Plant Mol Biol (2007) 65:667–676
DOI 10.1007/s11103-007-9231-2

The role of the novel adenosine 5′-phosphosulfate reductase in regulation of sulfate assimilation of *Physcomitrella patens*

Gertrud Wiedemann · Anna Koprivova · Melanie Schneider · Cornelia Herschbach · Ralf Reski · Stanislav Kopriva

Received: 22 May 2007 / Accepted: 18 August 2007 / Published online: 5 September 2007

Abstract Sulfate assimilation provides reduced sulfur for the synthesis of the amino acids cysteine and methionine and for a range of other metabolites. The key step in control of plant sulfate assimilation is the reduction of adenosine 5′-phosphosulfate to sulfite. The enzyme catalyzing this reaction, adenosine 5′phosphosulfate reductase (APR), is found as an iron sulfur protein in plants, algae, and many bacteria. In the moss *Physcomitrella patens*, however, a novel isoform of the enzyme, APR-B, has recently been discovered lacking the co-factor. To assess the function of the novel APR-B we used homologous recombination to disrupt the corresponding gene in *P. patens*. The knock-out plants were able to grow on sulfate as a sole sulfur source and the content of low molecular weight thiols was not different from wild type plants or plants where APR was disrupted. However, when treated with low concentrations of cadmium the APR-B knockout plants were more sensitive than both wild type and APR knockouts. In wild type *P. patens*, the two APR isoforms were not affected by treatments that strongly regulate this enzyme in flowering plants. The data thus suggest that in *P. patens* APS reduction is not the major control step of sulfate assimilation.

G. Wiedemann · A. Koprivova · M. Schneider · R. Reski
Plant Biotechnology, Faculty of Biology, University of Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany

C. Herschbach · S. Kopriva
Tree Physiology, Faculty of Forestry and Environmental Sciences, Institute of Forest Botany and Tree Physiology, University of Freiburg, Georges-Köhler-Allee 53, 79085 Freiburg, Germany

A. Koprivova · S. Kopriva (✉)
John Innes Centre, Norwich NR4 7UH, UK
e-mail: stanislav.kopriva@bbsrc.ac.uk

Keywords Adenosine 5′phosphosulfate reductase · Cadmium · Cysteine biosynthesis · Homologous recombination · *Physcomitrella patens* · Sulfate assimilation

Introduction

In plants sulfur is found in the amino acids cysteine and methionine as part of proteins and peptides, in many coenzymes and prosthetic groups, such as iron sulfur centers, thiamine, lipoic acid, etc., and in a variety of secondary metabolites, such as glucosinolates or alliins. Since most of the sulfur-containing compounds are reduced, the major form of sulfur available to plants, sulfate, has to be reduced and incorporated into organic compounds in the sulfate assimilation pathway. In this pathway sulfate is initially taken up into cells by sulfate transporters. Before reduction it has to be activated by adenylation to adenosine 5′-phosphosulfate (APS), catalyzed by ATP sulfurylase. APS is reduced to sulfite by APS reductase (APR). Yeast, fungi, some enteric bacteria and cyanobacteria need a second activation step, phosphorylation of APS to 3′phosphoadenosine 5′phosphosulfate (PAPS). PAPS can then be reduced to sulfite by thioredoxin dependent PAPS reductase. Sulfite is further reduced to sulfide by sulfite reductase and incorporated into the amino acid acceptor of *O*-acetylserine (reviewed in Leustek et al. 2000; Kopriva 2006).

Sulfate assimilation is highly regulated in a demand-driven manner and coordinated with assimilation of carbon and nitrogen (Lappartient et al. 1999; Leustek et al. 2000; Kopriva et al. 2002b; Kopriva and Rennenberg 2004). The key regulatory step in plants seems to be the reduction of APS catalyzed by APR (Vauclare et al. 2002). APR activity is strongly increased by sulfur starvation, exposure

to heavy metals or chilling and reduced rapidly when reduced sulfur is available to plants or by nitrogen or CO_2 deficiency (Brunner et al. 1995; Setya et al. 1996; Lee and Leustek 1999; Koprivova et al. 2000; Westermann et al. 2001; Kopriva et al. 2002b; Vauclare et al. 2002). The major role of APR in the regulation of sulfate assimilation was confirmed by control flux analysis of *Arabidopsis* root cultures exposed to thiols (Vauclare et al. 2002).

Plant APR is an iron sulfur protein with two domains, a sulfonucleotide reductase domain and a C-terminal thioredoxin-like domain (Bick et al. 1998; Kopriva et al. 2001). Analysis of *Physcomitrella patens* mutants lacking a functional APR gene led to the identification of a moss gene coding for a protein highly similar to bacterial and yeast PAPS reductases (Koprivova et al. 2002). However, a detailed enzymatic analysis of this protein revealed that the enzyme reduces APS despite lacking the FeS cluster, which was believed to be the determinant of specificity towards this substrate (Kopriva et al. 2002a, 2007a). The protein was named APR-B, as it uses a different reaction mechanism from the FeS chemistry of other plant APRs. APR-B lacks the thioredoxin-like domain and is therefore dependent on free thioredoxin. The enzyme is also capable of PAPS reduction, albeit at a much lower rate than reduction of APS (Kopriva et al. 2007a).

In order to explore the biological roles of the two APR isoforms in *P. patens* we extended our previous work on mutants lacking APR (Koprivova et al. 2002) by also disrupting the *PpAPR-B* gene by homologous recombination. We show that under normal conditions there is no effect of the disruption of *PpAPR-B* on moss growth and development. However, under heavy metal stress the *PpAPR-B* knockout plants seem to be more sensitive than the wild type or *PpAPR* knockout plants. Finally we show that in *P. patens* APR is regulated differently than in flowering plants.

Material and methods

Plant material

Physcomitrella patens (Hedw.) B.S.G. was cultured in liquid or solid Knop medium as described earlier (Reski and Abel 1985). Bioreactor cultures were grown semi-continuously in standard stirred-tank glass bioreactors in Knop medium as described (Hohe et al. 2002). To study the effect of cadmium on protonema, cultures in flasks were adjusted to a moss fresh weight of 400 mg/l and the Knop medium was supplemented with 5 or 10 μM $CdCl_2$ for 5 or 10 days or with 1 μM $CdCl_2$ for 3 and 6 days for the analysis of thiols. Cadmium treatments were performed in two independent repetitions each with three independently inoculated cultures of each genotype. For comparison, gametophors were grown for 15 days on solid Knop medium with or without addition of 15 μM $CdCl_2$. Treatments with 1 mM *O*-acetylserine, 1 mM GSH, 1 mM cysteine, and 1 mM buthionine sulfoximine were performed in 10 ml volume containing 100 mg wild type moss protonema. For sulfur deficiency, Knop nutrient solution was exchanged by a modified solution where sulfate was substituted with chloride.

