic activity, and how dual targeting is achieved at the molecular level, is not yet known.

The number of ATPS isoforms is generally lower in basal land plants and green algae, only one can be found in *S. moellendorffii* and two in *P. patens* and *C. reinhardtii,* all predicted to be targeted to plastids. On the other hand, *P. patens* and *S. moellendorffii* possess four genes for APS kinase, the same number as flowering plants. This is especially surprising given the absence of SOT, but would indicate either sulfotransferase enzymes unrelated to higher plant SOT or another function for PAPS. Indeed, in *P. patens* and *S. moellendorffii* PAPS in the chlo-

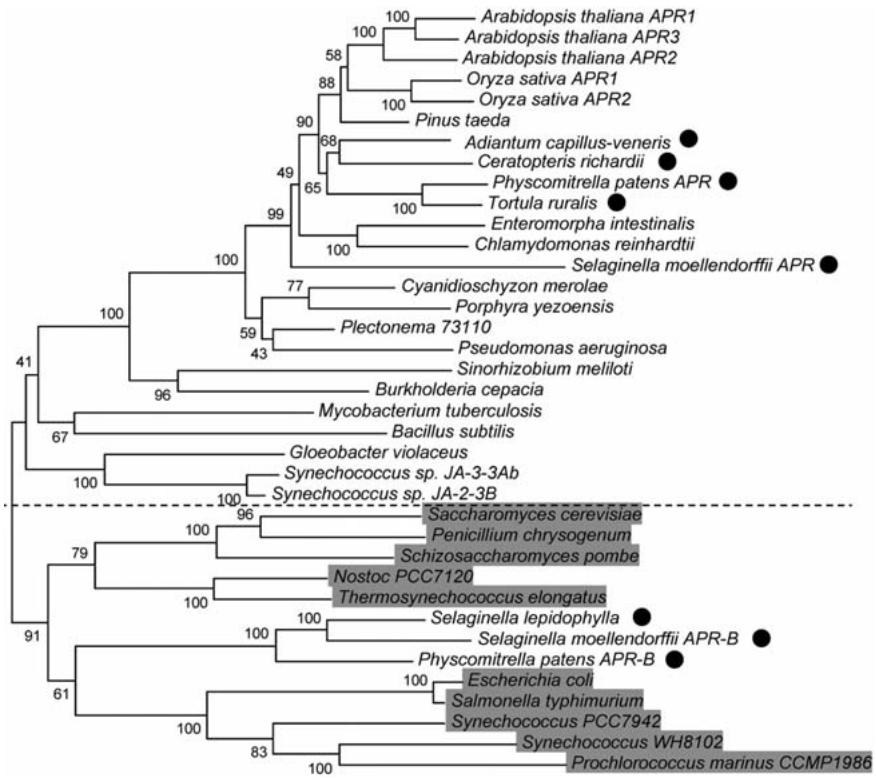

Fig. 3 Neighbor-joining tree of amino acid sequences of APS and PAPS reductases from *A. thaliana, S. moellendorffii, P. patens, C. reinhardtii,* and other lower plants and algae, together with some sequences from bacteria, cyanobacteria, and fungi. The sequences were retrieved from GenBank and aligned by CLUSTALW. The calculation of distances and tree construction were performed with the software Mega3.1 (Kumar et al., 2004). Numbers indicate scores of bootstrap analysis. The sequences of lower plants are marked by complete circles. PAPS reductases are marked with a grey background. The dashed line represents division between sequences containing the two cysteine pairs binding the FeS cluster (above the line) and proteins without the cofactor (below the line).

roplasts can be used in the sulfate assimilation pathway (Kopriva et al., 2007).

