Functional knockout of the adenosine 5´phosphosulfate reductase gene in Physcomitrella patens revives an old route of sulfate assimilation*

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Knockout of APS reductase gene in *Physcomitrella*

ABSTRACT
Reduction of adenosine 5’-phosphosulfate to sulfite catalyzed by adenosine 5’-phosphosulfate reductase is considered to be the key step of sulfate assimilation in higher plants. However, in analogy to enteric bacteria, an alternative pathway of sulfate reduction via phosphoadenosine 5’-phosphosulfate was proposed. Up to now the presence of the corresponding enzyme, phosphoadenosine 5’-phosphosulfate reductase, could be neither confirmed nor excluded in plants. In order to find possible alternative routes of sulfate assimilation we disrupted the adenosine 5’-phosphosulfate reductase single copy gene in *Physcomitrella patens* by homologous recombination. This resulted in complete loss of the correct transcript and enzymatic activity. Surprisingly, the knockout plants grew on sulfate as the sole sulfur source and the concentration of thiols in the knockouts did not differ from the wild type plants. However, when exposed to sub-lethal concentration of cadmium, the knockouts were more sensitive than wild type plants. When fed [35S]sulfate, the knockouts incorporated 35S in thiols; the flux through sulfate reduction was about 50% lower than in the wild type plants. PAPS reductase activity could not be measured with thioredoxin as reductant, but a cDNA and gene coding for this enzyme were detected in *P. patens*. The moss *Physcomitrella patens* is thus the first plant species where PAPS reductase was confirmed on the molecular level and also the first organism where both APS and PAPS dependent sulfate assimilation co-exist.
INTRODUCTION

Assimilatory sulfate reduction is a pathway used by plants, algae, fungi, and bacteria to convert inorganic sulfate to sulfide which is further incorporated into carbon skeletons of amino acids to form cysteine or homocysteine (1). For reduction, sulfate must be activated by ATP sulfurylase to adenosine 5’-phosphosulfate (APS). In higher plants APS is directly reduced by APS reductase (APR) to sulfite, which is subsequently reduced to sulfide by sulfite reductase and as such incorporated into O-acetylserine by O-acetylserine (thiol)lyase (Fig. 1) (2, 3). In fungi and some enteric bacteria, such as Escherichia coli and Salmonella typhimurium, APS must be phosphorylated by APS kinase to phosphoadenosine 5’-phosphosulfate (PAPS) in order to be reduced by PAPS reductase (4). Since the sulfate assimilation pathway was first resolved in bacteria which reduce sulfate via PAPS, the PAPS reductase pathway of sulfate assimilation was expected to exist also in plants (5, 6).

Although plants and algae utilize APS rather than PAPS as sulfonyl donor for reduction and APR was shown to play a key role in controlling sulfate reduction in plants (2), the existence of PAPS dependent pathway of sulfate assimilation was never excluded, especially since a partial purification of a PAPS reductase from spinach had been reported (7). The Arabidopsis genome does not contain any genes homologous to E. coli PAPS reductase other than those encoding three isoforms of APR (8, 9). Since a putative plant PAPS reductase may have a structure completely divergent from that of the bacterial enzyme, only analysis of plants lacking APR activity may prove or exclude the PAPS dependent sulfate assimilation in plants. However, until now, neither mutants nor transgenic plants devoid of APR activity were described, probably because APR is encoded by small multigene families of 2-3 isoforms in most plant species analyzed to date (8-11). Therefore, to produce and analyze plants lacking APR activity we turned to the moss Physcomitrella patens.
In the last few years, *P. patens* became an increasingly used model system to study function of plant genes (12-14). *Physcomitrella* can easily be stably transformed by PEG-mediated DNA transfer to protoplasts (15). Most importantly, it possess an efficient system of homologous recombination allowing exact gene targeting (16-19). Thus, *Physcomitrella* is the only plant where gene knockouts can be routinely produced by a highly efficient and straightforward approach (20). Using this approach, a function of an ancestral tubulin FtsZ in plastid division was revealed (17). The value of the targeted gene disruptions was further demonstrated by analysis of knockouts in genes encoding Δ6-acyl-group desaturase and multiubiquitin chain binding subunit MCB1 of the 26S proteasome illustrating their functions in fatty acid synthesis and plant development, respectively (18, 19). Here, we report an analysis of a functional knockout in a gene encoding APR in *P. patens* and the impact of this knockout on sulfate assimilation.