Overexpression of PpAPR and *PpAPR-B* in *E. coli*

The two APR isoforms from *P. patens* were produced in *E. coli* by the pET14b expression system and were purified to homogeneity by criteria of SDS polyacrylamide electrophoresis with the His Tag® System (Novagen) (Kopriva et al. 2007a).

Enzyme assays

APS reductase activity was measured as the production of [^{35}S]sulfite and assayed as acid volatile radioactivity. The reaction mixture contained 4 or 100 ng of recombinant PpAPR and PpAPR-B, respectively, 50 mM Tris–HCl pH 9, 500 mM $MgSO_4$, 5 mM DTE, 20 μM [^{35}S]APS (specific activity 1.67 kBq/nmol), and 10 μg *E. coli* thioredoxin (Sigma) in volume of 500 μl and was incubated for 30 min at 37°C (Kopriva et al. 2007a). Total APR activity was measured in crude protonema extracts using [^{35}S]APS and DTE as reductant as described previously (Koprivova et al. 2002). To measure APR-B activity the $MgSO_4$ was omitted from the assay and 10 μg *E. coli* thioredoxin was added. For the detection of both enzymes simultaneously 10 μg *E. coli* thioredoxin was added to the standard assay. [^{35}S]APS was synthesized enzymatically from [^{35}S]SO_4^{2-} as described in Li and Schiff (1991).

Molecular cloning

The sequence of the cDNA clone for *PpAPR-B* was described previously (Koprivova et al. 2002). The corresponding genomic DNA (3526 bp) was obtained by PCR and sequenced by primer walking. To create the knockout construct a fragment of 2113 bp was amplified from genomic DNA by PCR with primers PPAPR3 (AAGTTT CTCAGCGAAGTGG) and PPREC (CACCGGATCCTA TGTTCTACCACC) (Fig. 1) and cloned into pCRII vector (Invitrogen). This plasmid was digested with BstBI and AatII to cut out a 629 bp fragment, containing the sixth and

the seventh exon (Fig. 1), and replaced with the nptII selection cartridge which was amplified from the vector pRT101neo by PCR introducing the relevant restriction sites (Girke et al. 1998). For transformation, 30 µg of the knockout construct were cut with EcoRI, producing a 3 kb linear fragment which contained the nptII gene flanked by PpPAPR genomic sequences of 952 bp and 532 bp. The DNA was precipitated from the reaction mixture, redissolved in 90 µl water, and used for transformation of *P. patens* (Hohe et al. 2004).

Protoplast isolation, transformation and regeneration

Protoplasts were isolated and PEG-mediated transformation was performed as described previously (Strepp et al. 1998). The transformations were performed with 3×10^5 protoplasts and 30 µg of DNA. Regeneration and selection were performed as described. For selection the Knop media were supplemented with 50 µg/ml G418 (Frank et al. 2005a).

Molecular analysis of transgenic plants

For the pre-screening of G418-resistant plants, small pieces of gametophores were incubated for 30 min in PCR buffer with $(NH_4)_2SO_4$ at 45°C, frozen at –20°C and afterwards thawed at 45°C. Three micro liter of this extract were used for PCR with the following primers (Fig. 1): PPRKO1 (CG CATCTGTAAAGTTGAGC) and PPRKO2 (GCTCGGA AAGCCTTGGTCG) to detect a disruption of the original *PpAPR-B* gene, N1 (TACCGACAGTGGTCCCAAAG) and N2 (CCACCATGATATTCGGCAAG) to detect the presence of the *npt*II cassette, PPREN (AAAGCATATGG CATCTGCAGTGCCTG) and N3 (TGTCGTGCTCCACC ATGTT), and N4 (GGTGAGCATATAAGAAAC) and PPAPR1 (CTTACTTTGTACAATTAGAAG) to control the integration of the transgene at the 5′-end and the 3′-end, respectively. Eight plants that gave the expected fragments in all four PCR reactions were considered as putative knockouts and selected for further analysis.

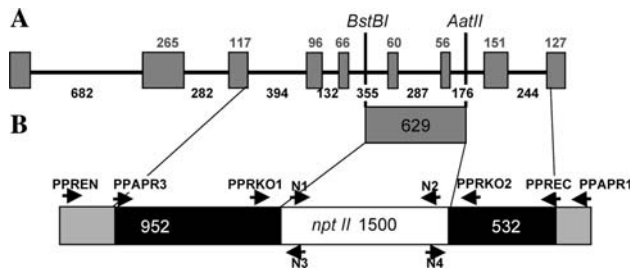

Fig. 1 Schematic representation of the *PpAPR-B* gene and knockout construct. (**A**) The *PpAPR-B* gene from *P. patens*. The rectangles represent exons, introns are presented as lines. The numbers represent the length in base pairs. The positions of the *BstBI* and *AatII* sites, used to cut out the 629 bp fragment and replace it with the *nptII* casette, are indicated. (**B**) The *PpAPR-B* disruption construct. The positions of the PCR primers are indicated by arrows

Total RNA from frozen moss tissue was isolated using the TRIZOL® Reagent (Invitrogen) according to the manufacturer's instructions. First strand synthesis was performed with Superscript II reverse transcriptase (Invitrogen) from 2 µg of total RNA. Reverse transcription PCR with primers PPRKO1 and PPRKO2 was performed according to the standard protocol (Sambrook et al. 1989). The correct cDNA synthesis was controlled by using primers for the mRNA of the ribosomal protein L21 (Reski et al. 1998) C45fw (GGTTGGTCATGGGTTGCG) and C45rev (GAGGTCAACTGTCTCGCC) which is constitutively expressed in moss protonema (Bierfreund et al. 2004; Koprivova et al. 2004).

