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Three members of a novel small gene-family from *Arabidopsis thaliana* able to complement functionally an *Escherichia coli* mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and "APS reductase" activity

(reductive sulfate assimilation/λYES cDNA library/phylogeny/gene expression)

JOSÉ F. GUTIERREZ-MARCOS, MICHAEL A. ROBERTS*, EDWARD I. CAMPBELL, AND JOHN L. WRAY†

Plant Sciences Laboratory, Research Division of Environmental and Evolutionary Biology, Sir Harold Mitchell Building, School of Biological and Medical Sciences, University of St. Andrews, St. Andrews, Fife KY16 9TH, United Kingdom

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ABSTRACT **Three different cDNAs, Prh-19, Prh-26, and Prh-43 [3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase homolog], have been isolated by complementation of an *Escherichia coli cysH* mutant, defective in PAPS reductase activity, to prototrophy with an *Arabidopsis thaliana* cDNA library in the expression vector λYES. Sequence analysis of the cDNAs revealed continuous open reading frames encoding polypeptides of 465, 458, and 453 amino acids, with calculated molecular masses of 51.3, 50.5, and 50.4 kDa, respectively, that have strong homology with fungal, yeast, and bacterial PAPS reductases. However, unlike microbial PAPS reductases, each PRH protein has an N-terminal extension, characteristic of a plastid transit peptide, and a C-terminal extension that has amino acid and deduced three-dimensional homology to thioredoxin proteins. Adenosine 5′-phosphosulfate (APS) was shown to be a much more efficient substrate than PAPS when the activity of the PRH proteins was tested by their ability to convert ^{35}S-labeled substrate to acid-volatile ^{35}S-sulfite. We speculate that the thioredoxin-like domain is involved in catalytic function, and that the PRH proteins may function as novel "APS reductase" enzymes. Southern hybridization analysis showed the presence of a small multigene family in the *Arabidopsis* genome. RNA blot hybridization with gene-specific probes revealed for each gene the presence of a transcript of ≈1.85 kb in leaves, stems, and roots that increased on sulfate starvation. To our knowledge, this is the first report of the cloning and characterization of plant genes that encode proteins with APS reductase activity and supports the suggestion that APS can be utilized directly, without activation to PAPS, as an intermediary substrate in reductive sulfate assimilation.**

Sulfur in its reduced form plays an important role in plant metabolism being involved in the biosynthesis of a wide range of primary and secondary S-containing metabolites. However the pathway of reductive assimilation of inorganic sulfate to sulfide in higher plants is the subject of some controversy (reviewed in ref. 1). After sulfate uptake, and its activation to adenosine 5′-phosphosulfate (APS) (5′-adenylylsulfate) by the enzyme ATP sulfurylase, it has been unclear whether APS is further metabolized by the enzyme APS sulfotransferase to "bound-sulfite" and then reduced to "bound sulfide" by a thiosulfonate reductase or whether the "free-sulfite" pathway, found in most enterobacteria and certain fungi and yeast, operates. In this latter pathway APS is converted to 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (3′-phosphoadenylylsulfate) by APS kinase and PAPS is reduced to free sulfite by PAPS reductase. Sulfite is then reduced to sulfide by sulfite reductase. Discovery of a thioredoxin-dependent PAPS reductase activity in spinach (2) and the more recent suggestion that APS sulfotransferase activity is a side-reaction of APS kinase (3) provide strong support for the notion that the "free-intermediate" pathway operates not only in enterobacteria and yeasts but also in higher plants. With the exception of PAPS reductase, cDNA species encoding all steps of the free-intermediate reductive sulfate assimilation pathway have been cloned from higher plants.

Here we describe the molecular cloning and characterization of three novel *Arabidopsis thaliana* cDNAs obtained by functional complementation of the *Escherichia coli* mutant *cysH*, defective in PAPS reductase, to prototrophy. The novel PRH proteins encoded by these cDNAs carry a thioredoxin-like domain (and thus may represent new members of the thioredoxin superfamily) and also prefer APS over PAPS as substrate. We speculate that the thioredoxin-like domain may be involved in catalytic function, and that the PRH proteins may function as novel APS reductase enzymes rather than as PAPS reductases. Our data support the idea (reviewed in ref. 1) that APS itself can be utilized directly, without activation to PAPS, as an intermediary substrate in reductive sulfate assimilation.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Bacterial Strains. *A. thaliana* ecotype *Columbia* was used for all experiments. Plants were grown in compost in pots or in liquid modified MS medium (4) in Erlenmeyer flasks in a shaker with agitation at 120 rpm under continuous light (10 μmol·m^{-1}·sec^{-1}) at 23°C. The modified MS medium contained nitrate (18.8 mM) as sole nitrogen source and sulfate salts were replaced in sulfate-free medium by the equivalent chloride salt. *E. coli* strain JM96 (*thr-1*, *leuB6*, *fhu*A2, *lacY1*, *su*pE44, *gal-6*, λ$^-$, *trp-1*, *hisG1*, *cysH56*, *galP63*, *gltB31*, *rpsl9*, *malT1*(λ^R), *xyl-7*, *mtl-2*, *argH1*, *thi-1*), defective in PAPS reductase activity and unable to grow on sulfate as sole sulfur source (5), was obtained from B. Bachmann (*E. coli* Genetics Stock Center, Yale University). An aliquot of the *A. thaliana* (Columbia ecotype) cDNA library in the λYES yeast–*E. coli* expression vector (6) and *E. coli* strain BNN132 containing λKC were gifts from J. T.