**EXPERIMENTAL PROCEDURES**

*Plant Material* - *Physcomitrella patens* (Hedw.) B.S.G. was cultured in liquid or solid modified Knop medium as described earlier (21). For protoplast isolation, protonema was grown in semi-continuous bioreactor cultures supplemented with 2.5 mM ammonium tartrate (22). To study the effect of cadmium the plants were grown for 5 days on solid Knop media supplemented with 5, 10, or 15 µM CdCl₂.

*Protoplast Isolation, Transformation, and Regeneration* - Protoplasts were isolated and transformed as described before (17). The transformations were performed with 3x10⁵ protoplasts and 30 µg DNA. Regeneration and selection were performed as described (17).
For selection, Knop media were supplemented with 50 µg/ml G418. Both selection and release media contained 5 µg /ml reduced glutathione (GSH).

Molecular Cloning - The cDNA clone for APR (PpAPR, pp004074261r) was obtained upon BLAST searching of P. patens EST library, generated in a joint project of University of Freiburg with BASF Plant Science GmbH (23), with the protein sequence of APR2 isoform of APR from A. thaliana (Accession No. U56921) as a query. The 1724 bp full-length PpAPR cDNA clone was sequenced on both strands. The corresponding genomic DNA (3616 bp) was obtained by PCR and sequenced by primer walking. For creating the knockout construct a fragment of 2006 bp was amplified from genomic DNA by PCR with primers R10 (TCTTTCACTATTCCGTTGACG) and R11 (CGACCACACATTAGATCC) (Fig. 2) and cloned into pCRII vector (Invitrogen, Groningen, The Netherlands). This plasmid was digested with HindII to cut out a 361 bp fragment, containing part of the fifth and the sixth exon (Fig. 2), and replace it with the nptII selection cartridge which was obtained from the vector pRT101neo (18). To clone the selection cassette pRT101neo was digested with HindIII and the protruding ends were filled with Klenow fragment of DNA polymerase (24). 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 APR genomic sequences of 874 bp and 771 bp length. The DNA was precipitated from the reaction mixture, redissolved in 90 µl water, and used for transformation of P. patens. P. patens PAPS reductase sequences (Accession Nos. BJ166495 and BJ173474) were retrieved from the GenBank after TBLASTN search of the EST database with the PpAPR protein sequence as query. Oligonucleotide primers PR1 (CTTACTTTGTACAATTAGAAGG) and PR2 (TAAGTTTCTCAGCGAAGTGG) were derived from these EST sequences and utilized to
amplify the cDNA from *P. patens* total RNA by RT-PCR. The identity of the PCR fragment was confirmed by sequencing.

*Molecular Analysis of Transgenic Plants* - For the pre-screening of G418 resistant plants small pieces of gametophores (1-5 mg) were treated for 30 min at 45°C in 75 mM Tris-HCl, pH 8.8 containing 20 mM (NH₄)₂SO₄ and 0.1 % Tween 20, and 3 µL of this extract was used for PCR with four pairs of primers (Fig. 2): R14 (CGGAACGCGTGCCAACGTTC) and R15 (GCAACACCATCAAGGTTACC) to detect a disruption of the original *apr* gene, N1 (TACCGACAGTGGTCCCAAAG) and N2 (CCACCATGATATTCCGGCAAG) to detect the presence of the *nptII* cassette, R20 (CAAAGCACCCTTGATATCC) and N3 (TGTCGTGCTCCACCAGTGT) to control the integration of the transgene at the 5’ end, and N4 (GTTGAGCATATAAGAACAA) and R21 (TGCGTAGGCTTTCTGAGC) to check the integration at 3’ end. Plants that gave expected fragments by all four PCR reactions were considered as putative knockouts and selected for further analysis. For Southern analysis genomic DNA was isolated from the selected plants with the Nucleospin plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instruction and the analysis was performed with 2 µg DNA according to standard protocol (24). The integration regions of the transgene were isolated from transformants 11-3-3, 11-3-11, 11-3-12, 12-4-29, and 12-7-7 by PCR with primer pairs R20 and N3, and R21 and N4, for the 5’ and 3’ ends, respectively. The PCR fragments were cloned in pCRII vector and sequenced.