Expression analysis

Semiquantitative PCR reactions with 1.25 µl cDNA (corresponding to 125 ng total RNA) in a total volume of 25 µl were amplified for 24, 25, 26, and 27 cycles. Primers PPRKO1and PPRKO2 were used to detect the transcript of *PpAPR-B*, APR14 (CGGAACGCGTGCAACGTTC) and APR15 (GCAACACCATCAAGGTTACC) for *PpAPR*. Amplification with primers for the constitutively expressed TATA-binding protein TBPfw (GCTGAGGCAGTCTTG-GAG) and TBPrev (TCGAGCCGGATAGGGAAC) was used for normalization. The whole volume of the PCR product was subjected to electrophoresis on ethidium bromide containing 1% agarose gels. The resulting band intensity on an UV transilluminator was calculated with the Quantity One® software package (Bio-Rad). Northern blot analysis was performed according to standard protocol (Sambrook et al. 1989) with ^{32}P labeled cDNA fragments of *PpAPR*, *PpAPR-B*, and L21 obtained by RT-PCR amplification from total RNA with primers APR14 and APR15, PPAPR3 and PPREC, and C45fw and C45rev, respectively.

Chlorophyll measurements

Fifty to 100 mg plant material was placed in a 2 ml Eppendorf tube and ground up in liquid nitrogen using a ball mill to obtain a homogenous fine powder. The chlorophyll was extracted with 2 ml of 80% (v/v) acetone. To remove cell debris the samples were centrifuged for 5 min at 13,200 rpm. For chlorophyll quantification the absorbance of the supernatant was measured at 645 and 663 nm.

The dry weight of the remaining plant material in the initial Eppendorf tube was obtained by incubation at 105°C for 2 h. The total chlorophyll (mg chlorophyll/g dry weight) was calculated as follows: $((A_{663})(0.00802) + (A_{645})(0.0202)) \times 2$/dry weight (Frank et al. 2005b).

HPLC analysis of low molecular weight thiols

The analysis of cysteine, γ-glutamylcysteine and GSH was performed as described in (Koprivova et al. 2002). 50–70 mg of plant material from liquid culture was ground in liquid nitrogen and extracted in 1.5 ml 0.1 M HCl containing 100 mg insoluble PVPP (Sigma, Deisenhofen, Germany) pre-cooled at 4°C. After centrifugation, 120 µl of supernatant were mixed with 180 µl of CHES buffer, pH 9.3, and 30 µl of 5 mM dithiothreitol were added to reduce disulfides. After 30 min, 20 µl of 30 mM monobromobimane (Thiolyte® MB, Calbiochem) was added, and derivatization of thiols was allowed to proceed for 15 min in the dark. The reaction was stopped and the conjugates were stabilized by the addition of 240 µl of 5% acetic acid. Bimane conjugates were separated by HPLC (SUPELCOSIL™ LC-18, 25 cm × 4.6 mm, 5 µm, Sigma-Aldrich) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 9.3) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 9.3) as solvent B. The elution protocol employed a linear gradient from 96% to 82% A in B within 20 min, and the flow rate was kept constant at 1 ml/min. Bimane derivates were detected fluorimetrically (RF535, Shimadzu, Kyoto, Japan) with excitation at 380 nm and emission at 480 nm.

Statistical analysis

Statistical analysis was performed using Tukey test ($P \leq 0.05$) or *T*-test ($P \leq 0.05$) with SPSS for Windows, Release 9.0.

Results

Disruption of the *PpAPR-B* gene

The *PpAPR-B* cDNA sequence was described previously (Koprivova et al. 2002). It was used to define oligonucleotide primers for amplification of the gene from the genomic DNA. The 3526 bp gene fragment was then completely sequenced. Fig. 1 shows the exon/intron structure of *PpAPR-B*, the gene contains 9 exons and 8 introns. Southern analysis, an analysis of trace files from the *P. patens* genome sequencing in the NCBI WGS database (approx. 8-fold coverage), and the analysis of the assembled genome at US Department of Energy Joint Genome Institute revealed that it is a single copy gene. Therefore, no gene coding for PpAPR-B homologous protein other than the single copy *PpAPR* (Koprivova et al. 2002) occurs in the *P. patens* genome.

To synthesize the disruption construct, a 2113 bp central fragment of the *PpAPR-B* gene was amplified from the genomic DNA (Fig. 1), cloned into pCR plasmid and a 629 bp fragment including exons 6 and 7 was replaced by the *nptII* gene as a selection marker. Forty-four regenerated G418 resistant plants were screened by PCR with four different primer combinations to detect the *nptII* cassette, disruption of the *PpAPR-B* gene, and correct integration of the transgene on both 5′ and 3′ ends to identify positive recombination events. For eight transformants all four PCR reactions resulted in the expected products indicating that they represent true knockouts of the *PpAPR-B* gene. Indeed, no *PpAPR-B* transcript was detected in the transformants by RT-PCR with primers PPRKO1 and PPRKO2 (Fig. 2). The expression of the control gene for L21 (Reski et al. 1998) was not affected in the transformants.

Analysis of PpAPR-B knockout plants

Similar to the Δ*APR* plants (Koprivova et al. 2002) the Δ*APR-B* plants have not displayed any observable difference in growth or developmental phenotype under standard conditions. However, the content of GSH, the major low molecular weight thiol, was consistently approx. 15% lower in 4 out of 5 Δ*APR-B* knock-out lines, with the other thiols, cysteine and γ-glutamylcysteine (data not shown), not being affected (Table 1). Surprisingly, the GSH content in two Δ*APR* lines was even higher than in wild type moss. It seems that each of the enzymes, APR and APR-B, possess enough capacity to reduce sulfate in the absence of the other enzyme.

For APS reductase it was shown, however, that disruption of *PpAPR* leads to higher cadmium sensitivity

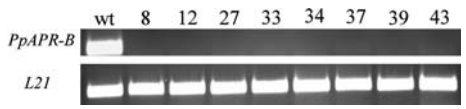

Fig. 2 Expression analysis of *PpAPR-B*. Total RNA was extracted from protonema tissue of wild type *P. patens* (WT) and eight putative Δ*APR-B* lines: (P8, 12, 27, 33, 34, 37, 39 and 43). Two micro gram RNA was reverse transcribed and an equivalent of 125 ng utilized for PCR with primers PPRKO1 and PPRKO2 to detect the *PpAPR-B* transcript. The PCR fragments were resolved on 1% agarose/TBE gel. As a control, PCR was performed with primers derived from the sequence of the constitutively expressed gene for ribosomal protein L21

Table 1 Thiol content of *PpAPR* and *PpAPR-B* knockout plants

	WT	A11-3-12	A12-4-3	A12-7-7	P8	P12	P27	P34	P43
Cysteine	96 ± 20	111 ± 29	95 ± 11	126 ± 13	100 ± 19	89 ± 9*	92 ± 13	93 ± 9	84 ± 15
GSH	449 ± 89	600 ± 49*	398 ± 30	627 ± 110*	452 ± 95	363 ± 48*	364 ± 52*	389 ± 66*	334 ± 26*