Sulfate and Sulfite Reduction

The greatest difference in sulfate assimilation between basal and advanced land plants seems to be the reduction of activated sulfate to sulfite. APR has been shown to possess a high control over the flux through the sulfate assimilation pathway in flowering plants (Vauclare et al., 2002; Tsakraklides et al., 2002). However, largely for historical reasons, the presence of a PAPS-dependent pathway in plants was discussed and has never been excluded (Schmidt and Jäger, 1992), especially after the purification of PAPR from spinach was reported (Schwenn, 1989). Bacterial and yeast PAPR share a moderate sequence similarity to the N-terminal part of APR, but the two enzymes can be distinguished based on the sequence. The FeS cofactor is bound by two cysteine pairs that are invariant in all APR enzymes from plants and bacteria, so the cysteine residues seem to serve as a marker for APR (Kopriva et al., 2002; Kopriva and Koprivova, 2004). Based on this criterion, no PAPR genes were found in any sequenced genomes from plants or chlorophytes. Since plant PAPR may have a completely divergent structure, and the two sulfonucleotides are readily interchangeable by actions of APS kinase and a phosphatase, only the analysis of plants devoid of APR activity may prove or exclude PAPS-dependent sulfate assimilation. As evident from Table **1**, flowering plants possess multiple isoforms of APR, therefore, the ability of gene targeting in *P. patens* has been exploited. The single copy APR gene was disrupted by homologous recombination, resulting in the complete loss of correct transcript and enzymatic activity (Koprivova et al., 2002). Surprisingly, however, the knockout plants grew on sulfate as the sole sulfur source. PAPR activity could not be measured in moss extracts, however, a cDNA and gene highly similar to bacterial and fungal PAPR were isolated (Koprivova et al., 2002). The capability of the enzyme to reduce PAPS was confirmed with a recombinant protein (Kopriva et al., 2007). Surprisingly, it also catalysed the reduction of APS to sulfite at a much higher rate than the reduction of PAPS. As this protein does not bind a FeS cluster, it represents a novel form of APR (Kopriva et al., 2007). Disruption of the novel APS reductase gene, *PpAPR-B,* does not affect the ability of moss to grow on sulfate as the sole sulfur source, so that it is evident that both enzymes participate in sulfate assimilation (Wiedemann G., Koprivova A., Reski R., Kopriva S., unpublished results).

Importantly, orthologues of the novel APR-B were also found in the genome of *S. moellendorffii* and in the EST library from another spike-moss species, *S. lepidophylla. S. moellendorffii* also possesses a gene for APR; however, the sequence of the active centre of the protein is altered and the enzyme will most probably be inactive. A phylogenetic analysis with a limited number of APR and PAPR sequences shows a clear division into an APR cluster and a PAPR cluster, the latter containing the novel APR-B isoform (Fig. **3**). In the former, sequences of plants and algae are clustered with APR sequences from bacteria and cyanobacteria. Due to their similarity to plant APR, these bacterial enzymes, e.g., from *Pseudomonas* and *Rhizobia,* were recognised as APR, although it was believed that heterotrophic prokaryotes require PAPS for reduction (Abola et al., 1999; Bick et al., 2000; Kopriva et al., 2002). Probably because of the inactivation of the active site, the *S. moellendorffii* APR branches before the separation of chlorophytes and plants, which certainly does not agree with the evolutionary history of this species. As expected, other lower plants are positioned between algae and

higher plants. The novel APR-B from basal plants is clustered with known PAPRs from yeast, bacteria, and cyanobacteria, and is more closely associated with prokaryotes than with eukaryotes. It is impossible to determine the origin for both plant genes, since the major prokaryotic groups are polyphyletic and dispersed throughout the tree. It is evident, however, that the new APR-B isoform evolved from a PAPR and changed the substrate specificity to APS while retaining the FeS independent reaction mechanism. This seems to be the most efficient way of reducing activated sulfate, as the energy costs for building the cofactor are eliminated as well as the necessity for a second activation step by ATP, which is the price organisms possessing PAPR pay for saving the cluster.