Total RNA was isolated from moss tissue using the RNeasy kit (Qiagen, Hilden, Germany). RT-PCR with primers R14 and R11 was performed according to the standard protocol (24). As control, the reverse transcribed RNA was subjected to PCR with primers C45F (GGTTGGTCATGGGTTGCG) and C45R (GAGGTCAACTGTCTCGGC)
corresponding to the constitutively expressed gene for ribosomal protein L21 (25). For northern analysis 5 µg of total RNA were subjected to electrophoresis on formaldehyde-agarose gels at 120 V, transferred onto Hybond-N nylon membranes (Amersham, Freiburg, Germany) and hybridized with 32P-labelled cDNA probes for APR, L21, and PAPS reductase. The membranes were washed four times at different concentrations of SSC in 0.1% SDS for 20 min at 65°C, the final washing step being 0.5 x SSC, 0.1% SDS at 65°C, and exposed to a X-ray film (Fuji medical RX) at -80°C for 2 to 3 days.

**Enzyme Assays** - Plant extracts were prepared from 200 mg of protonema tissue by homogenization in 2 ml of 50 mM Na/KPO4 buffer (pH 8) supplemented with 30 mM Na2SO3, 0.5 mM 5’-AMP, and 10 mM DTE (26). APR activity was measured in extracts as the production of [35S]sulfite, assayed as acid volatile radioactivity formed in the presence of [35S]APS and DTE (27). PAPS reductase was measured using [35S]PAPS, DTE, and recombinant thioredoxin m or f from spinach, thioredoxin from E. coli, or GSH and glutaredoxin from E. coli (28). Protein concentration in the extracts was determined according to Bradford (29) with bovine serum albumin as standard.

**HPLC Analysis of Low Molecular Weight Thiols** - For in vitro labeling, 50-100 mg plant material from liquid culture was ground in liquid nitrogen and extracted in 750 µl 0.1 N HCl. After centrifugation 120 µl supernatant were mixed with 180 µl 0.2 M CHES-buffer, pH 9.3, and 30 µl 5 mM DTT were added to reduce disulfides. After reduction 20 µl of 30 mM monobromobimane (Thiolyte® MB, Calbiochem, Bad Soden, Germany) was added and derivatization of thiols allowed to proceed for 15 min in the dark. The reaction was stopped
and the conjugates stabilized by adding 240 µl 10% acetic acid. Bimane conjugates were separated by HPLC (SUPELCOSIL™ LC-18, 25 cm x 4.6 mm, 5 µm, Sigma-Aldrich, Deisenhofen, Germany) using 10% (v/v) methanol/0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol/0.25% (v/v) acetic acid (pH 3.9) as solvent B. The elution protocol employed a linear gradient from 96% A to 82% A in 20 min and the flow rate was kept constant at 1 ml/min. Bimane derivatives were detected fluorimetrically (RF535, Shimadzu, Kyoto, Japan) with excitation at 390 nm and emission at 480 nm.

**Feeding of \(^{35}\)SO\(_4^{2-}\) and Determination of \(^{35}\)S in Thiols** - Three cultures each of wild type moss and the 11-3-12 knockout line, containing approximately 100 mg protonema in 10 ml Knop medium with 0.06 mM SO\(_4^{2-}\), were supplemented with 150 µCi \(^{35}\)SO\(_4^{2-}\) (specific activity 9.25 x 10\(^{12}\) Bq mmol\(^{-1}\)) and further cultivated for two hours. Plant material was extracted with 1.2 ml of 0.1 M HCl containing 1 mM Na\(_2\)EDTA. After centrifugation the thiols were reduced with DTT and labelled with monobromobimane as described (30). A 100 µl aliquot of each sample was separated by HPLC and fractions of 1 ml were collected in scintillation vials. The \(^{35}\)S radioactivity was determined in a liquid scintillation counter (Wallac, Turku, Finland). Total cysteine, \(\gamma\)-glutamylcysteine, and GSH were analyzed by the same HPLC system as described above.

**RESULTS**

**Cloning and Characterization of APS Reductase Gene**

The sequence of APR2 isoform of APS reductase from *A. thaliana* was used as a query to
search the *P. patens* EST bank, generated in a joint project of University of Freiburg with BASF Plant Science GmbH (23), by the BLAST software. The analysis identified a single contig composed from 16 sequences. The longest cDNA clone, pp004074261r, was fully sequenced. This cDNA was 1724 base pairs long and 57.9% identical to the APR2 cDNA. It contained a single open reading frame encoding a protein of 465 amino acids. Similar to APR from *Arabidopsis*, the protein from *P. patens* consisted of three domains: a chloroplast targeting peptide, a part similar to bacterial PAPS reductase, and a C-terminal thioredoxin-like domain (Fig. 2). The APR of *P. patens* was 60.6% identical with the APR2 at the amino acid level, the identity raised to 71.3% when the mature proteins were compared.