Content of low-molecular weight thiols in moss protonema grown in Knop medium was determined by HPLC. The data in (nmol/g fresh weight) are presented as average ± SD from 18 independent cultures of wild type moss (WT), and nine independent cultures each of *ΔAPR* lines (A11-3-12, A12-4-3, A12-7-7 from Koprivova et al. 2002) and *ΔAPR-B* lines (P8, P12, P27, P34, P43). Asterisks denote values significantly different from wild type at $P \leq 0.05$

(Koprivova et al. 2002). Therefore, the effect of Cd was compared in Δ*APR-B* and Δ*APR* plants (Fig. 3). After 15 days on solid media supplemented with 15 µM Cd both types of knockouts displayed a Cd-sensitive phenotype compared to WT moss. Both Δ*APR-B* lines, however, were visibly more affected than the Δ*APR* lines (Fig. 3A). To obtain quantitative data protonema of different genotypes was grown in liquid culture with or without addition of $CdCl_2$ and their chlorophyll content was determined. Already after 5 days at 5 µM $CdCl_2$ the chlorophyll content of all genotypes was reduced compared to control media. In both Δ*APR* and Δ*APR-B* plants the reduction was more profound than in WT moss with the exception of Δ*APR12* plants treated with 10 µM Cd. After 10 days, however, the decrease in chlorophyll content in Δ*APR* plants was similar to the one in wild type moss, whereas the cultures of Δ*APR-B* plants were more affected at both concentrations of $CdCl_2$ (Fig. 3C). Therefore, it seems that the *PpAPR-B* is more important in defense from Cd than *PpAPR*.

To obtain an insight into the effect of Cd on the two enzymes we determined the steady state levels of *PpAPR* and *PpAPR-B* transcripts in all three genotypes under Cd stress (Fig. 4). Whereas the *PpAPR-B* mRNA was not

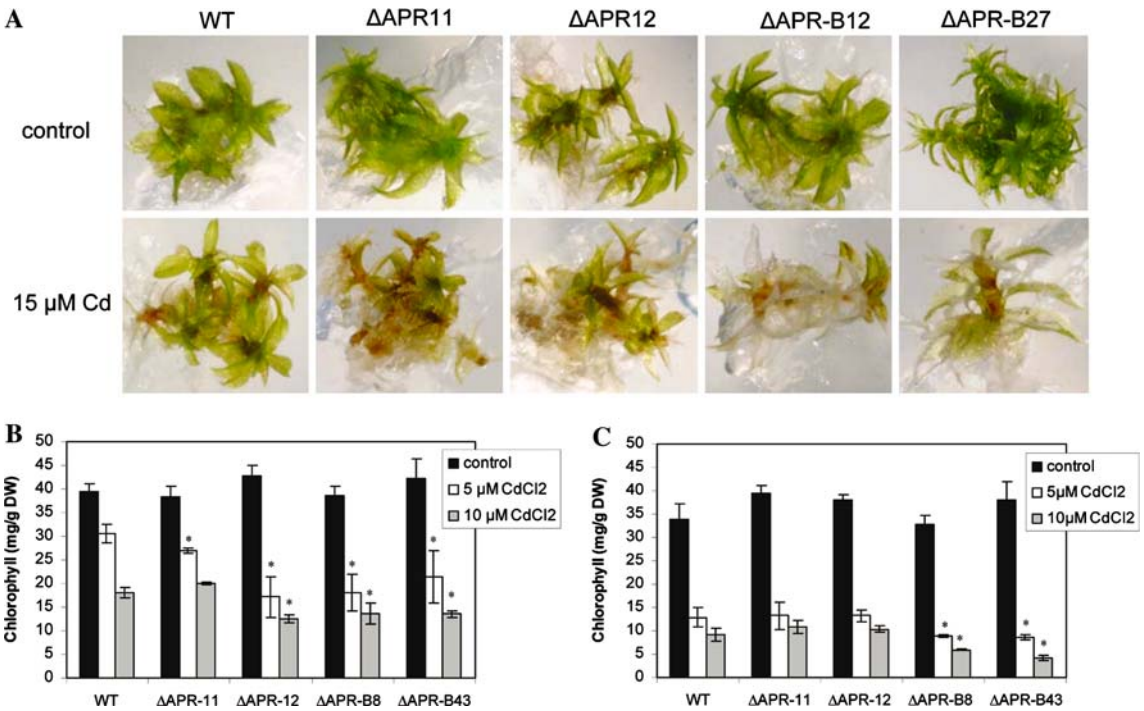

Fig. 3 Enhanced cadmium sensitivity in Δ*APR-B* plants. (**A**) Wild type *P. patens*, two Δ*APR* lines (corresponding to A11–3-12 and A12-4-3 from Koprivova et al. 2002) and two Δ*APR-B* knockouts (P12 and P27) were incubated for 15 days on solid Knop medium (control) or Knop medium with addition of 15 µM $CdCl_2$. (**B**) Synchronized liquid cultures of wild type moss (WT), two Δ*APR* lines and two Δ*APR-B* lines with a fresh weight of 4 mg/10 ml were distributed to flasks containing 30 ml Knop medium and shaken for further 3 days. Cadmium stress was applied by addition of 5 or 10 µM of $CdCl_2$ (dissolved in water) to the flasks, the control cultures were untreated. The tissue was harvested after five days. The material was ground up in the ball mill and chlorophyll was extracted with 80% (v/v) acetone. After centrifugation the absorbance of the supernatant was measured at 645 and 663 nm. The chlorophyll content is related to the dry weight of the sample. The whole experiment was carried out twice on three independent cultures with similar results. Data from one experiment are presented. (**C**) Chlorophyll content of the samples after 10 days. Data are presented as means ± SD. Values indicated by asterisks are different from wild type values at $P \leq 0.05$