The finding of the novel APR-B in the two *Selaginella* species is important, since it excludes the origin of the gene in *P. patens* from a recent lateral gene transfer. The gene must have been present in the common ancestor of bryophytes and vascular plants. In *P. patens* both forms of APS reductase are clearly expressed and functional, whereas in *S. moellendorffii* the plant-like APR does not seem to be functional and no EST for APR was found for *S. lepidophylla*. The two spike-moss species thus might possess only one functional gene encoding the novel APR-B, leaving *P. patens* (and possibly other bryophytes) as the only organism where both enzymes are functional. This provokes questions on the regulation of the two enzymes, since the reduction of activated sulfate to sulfite is the major control point of the sulfate assimilation pathway. We have evidence, however, that in *P. patens* APR is not regulated in the same way as in flowering plants, as it is not induced by OAS and not feedback-regulated by GSH (Wiedemann G., Koprivova A., Reski R., Kopriva S., unpublished results). Also, the mRNA levels of the two APS reductases were not significantly altered after stimuli leading to high regulation of higher plant APR. This indicates that the control point of sulfate assimilation in basal plants has moved away from this reaction. This conclusion was corroborated by studies on the regulation of sulfate assimilation in *P. patens* by cadmium (Rother et al., 2006). Although all genes of the pathway were induced by Cd treatment, the gene responding to the highest level was sulfite reductase. It is thus tempting to hypothesise that, in mosses, SiR possesses the highest degree of control over sulfate reduction.

Sulfite reductase is strictly a plastidic protein. Therefore, it is often encoded by a single copy gene, as in *A. thaliana* or *S. moellendorffii*. Surprisingly, however, three closely-related isoforms exist in *P. patens*. The deduced amino acid sequences are very similar to those of higher plants and the sequence comparison does not indicate any differences in function. All three isoforms are expressed, as EST sequences exist for all three. Why such complexity emerged in *P. patens* is not clear; however, one or more of the SiR isoforms may have a completely different role, unrelated to sulfate assimilation. SiR has been described in several species as a DNA-binding protein in chloroplast nucleoids (Sato et al., 2001). Whether this is also true for basal land plants remains to be demonstrated. An important role of SiR for moss metabolism can, however, be inferred from our preliminary data, showing developmental phenotypes in *P. patens* disrupted in one of the SiR isoforms (Wiedemann G., Reski R., Kopriva S., unpublished results).

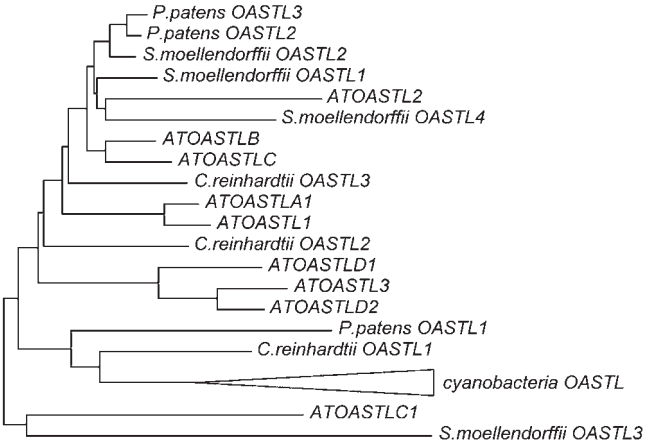

Fig. 4 Neighbor-joining tree of amino acid sequences of OASTL from *A. thaliana, S. moellendorffii, P. patens,* and *C. reinhardtii*, indicating the position of OASTLs from cyanobacteria. The sequences were retrieved from GenBank and aligned by CLUSTALW. The calculation of distances and tree construction were performed with the software Mega3.1 (Kumar et al., 2004).