Surprisingly, the thioredoxin-like active site CXXC was modified to CXXS. Southern analysis confirmed that, in contrast to higher plants, APR is encoded by a single gene in *P. patens* (data not shown). The APR cDNA sequence was used to define oligonucleotide primers for amplification of the gene from the genomic DNA. The gene was then completely sequenced. Figure 2 shows the exon/intron structure of APR encoding gene from *P. patens* and its comparison to the *apr2* gene from *A. thaliana* (31). The three introns present in the *A. thaliana apr2* gene were found at identical positions in *P. patens*. However, whilst the major part of the protein is encoded by a single exon in *Arabidopsis*, five additional introns are present in the *P. patens* gene (Fig. 2).

**Gene Targeting**

To synthesize the disruption construct, a 2006 bp central part of APR gene was amplified from the genomic DNA (Fig. 2), cloned into pCR plasmid and a 361 bp fragment comprising part of exon 5, intron 5, and exon 6 was replaced by the *nptII* gene as a selection marker. 130 regenerated G418 resistant plants were pre-screened by PCR with four different primer
combinations to detect the nptII cassette, disruption of apr gene, and correct integration of the transgene on both 5’ and 3’ ends to identify positive recombination events. For 55 transformants all four PCR reactions resulted in the expected products indicating that these represent true knockouts of the apr gene. From these, 8 individual transformants were selected for further analysis. Southern blot analysis using the 361 bp fragment, which had been replaced in the construct by the nptII gene, as a probe verified that in all 8 transformants the original APR gene was disrupted (Fig. 3). Sequencing of 5’ and 3’ integration sites of the transgene from five transformants revealed precise integrations without any insertions or deletions. Flow cytometry measurements revealed that the transformant 12-4-2 was a diploid, while the other 7 mutants were haploids.

No APR transcript was detected in the transformants by RT-PCR with primers R14 and R15 (Fig. 4A). Northern analysis detected the correct 1.6 kb APR transcript solely in the wild type, whereas the transformants possessed only aberrant transcripts of higher or lower molecular weight (Fig. 4B). The expression of the control gene for L21 (25) was not affected in the transformants.

Analysis of Sulfate Assimilation

To answer the question whether the disruption of apr gene indeed resulted in loss of function, the APR activity was measured in the transformants. While the APR activity reached 6.1 nmol min⁻¹ mg protein⁻¹ in extracts of wild type plants, sulfite production from APS could not be measured in the extracts of the transformants. The transgenic plants were thus real functional knockouts in APS reductase.

Since we expected, that the transgenic plants lacking APR would not be able to grow on sulfate as a sole sulfur source, like yeast or E. coli PAPS reductase mutants (32, 33), the
selection media were supplemented with GSH. Surprisingly however, when transferred to a normal Knop medium without a reduced sulfur source, the transgenic plants survived and showed no obvious phenotypic effects compared to the wild type. Determination of low molecular weight thiols revealed no significant difference in concentration of cysteine and glutathione between wild type and mutant plants (data not shown). Evidently, an alternative pathway of sulfate reduction independent of APR must exist in *P. patens*.

To test whether this alternative pathway is capable of providing enough reduced sulfur also under conditions of increased demand, the transformants were exposed to three different sublethal concentrations of cadmium. After 5 days incubation on 15 µM CdCl₂, the wild type plants were seriously damaged but still viable whereas the transformants were dead (Fig. 5). At lower Cd concentrations the transformants were also more injured than the wild type plants. In order to obtain quantitative data about the flux through the sulfate assimilation pathway, plants of the wild type and the 11-3-12 Δapr line were fed [³⁵S] sulfate and the radioactivity incorporated into cysteine and GSH was determined (Fig. 6). The knockout was indeed able to reduce sulfate but at a significantly lower rate. The flux from sulfate towards thiols was 40% lower in the knockout line than in the wild type plants.