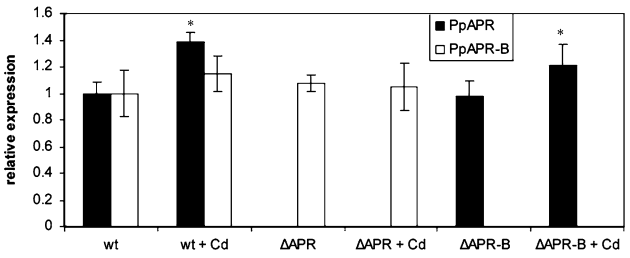

Fig. 4 Relative expression level of *PpAPR* and *PpAPR-B* after cadmium stress. Total RNA was isolated from protonema tissue of *P. patens* wildtype (WT) two Δ*APR* lines (corresponding to A11-3-12 and A12-4-3) and two Δ*APR-B* lines (P8 and P43) grown for 10 days with or without addition of 10 μM $CdCl_2$. The RNA was reverse transcribed and subjected to semi-quantitative RT-PCR with primers APR14 and APR15 to quantify the transcript of *PpAPR*, PPRKO1and PPRKO2 for *PpAPR-B*, and primers for the constitutively expressed TATA-binding protein for normalization. For each sample and primer combination four PCR reactions were run with 24, 25, 26 and 27 cycles. The fragments were resolved on TBE/agarose gel and visualise with ethidium bromide. The resulting band intensity on an UV transilluminator was calculated with the Quantity One® software package (Bio-Rad). The expression of wt moss without Cd treatment was set as 1. Data are presented as means ± SD from the four PCR amplifications on three independent RNA preparations, the two Δ*APR* and Δ*APR-B* lines were calculated together. Values indicated by asterisks are different at $P \leq 0.05$

affected by the Cd treatment, the *PpAPR* mRNA level was increased by 30–40%. The genes were regulated in the same way in wild type moss and the knockout lines. Thus, despite the capacity to induce mRNA levels of *PpAPR*, the Δ*APR-B* plants seem to be more sensitive to Cd than Δ*APR*. A possible explanation could be the difference in direct effect of Cd on the proteins, since *PpAPR* possess the FeS cofactor and *PpAPR-B* does not. Therefore, we expressed the two proteins in *E. coli* and added Cd into reaction assays with purified recombinant proteins. No inhibition of enzyme activity by up to 2 mM Cd was detected (data not shown).

Treatment with a low concentration of Cd (1 μM) induced accumulation of thiols in all genotypes to a similar extent (Fig. 5), revealing that despite differences in the regulation of mRNA levels both enzymes are independently capable of supporting, at least partially, the increased demand for reduced sulfur during moderate heavy metal stress.

APR and APR-B activity in *P. patens*

In the Δ*APR* plants no APR activity was measured, despite the presence of the second APR-B isoform (Koprivova et al. 2002). To investigate this discrepancy we measured APR activity under three assay conditions designed to be specific for APR (without thioredoxin), preferable for APR-B (without $MgSO_4$), and allowing detection of both activities (standard assay with thioredoxin) (Kopriva et al. 2007a). Indeed, without addition of thioredoxin hardly any APR activity can be measured in Δ*APR* plants, whereas the activity in Δ*APR-B* plants remains the same as in WT (Fig. 6). In reaction conditions preferred by the novel APR-B enzyme, the activity is much lower but clearly detectable in all genotypes. Assays under optimal conditions for both enzymes revealed that the in vitro APR activity in moss extracts is several-fold higher than APR-B activity. Disruption of the genes led to reduction of total APR activity in both types of knockouts but to a very different extent (Fig. 6). It seems that the APR-B contributes only approx. 12% of total APS reductase activity.

Since *PpAPR* and *PpAPR-B* were regulated differently by Cd, we analyzed the regulation of the two genes at conditions that were shown to strongly affect mRNA accumulation and activity of APR in higher plants (Leustek et al. 2000; Kopriva 2006). The moss protonema were treated with *O*-acetylserine and buthionine sulfoximine and subjected to sulfur deficiency, which strongly upregulate APR, and with glutathione and cysteine which act as strong negative regulators of the gene. Surprisingly, the APR activity was not affected by any treatment (Fig. 7). Accordingly, *PpAPR* mRNA levels were not affected, whereas the *PpAPR-B* transcript levels were only reduced after addition of glutathione but not by the other treatments. These results clearly show a different regulation of APS reductase in *P. patens* and flowering plants and indicate that contrary to higher plants, APS reduction in moss may not possess high control over the flux through sulfate assimilation.

Discussion

Physcomitrella and other mosses seem to be unique in possessing a novel APS reductase isoform without an iron sulfur cluster. The APR-B is closely related to PAPS reductases from cyanobacteria, some γ-proteobacteria, fungi and, accordingly, was originally considered to catalyze reduction of PAPS (Koprivova et al. 2002). Since a gene orthologue of *PpAPR-B* has been found in several basal land plants, such as *Selaginella lepidophylla*, *S. moellendorffii*, and *Marchantia polymorpha*, this isoform of APS reductase, despite being lost in higher plants, must have played an important role in the evolution of plant sulfate assimilation (Kopriva et al. 2007a, b). In order to assess whether *PpAPR-B* has a specific function in *P. patens* we disrupted the gene by homologous recombination. *P. patens* is unique in its high efficiency of gene targeting (Schaefer 2001; Kamisugi et al. 2006). The disruption of *PpAPR-B* resulted in the disappearance of transcripts for *PpAPR-B*, as judged by RT-PCR analysis.