Cysteine synthesis

Two enzymes, SAT and OASTL, are involved in cysteine synthesis from sulfide and OAS. Metazoa and some microorganisms are capable of cysteine synthesis by transsulfuration from methionine, but this pathway does not appear to be present in plants. Neither were the genes found in either genome of the two basal plants. Cysteine synthesis takes place in all three compartments capable of protein synthesis, i.e., cytosol, plastids, and mitochondria. Not surprisingly, therefore, both enzymes are encoded by multigene families. SAT is encoded by five genes in flowering plants, with different localisation and regulation (Kawashima et al., 2005). The recombinant SAT isoforms also differ in their kinetic parameters, indicating special functions (Kawashima et al., 2005). Four SAT genes are found in the *P. patens* genome, while *S. moellendorffii* possesses three SAT isoforms. The number of genes in the OASTL family in flowering plants (9 – 10) indicates either redundancy or a high level of specialisation of the individual isoforms. In the basal plants, the OASTL family is much smaller, comprising only four genes (Table **1**). Nevertheless, the number of genes for both SAT and OASTL allows for cysteine synthesis in each of the three compartments, as in higher plants. Phylogenetic analysis of OASTL protein sequences from *P. patens, S. moellendorffii, C. reinhardtii,* and *A. thaliana* shows that, for at least two genes, another function can be predicted (Fig. **4**). The AtOASTLC1 isoform has been identified as β-cyanoalanine synthase (Jost et al., 2000). The close branching of *S. moellendorffii* OASTL3 with this protein strongly indicates the same function.

GSH synthesis

Two enzymes are involved in GSH synthesis, γ-ECS and GSHS. Since GSH is an essential metabolite in plants, it is not surprising that both genes are present in the basal plants and algae. *S. moellendorffii* possesses two copies of γ-ECS, whereas, in the *P. patens* genome, three γ-ECS genes are present, and both species have a single-copy GSHS gene (Table **1**). GSH synthesis was shown to take place in the cytosol and plastids (Hell and Bergmann, 1990). However, Wachter et al. (2005) recently

showed that, in *A. thaliana,* which possesses only single-copy genes for both enzymes, the γ-ECS is confined to the plastids but GSHS is dual-targeted to the cytosol and plastids, with the majority of activity in the cytosol. Whether *A. thaliana* and perhaps other *Brassicales* are exceptions remains to be shown, but since in other higher plant species multiple γ-ECS and GSHS genes are present, this seems to be the case. The multiple genes for γ-ECS in lower plants, although all with putative transit peptides, imply that at least the first step of GSH synthesis occurs in both compartments. However, similar mechanisms for dual targeting of GSHS, as found in *A. thaliana,* may exist in basal land plants, especially as dual targeting between plastids and mitochondria has been proven for Phage-type RNA polymerases in *P. patens* (Richter et al., 2002), as well as dual targeting of ancestral tubulin FtsZ between the plastids and cytosol (Kiessling et al., 2004). Alternatively, the γ-ECS proteins are all targeted to the same compartments, presumably the plastids, where they fulfil specialised functions. This might be a plausible explanation since, apart from its role in GSH synthesis, γ-ECS in *A. thaliana* plays an important role in chloroplast nucleus signalling. The *rax1* mutant, which constitutively expresses ascorbate peroxidase 2 and is affected in response to high-light signalling, is caused by a mutation in γ-ECS (Ball et al., 2004). Whereas in *A. thaliana* the metabolic and signalling function reside on a single polypeptide, other plants may have a dedicated isoform for each function. *P. patens* offers an attractive possibility to test this hypothesis by gene targeting.

Conclusions

The genomics of basal land plants is still in its infancy. Nevertheless, analysis of the sequenced genomes of one moss and one spike-moss revealed a reduced complexity in some aspects of sulfur metabolism, such as cysteine synthesis or synthesis of sulfated metabolites, and increased complexity in others. The increased number of genes for SiR and γ-ECS in *P. patens* opens the possibility of addressing a structure-function relationship *in vivo,* using targeted knockouts of the individual genes. Perhaps the most interesting feature of sulfur metabolism in basal land plants is the existence of the two parallel sulfate-reducing systems. This unexpected complexity is in line with the global view that *P. patens* harbours an enormous amount of metabolic genes (Lang et al., 2005), and may specifically account for the relatively high tolerance to abiotic stress of this moss (Frank et al., 2005). Understanding the coordination, regulation and distribution of genes from the two parallel sulfate-reducing systems among other species will be crucial for our comprehension of the evolution of plant sulfate assimilation.

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