*Identification of PAPS Reductase in Physcomitrella patens*

In order to identify the enzyme(s) involved in the alternative sulfate assimilation pathway of the knockouts we first measured the PAPS reductase activity. Neither the wild type moss nor the transformants possessed measurable activities of this enzyme using DTT and recombinant thioredoxin m and f from spinach, thioredoxin from *E. coli*, or glutaredoxin and GSH as reductants (data not shown). However, a new search of the GenBank database with
the *P. patens* APR as a query identified two recently submitted EST clones (Accession Nos. BJ166495 and BJ173474) as putative PAPS reductase. The deduced amino acid sequence is 24.2% and 31.1% identical with *P. patens* APR and *E. coli* PAPS reductase, respectively (Fig. 7). The putative protein contains the (P)APS reductase signature ECG(IL)H but not the two cysteine pairs binding the FeS cluster in APR (34). The corresponding cDNA was amplified from *P. patens* RNA and used as a probe for northern analysis. PAPS reductase was indeed expressed in wild type and all "apr knockout lines analyzed. In most of the knockouts the mRNA level was 2-3 times higher than in the wild type moss (Fig. 8). *P. patens* is thus the first plant species with molecular evidence for a PAPS reductase and also the first organism where both APS and PAPS dependent reductases exist.

**DISCUSSION**

APS reductase is an enzyme possessing the major control over the flux through sulfate assimilation in plants and as such, it is extensively regulated (1, 2). No mutants or transgenic plants lacking the APR have been described, however, the deletion of its counterpart in bacteria and yeast, the PAPS reductase, led to cysteine auxotrophy of the mutants (32, 33). In most higher plants APR is encoded by a small multigene family (8-11). This redundancy might explain why no APR deficient mutants have been identified to date. Here we have shown that the moss *P. patens* contains only a single gene for APR. The apr gene structure from *P. patens* differs significantly from the apr genes of *A. thaliana*. The three introns present in the *A. thaliana* apr2 and apr3 genes (apr1 is lacking intron 2) were found at identical positions in *P. patens* but 5 additional introns are present in the *P. patens* gene. The mature APR protein is composed of two domains catalyzing separate steps in reduction of APS (35). It was therefore expected that the protein originated by a fusion of the individual
proteins and these domains would be encoded by discrete exons (31). However, surprisingly, the major part of the protein is encoded by a single exon in Arabidopsis (31). In the P. patens apr gene the two domains are separated by an intron (Fig. 2), thus, the apr gene organization in P. patens but not in A. thaliana corresponds to its probable evolutionary history, i.e. fusion of genes for bacterial type APR and thioredoxin (36).

P. patens seems to be an excellent object to construct plants lacking APR activity due to the possibility of gene targeting in this organism (13, 16, 20) and presence of only a single apr gene in the genome. The apr gene was disrupted by substitution of a 361 gene fragment by the nptII cassette via homologous recombination. In 55 from 130 G418 resistant plants analyzed all four criteria to select putative knockouts were met. The efficiency of the gene targeting (42%) was thus higher than in experiments in which cDNA was used for the disruption constructs (17, 19) and comparable or lower than described for constructs with genomic DNA (18, 36). In all 8 randomly selected putative knockouts Southern analysis (Fig. 3) revealed the disruption of the wild type apr locus and sequencing of the junction regions of five transformants confirmed a correct integration of the transgene in the genome, similar as described previously (17, 18, 37). The disruption of the apr gene led to a loss of a correct transcript as revealed by the RT-PCR and northern analysis (Fig. 4). The latter method detected a single APR transcript in the wild type P. patens and strong but aberrant signals of both greater and lower molecular weight in the knockouts (Fig. 4). The aberrant transcripts, which were also observed by Strepp et al., (17), were most probably derived from the still functioning native apr promoter or from insertion of multiple copies of the transgene. The lack of correct transcript thus corroborates the disruption of apr gene. However, the absence of APR transcript still was not sufficient evidence for a full lack of APR function, since the possibility of a second isoform with different primary structure
could not be excluded. Therefore, only the absence of detectable APR activity represented the final prove, that the Δapr knockouts obtained indeed represent mutants with complete lack of function.