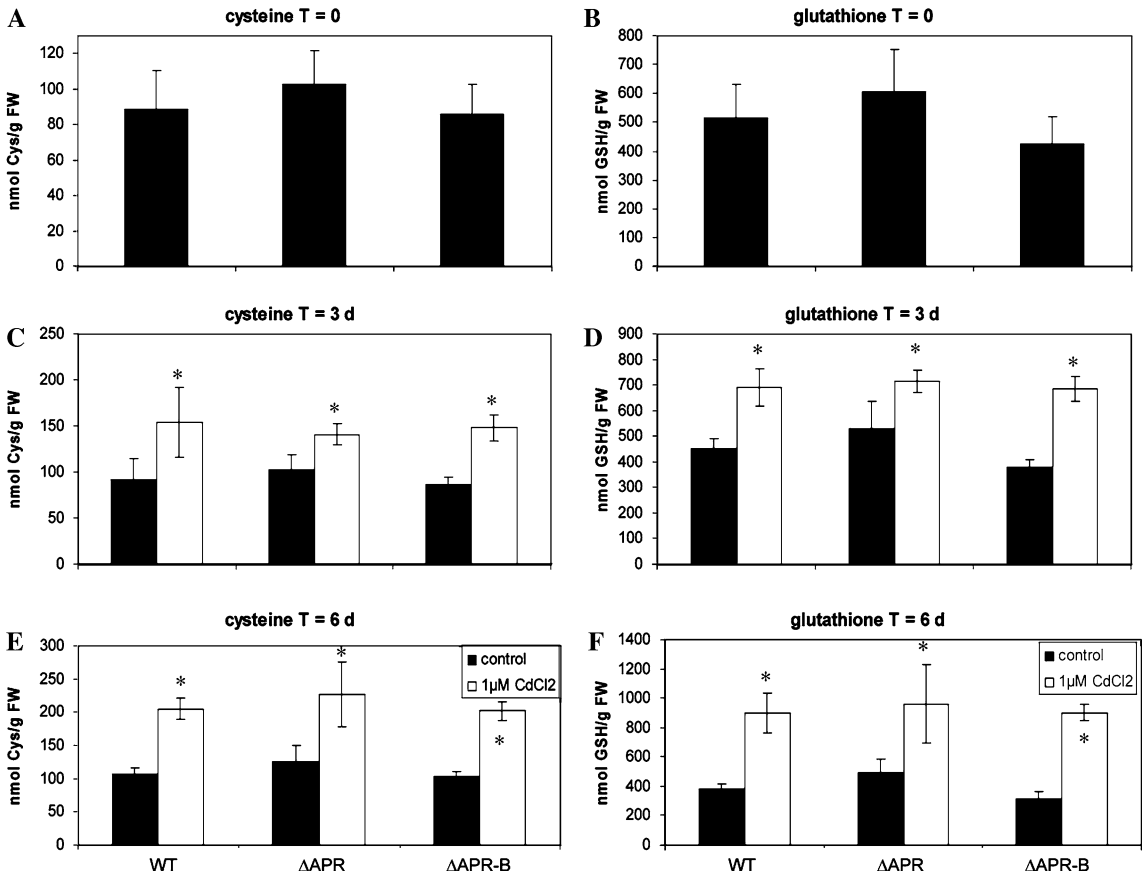

Fig. 5 Induction of thiol content by Cd. Synchronized liquid cultures of wild type moss (WT), two Δ*APR* lines (corresponding to A11-3-12 and A12-4-3 from Koprivova et al. 2002) and two Δ*APR-B* lines (P8 and P43) were treated with 1 µM $CdCl_2$. Cysteine (**A**, **C**, **E**) and glutathione (**B**, **D**, **F**) content was determined before the treatments (**A**, **B**), after 3 (**C**, **D**) and 6 days (**E**, **F**). Data are presented as means ± SD. Values indicated by asterisks are different at $P \leq 0.05$. The whole experiment was carried out twice with three independent cultures per line

This enabled us to perform a side by side comparison of knockout mutants of both APS reductase forms, *PpAPR-B* and *PpAPR* (Koprivova et al. 2002). As expected from the results with Δ*APR* plants, the Δ*APR-B* lines grew on medium with sulfate as a sole sulfur source without any growth or developmental alterations. However, the content of GSH was slightly lower than in Δ*APR* and wild type moss. Clearly, each of the enzymes is independently capable of providing enough sulfite to meet the demands under standard growth conditions.

This was corroborated also during an increased demand for reduced sulfur by exposure of the different moss genotypes to 1 µM Cd. Under this condition thiol content increases (Fig. 6; see also Rother et al. 2006). No differences in the level of induction have been found between wild type moss and the knockout lines, which again confirms that each isoform alone has enough capacity to sustain the increased flux through sulfate assimilation. Whereas the *PpAPR* mRNA level was increased by the Cd treatment, *PpAPR-B* was not affected. This seems to be in contrast to the results of Rother et al. (2006), who showed a coordinated increase in transcript levels for all enzymes of the sulfate reduction pathway. These authors, however, observed a concentration-dependent response, with higher induction with increasing Cd concentration from 5 to 10 µM. The 1 µM Cd used in our experiments was, therefore, sufficient to induce synthesis of thiols but does not appear to be high enough to provoke the strong upregulation in the pathway as seen in Rother et al. (2006). As expected, increasing the Cd concentration to 5 and 10 µM resulted in significantly higher injury of the moss plants. The injury after 5 days, quantified as chlorophyll content, was very similar in our experiments to Rother et al. (2006). Comparing the two classes of knockout lines with wild type moss revealed that Δ*APR-B* plants were more sensitive to Cd than Δ*APR*. The Δ*APR* plants were already characterized as Cd sensitive, based on cultivation on 15 µM $CdCl_2$ (Koprivova et al. 2002). It is evident that the deficiency in one APS reductase isoform affects the survival of moss only at high Cd level. The disruption of *PpAPR-B* seems to have a higher effect on moss than the lack of *PpAPR*. At these high Cd concentrations mRNA

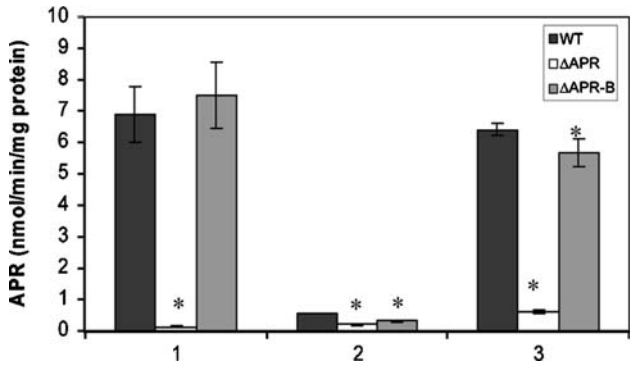

Fig. 6 APS reductase activity in WT moss, Δ*APR* and Δ*APR-B* plants. APS reduction rate was measured in crude extracts of protonema from WT moss, 3 independent Δ*APR* lines (corresponding to A11-3-12, A12-4-3 and A12-7-7 from Koprivova et al. 2002)and three Δ*APR-B* lines (P8, P27, and P43). The activity was measured under standard reaction conditions specific for APR (1), omitting $MgSO_4$ and adding thioredoxin to primarily detect APR-B (2), and in standard conditions plus thioredoxin to detect both APR and APR-B (3). Data are presented as means ± SD. Values indicated by asterisks are different from WT at $P \leq 0.05$