The primary reason for construction of the APR knockouts was to bring evidence for or against existence of PAPS reductase in plants. This enzyme was proposed to be present in plants in analogy with enteric bacteria and yeast (5, 6) and its activity was measured in spinach (7), its existence is, however, rather doubted (2). Indeed, in the Arabidopsis genome no homologue of E. coli PAPS reductase other than the three APR isoforms was found. However, as discussed above, the lack of homologue proteins does not exclude an existence of a protein with completely unrelated primary structure. Due to the presence of APR the PAPS reductase activity could be masked, since the two sulfonucleotides are easily interchangeable by catalytic action of APS kinase and 3'(2'),5'-bisphosphate nucleotidase, or the enzyme synthesis might be repressed. Therefore, by disruption of apr gene we hoped to remove the possible repression of PAPS reductase. We expected that the Δapr knockouts would be disturbed in sulfate assimilation and therefore might be unable to grow on sulfate as the sole sulfur source like mutants of E. coli or S. cerevisiae lacking functional enzymes of the sulfate assimilation pathway (32, 33). Surprisingly, however, the knockout plants grew also on standard medium without addition of reduced sulfur and under normal grow conditions they were undistinguishable from wild type plants. Clearly, another route of sulfate reduction independent from APR must exist in P. patens. This new pathway seems to be as efficient as the APR dependent sulfate assimilation since the knockouts did not exhibit any phenotypical changes when grown on a standard nutrient solution with sulfate as the sole sulfur source. It was thus interesting to test the efficiency of the novel pathway under conditions of increased demand of reduced sulfur. In higher plants exposure to cadmium
leads to induction of synthesis of phytochelatins, small peptides formed from repetitions of
\( \gamma \)-glutamylcysteine units (38). In most bryophytes, phytochelatins are not synthesised, but the
glutathione pool increases significantly upon addition of Cd (39). In any case, exposure of
plants to heavy metals leads to an increased demand for reduced sulfur compounds. The
treatment of the \( \Delta \)april knockouts with cadmium revealed strong phenotypical differences with
the wild type plants under these conditions. Thus, the new pathway was not as effective to
cope with the higher demand for glutathione as the pathway via APR. The confirmation that
Cd sensitive phenotype of the \( \Delta \)april knockouts indeed is caused by a reduced capacity for
sulfate reduction was achieved by \textit{in vivo} measuring of flux through sulfate assimilation
(Fig. 6).

The most obvious explanation for the alternative route of sulfate reduction would be the
presence of PAPS reductase. In the \( \Delta \)april knockouts, however, no PAPS reductase activity
could be measured. Nevertheless, an EST sequence from \( P. \) \textit{patens} was identified which
most probably codes for PAPS reductase (Fig. 7) because (i) the deduced amino acid
sequence is 31.1 % identical to PAPS reductases from \( E. \) \textit{coli} and yeast, (ii) the sequence
contains the (P)APS reductase signature ECG(IL)H (36) but not the two cysteine pairs
binding the FeS cluster in APR (34, 36), and (iii) the mRNA level of this EST was increased
in most of the \( \Delta \)april knockouts (Fig. 8). Unfortunately, the final evidence that the EST indeed
codes for PAPS reductase, i.e. the detection of PAPS reductase activity in moss extracts, is
still missing. In \( P. \) \textit{patens} extracts, both wild type and \( \Delta \)april knockouts, sulfite production
from PAPS was detectable neither with \( E. \) \textit{coli} thioredoxin and glutaredoxin nor with
recombinant thioredoxins m and f from spinach. Alternative cofactors might thus be required
for the PAPS reduction, or the enzyme might be inhibited by DTE used in the assay to keep
the thioredoxin reduced, since \textit{in vivo} thioredoxin is reduced by ferredoxin-thioredoxin.
reductase (40). A necessity of another unknown factor for the enzyme activity also cannot be excluded. The establishment of an assay for the PAPS reductase from *P. patens* will be a most important aim of further experiments.

*P. patens* is the first plant species where molecular evidence for PAPS reductase exists. No homologues genes are found in *Arabidopsis* genome and in any plant EST database, although from several species there are much more EST sequences in the GenBank than from *P. patens*. It is thus plausible to consider the PAPS reductase in *P. patens* an exception and not the rule for organization of sulfate assimilation in plants. Both APR and PAPS reductase probably occurred in the last common ancestor of mosses and vascular plants. The latter was then probably lost in evolution and diversification of higher plants, but both genes survived in *P. patens* and perhaps other lower plants. Therefore it would be very interesting to survey other mosses and liverworts for the presence of PAPS reductase to confirm this theory. Alternatively, the PAPS reductase gene could have been transferred to *P. patens* genome by horizontal gene transfer, as proposed for the evolution of APS and PAPS reductases in cyanobacteria (36). In any case, sulfate assimilation in *P. patens* differs significantly from that in higher plants and the regulation of this pathway, above all the coordination of APS and PAPS dependent routes, will certainly become a subject of new studies.
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REFERENCES


FOOTNOTES

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1 The abbreviations used are: APR, adenosine 5’-phosphosulfate reductase; APS, adenosine 5’-phosphosulfate; PAPS, phosphoadenosine 5’-phosphosulfate.

2 The nucleotide sequences reported in this paper were submitted to the GenBank™/EMBL Data Bank with accession numbers AJ429099 (APS reductase) and AJ489220 (PAPS reductase).
FIGURE LEGENDS

Fig. 1 Pathway of assimilatory sulfate reduction in plants.

Fig. 2 Schematic representation of the APR gene and knockout construct. A Schematic representation of the 3 domains of APR protein (ch-TP, chloroplast targeting peptide; TRX thioredoxin). B The apr2 gene from A. thaliana. The rectangles represent exons, the colours correspond to the respective domains, white colour represent 5’ and 3’ non-translated regions. Introns are presented as lines. The numbers represent lengths in base pairs. C The apr gene from P. patens. The position of the two HindII sites, utilized to cut out the 361 fragment are indicated. D The apr disruption construct. The position of the PCR primers is indicated by arrows.

Fig. 3 Southern blot analysis. Genomic DNA was extracted from protonema tissue of wild type P. patens (WT) and 8 Δapr knockouts: 11-3-3 (1), 11-3-11 (2), 11-3-12 (3), 12-4-2 (4), 12-4-3 (5), 12-4-14 (6), 12-4-29 (7), and 12-7-7 (8). The DNA was cleaved with HindII, separated on 1% agarose, blotted onto Hybond-N nylon membrane, and hybridized with 32P-labelled 361 bp fragment of apr gene, which was replaced in the disruption construct by the nptII cassette.

Fig. 4 Expression analysis of APR. Total RNA was extracted from protonema tissue of wild type P. patens (WT) and 8 Δapr knockouts: 11-3-3 (1), 11-3-11 (2), 11-3-12 (3), 12-4-2 (4), 12-4-3 (5), 12-4-14 (6), 12-4-29 (7), and 12-7-7 (8). A Two µg RNA was reverse transcribed and an equivalent of 200 ng utilized for PCR with primers R14 and R11 to detect the APR transcript. The PCR fragments were resolved on 1% agarose/TBE gel. As control, PCR was performed with primers derived from the sequence of gene for ribosomal protein L21. B 5 µg RNA was separated on 1% agarose in presence of formaldehyde, blotted onto
Hybond-N nylon membrane and hybridized with $^{32}$P-labelled cDNA fragments of APR and L21.

Fig. 5 **Cadmium sensitivity of Δapr knockouts.** Wild type *P. patens* and three Δapr knockouts were incubated for 5 days on solid Knop medium (control) or Knop medium with addition of 15 µM CdCl$_2$ (Cd).

Fig. 6 **Incorporation of $^{35}$S from [35S]sulfate into thiols.** Wild type moss and the 11-3-12 Δapr knockout were incubated in Knop medium containing 0.06 mM sulfate supplemented with $^{35}$SO$_4^{2-}$ for two hours. Radioactive sulfur in cysteine and GSH was measured. Mean values ± SD from 3 measurements are presented. Values indicated by asterisks are different at $p \leq 0.005$.

Fig. 7 **Comparison of amino acid sequences of APS and PAPS reductases.** The sequences of APR2 from *A. thaliana* (AtAPR2), APR (PpAPR) and PAPS reductase (PpPAPR) from *P. patens*, and PAPS reductase from *E. coli* (EcPAPR) were aligned with the program CLUSTAL. Asterisks identify identical residues, arrows mark the additional Cys in APS reductases. The conserved APR and PAPS reductase signature is underlined.

Fig. 8 **Expression of PAPS reductase.** Total RNA was extracted from protonema tissue of wild type *P. patens* (WT) and 7 Δapr knockouts: 11-3-3 (1), 11-3-11 (2), 11-3-12 (3), 12-4-2 (4), 12-4-3 (5), 12-4-29 (6), and 12-7-7 (7). 5 µg RNA was separated on 1% agarose in presence of formaldehyde, blotted onto Hybond-N nylon membrane and hybridized with $^{32}$P-labelled cDNA fragments of PAPS reductase (PAPR) and L21.
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