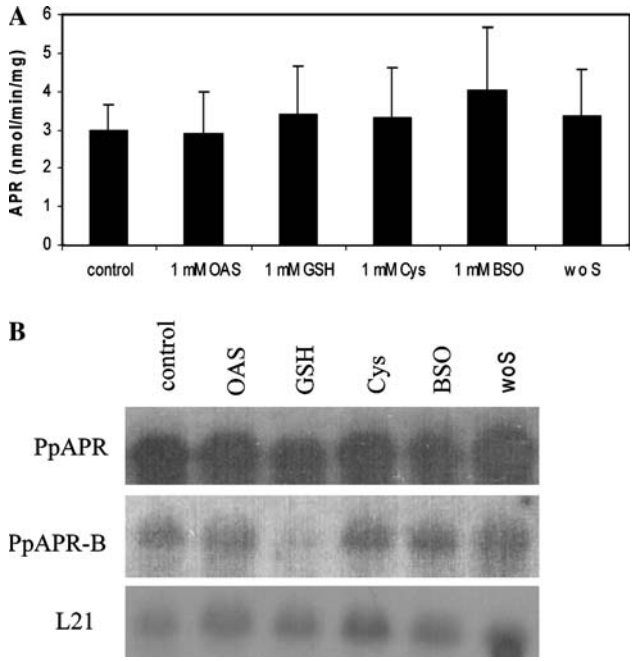

Fig. 7 Regulation of PpAPR and PpPAPR in *P. patens*. Wild type *P. patens* protonema cultures were treated with 1 mM *O*-acetylserine (OAS), 1 mM GSH, 1 mM cysteine for 4 hours, or treated with 1 mM buthionine sulfoxime (BSO) or transferred into a medium where sulfate was exchanged for chloride (woS) for 24 h. (**A**) APS reductase activity was measured in crude extracts as production of sulfite from [^{35}S]APS in the presence of DTE. (**B**) Total RNA was isolated and analyzed by northern blotting with ^{32}P labeled cDNA fragments of *PpAPR*, *PpAPR-B*, and L21 ribosomal protein as probes

levels of both isoforms are induced to the same level (Rother et al. 2006). An attractive hypothesis to explain this observation was the different level of direct inhibition of the enzymes by Cd. Since PpAPR possess the FeS cluster, it could be more prone to inactivation by Cd than the cofactor-less PpAPR-B. However, analysis of recombinant proteins revealed that the enzymes are not affected by Cd even at concentrations far higher than used in the plant treatments. The higher sensitivity to Cd in Δ*APR-B* plants might however be connected to their slightly but significantly lower GSH content compared to wild type and Δ*APR* plants. Why disruption of the *PpAPR-B*, which contributes much less to total APS reductase activity than *PpAPR* has a larger effect on the thiol accumulation is not clear and will be subject of further investigations.

In contrast to our previous report, where *PpAPR-B* mRNA was increased in Δ*APR* plants (Koprivova et al. 2002), we have not observed any compensation of transcript accumulation. This may be due to slight differences in growth conditions (light intensity, temperature), as in higher plants APS reductase is very highly regulated and its mRNA levels react rapidly to changes in environment (Leustek et al. 2000; Kopriva and Koprivova 2004). This finding has an important implication. If each of the enzymes alone provides enough sulfite, sulfite production must be tightly regulated in wild type moss, which possesses both isoforms, otherwise toxic amounts of reduced sulfur compounds would accumulate. The PpAPR or PpAPR-B may be inhibited by a post-translational feedback inhibition by reduced sulfur compound. This is the case in higher plants, where feeding with GSH strongly reduces APS reductase activity by both reducing mRNA levels and by redox regulation of the protein (Bick et al. 2001; Vauclare et al. 2002; Kopriva and Koprivova 2004). However, since higher concentrations of GSH are necessary for regulation and there is no significant difference between its content in wild type moss and the knockout plants, this does not seem to be the most probable case. APR was shown to possess the highest control of flux through sulfate reduction in higher plants (Vauclare et al. 2002). However, if the control were to be moved to another enzyme, ATP sulfurylase or sulfite reductase, even large changes in the rate of sulfite production would not affect the flux to sulfide and would not lead to an increase in thiol levels. This indeed seems to be the case, since neither mRNA level nor activity of PpAPR was affected by treatments strongly regulating APR in higher plants (Fig. 7). This was a very surprising finding since in various higher plants feeding with the precursor of cysteine, *O*-acetylserine, strongly induced the activity and mRNA level of APR and, on the other hand, in many experimental systems APR was strongly repressed by thiols (Leustek et al. 2000; Kopriva and Koprivova 2004; Kopriva 2006). Similar to the results with Cd treatment, it was *PpAPR-B* and not *PpAPR*, which was downregulated by GSH indicating that this gene is subjected to tighter regulation than

PpAPR. This is very surprising, since the reaction velocity of PpAPR-B is about two orders of magnitude lower than that of PpAPR (Kopriva et al. 2007a) and the enzyme contributes much less to total APS reductase activity than PpAPR (Fig. 6). However, this conclusion is supported by the results of Cd treatments, since the loss of *PpAPR-B* caused higher injury to the moss plants than the loss of *PpAPR* despite a much higher total APS reductase activity in the latter plants. In addition, treatment with 1 μM Cd led to only a minor increase in *PpAPR* mRNA level but to increase in thiol content in all three genotypes. These findings indeed suggest that sulfate assimilation in *P. patens* is not controlled by APS reductase as in flowering plants (Vauclare et al. 2002). Since ATP sulfurylase provides substrates not only for sulfate reduction but also for further activation by APS kinase to PAPS and subsequently synthesis of secondary metabolites, the likely candidate for flux controlling enzyme seems to be sulfite reductase. Remarkably, in the Cd treatments of Rother et al. (2006), sulfite reductase mRNA was increased to the highest levels from all enzymes of sulfate assimilation. The change in control point of sulfate assimilation from sulfite reductase to APR in the evolution of flowering plants could than be explained by the fact that in the latter sulfite reductase acquired a second function as a DNA binding protein in plastid nucleoids (Sato et al. 2001). Further experiments are necessary to analyze the role of sulfite reductase in the control of moss sulfate reduction. Altogether, it seems that sulfate assimilation in moss differs from higher plants not only by the presence of an additional and unusual gene for APS reductase, but the pathway seems to be regulated differently.

Acknowledgements This work was supported by the German Research Council (DFG) grant KO2065/3 within the research group FOR 383 "Sulfur metabolism in plants: junction of basic metabolic pathways and molecular mechanisms of stress resistance". We thank Anne Katrin Prowse for help with the manuscript.

References

Bick J-A, Åslund F, Chen Y, Leustek T (1998) Glutaredoxin function for the carboxyl-terminal domain of the plant-type 5′-adenylyl-sulfate reductase. Proc Natl Acad Sci USA 95:8404–8409

Bick JA, Seterdahl AT, Knaff DB, Chen YC, Pitcher LH, Zilinskas BA, Leustek T (2001) Regulation of the plant-type 5′-adenylyl sulfate reductase by oxidative stress. Biochemistry 40: 9040–9048

Brunner M, Kocsy G, Ruegsegger A, Schmutz D. Brunold C (1995) Effect of chilling on assimilatory sulphate reduction and glutathione synthesis in maize. J Plant Physiol 146:743–747

Bierfreund NM, Tintelnot S, Reski R, Decker EL (2004) Loss of GH3-function does not affect phytochrome-mediated development in a moss, *Physcomitrella patens*. J Plant Physiol 161:823–835

Frank W, Decker EL, Reski R (2005a) Molecular tools to study *Physcomitrella patens*. Plant Biol 7:220–227

Frank W, Ratnadewi D Reski R (2005b) *Physcomitrella patens* is highly tolerant against drought, salt and osmotic stress. Planta 220:384–394

Girke T, Schmidt H, Zäringer U, Reski R, Heinz E (1998) Identification of a novel Delta 6 acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. Plant J 15:39–48

Hohe A, Decker EL, Gorr G, Schween G, Reski R (2002) Tight control of growth and cell differentiation in photoautotrophically growing moss *Physcomitrella patens* bioreactor cultures. Plant Cell Rep 20:1135–1140

Hohe A, Egener T, Lucht J, Holtorf H, Reinhard C, Schween G, Reski R (2004) An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene knockouts in a moss, *Physcomitrella patens*. Curr Genet 44:339–347

Kamisugi Y, Schlink K, Rensing SA, Schween G, Stackelberg M v, Cuming AC, Reski R, Cove DJ (2006) The mechanism of gene targeting in *Physcomitrella patens*: homologous recombination, concatenation and multiple integration. Nucl Acids Res 34:6205–6214

Kopriva S (2006) Regulation of sulfate assimilation in Arabidopsis and beyond. Ann Bot 97:479–495

Kopriva S, Koprivova A (2004) Plant adenosine 5′phosphosulfate reductase—the past, the present, and the future. J Exp Bot 55:1775–1783

Kopriva S, Rennenberg H (2004) Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot 55:1831–1842

Kopriva S, Büchert T, Fritz G, Suter M, Weber M, Benda R, Schaller J, Feller U, Schürmann P, Schünemann V, Trautwein AX, Kroneck PMH, Brunold C (2001) Plant adenosine 5′-phosphosulfate reductase is a novel iron–sulfur protein. J Biol Chem 276:42881–42886

Kopriva S, Büchert T, Fritz G, Suter M, Benda R, Schünemann V, Koprivova A, Schürmann P, Trautwein AX, Kroneck PMH, Brunold C (2002a) The presence of an iron-sulfur cluster in adenosine 5′-phosphosulfate reductase separates organisms utilizing adenosine 5′-phosphosulfate and phosphoadenosine 5′-phosphosulfate for sulfate assimilation. J Biol Chem 277: 21786–21791

Kopriva S, Suter M, von Ballmoos P, Hesse H, Krähenbühl U, Rennenberg H, Brunold C (2002b) Interaction of sulfate assimilation with carbon and nitrogen metabolism in *Lemna minor*. Plant Physiol 130:1406–1413

Kopriva S, Fritzemeier K, Wiedemann G, Reski R (2007a) The putative moss 3′phosphoadenosine 5′phosphosulfate reductase is a novel form of adenosine 5′phosphosulfate reductase without iron sulfur cluster. J Biol Chem 282:22930–22938

Kopriva S, Wiedemann G, Reski R (2007b) Sulfate assimilation in basal land plants—what does genomic sequencing tell us? Plant Biol. doi:10.1055/s-2007-965430

Koprivova A, Suter M, Op den Camp R, Brunold C, Kopriva S (2000) Regulation of sulfate assimilation by nitrogen in *Arabidopsis*. Plant Physiol 122:737–746

Koprivova A, Meyer A, Schween G, Herschbach C, Reski R, Kopriva S (2002) Functional knockout of the adenosine 5́phosphosulfate reductase gene in *Physcomitrella patens* revives an old route of sulfate assimilation. J Biol Chem 277:32195–32201

Koprivova A, Stemmer C, Altmann F, Hoffmann A, Kopriva S, Gorr G, Reski R, Decker EL (2004) Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. Plant Biotechnol J 2:517–523

Lappartient AG, Vidmar JJ, Leustek T, Glass ADM, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J 18:89–95

Lee SM, Leustek T (1999) The affect of cadmium on sulfate assimilation enzymes in *Brassica juncea*. Plant Sci 141:201–207

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51:141–165

Li JY, Schiff JA (1991) Purification and properties of adenosine 5′-phosphosulphate sulphotransferase from *Euglena*. Biochem J 274:355–360

Reski R, Abel WO (1985) Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. Planta 165:354–358

Reski R, Reynolds S, Wehe M, Kleber-Janke T, Kruse S (1998) Moss (*Physcomitrella patens*) expressed sequence tags include several sequences which are novel for plants. Bot Acta 111:145–151

Rother M, Krauss GJ, Grass G, Wesenberg D (2006) Sulphate assimilation under Cd^{2+} stress in *Physcomitrella patens*—combined transcript, enzyme and metabolite profiling. Plant Cell Environ 29:1801–1811

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sato N, Nakayama M, Hase T (2001) The 70-kDa major DNA—compacting protein of the chloroplast nucleoid is sulfite reductase. FEBS Lett 487:347–350

Schaefer DG (2001) Gene targeting in *Physcomitrella patens*. Curr Opin Plant Biol 4:143–150

Setya A, Murillo M, Leustek T (1996) Sulfate reduction in higher plants: molecular evidence for a novel 5′-adenylsulfate reductase. Proc Natl Acad Sci USA 93:13383–13388

Strepp R, Scholz S, Kruse S, Speth V, Reski R (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. Proc Natl Acad Sci USA 95:4368–4373

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos O, Krähenbuhl U, Op den Camp R, Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible to negative control by thiols than ATP sulphurylase. Plant J 31:729–740

Westerman S, Stulen I, Suter M, Brunold C, De Kok LJ (2001) Atmospheric H_2S as sulphur source for *Brassica oleracea*: consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. Plant Physiol Biochem 39:425–432