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Abbreviations: APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; IPTG, isopropyl β-D-thiogalactosidase; PDI, protein disulfide isomerase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U53864 (Prh-19), U53865 (Prh-26), and U53866 (Prh-43)].

*Present address: Cereals Research Department, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, United Kingdom.

†To whom reprint requests should be addressed.

Mulligan (Stanford University School of Medicine). *E. coli* strain M15[pREP4] (*Nal^{s}Str^s, rif^s, lac$^-$, ara$^-$, gal$^-$, mtl$^-$, F$^-$, recA$^+$, uvr$^+$*) (Qiagen, Chatsworth, CA) was used as host for expression of recombinant 6×His PRH protein.

Functional Complementation. JM96/λKC lysogens were transfected with the λYES library, and JM96/λKC/pYES-complemented colonies were selected on M9 minimal medium supplemented with 0.2% mannitol, 50 μg/ml ampicillin, 240 μg/ml isopropyl β-D-thiogalactosidase (IPTG) and appropriate amino acids, but without cysteine (sulfate as sole sulfur source). Selected clones were subcloned into pBlueScript II KS(+) (Stratagene), and the DNA sequence was determined on both strands by the dideoxynucleotide chain-termination method with a series of synthetic primers using a 7-deaza Sequenase kit, Version 2.0 (Amersham).

Enzyme Assays. *E. coli* strains were grown at 37°C to an OD_{600} of 0.7 in Luria–Bertani broth supplemented where appropriate with 100 μg/ml ampicillin, induced with 1 mM IPTG for 3 h, pelleted, washed in 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, recentrifuged, and frozen at −20°C. Crude protein extracts were obtained from resuspended cells disrupted with a probe sonicator and then centrifuged. PAPS reductase and APS reductase activity was assayed at 25°C by measuring acid-volatile ^{35}S-sulfite formed from either ^{35}S-PAPS or ^{35}S-APS, as described (7). The assay mixture contained 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, 25 mM NaF, 5 mM DTT, 20 mM Na_2SO_3, 4.5 μg recombinant *E. coli* thioredoxin (Promega), 5.6 μM [^{35}S]PAPS or [^{35}S]APS (specific radioactivity, 5.38×10^{10} Bq/mmol; New England Nuclear) and crude extract in a final volume of 100 μl. Activity is expressed as pmol acid-volatile [^{35}S]sulfite produced/min^{-1}·mg protein^{-1}. [^{35}S]APS was prepared from [^{35}S]PAPS by alkaline phosphatase treatment (8). Protein was estimated according to Bradford (9) using bovine serum albumin as a standard.

Expression of Protein PRH-19 in *E. coli*. pQE-PRH-19, obtained by cloning Prh-19 cDNA into pQE30 to generate a fusion protein with a 6×His N-terminus, was transformed into *E. coli* strain M15[pREP4]. After growth at 37°C to an OD of 1.6 in Luria–Bertani broth containing kanamycin (50 mg/ml) and ampicillin (100 mg/ml) induction was carried out for 3 h with 1 mM IPTG and, after cell lysis, the recombinant protein was purified under nondenaturing conditions by nickel-affinity chromatography according to the manufacturer's instructions (CLONTECH).

Genomic Southern and RNA Blot Analysis. Genomic DNA (10 μg), isolated from green leaves of *A. thaliana* (10), was digested with the appropriate enzymes, fractionated by agarose gel electrophoresis, and transferred to Hybond-N (Amersham). Filters were hybridized at 65°C with either [α-^{32}P]dCTP-labeled full-length cDNAs or gene-specific probes derived from the 5′ end of each cDNA. The hybridization solution for Southern blots contained 5× SSPE (120 mM NaCl/5 mM sodium citrate/20 mM sodium phosphate, pH 6.8), 0.1% SDS, 5× Denhardt's solution, and denatured salmon sperm DNA at 200 μg·ml^{-1}. Filters were washed to a final stringency of 1× SSPE/0.1% SDS at 65°C (high stringency) and exposed to X-Omat AR film (Kodak). RNA was extracted by the guanidine hydrochloride method (11), subjected to denaturing formaldehyde/agarose gel electrophoresis, and transferred to Hybond-N. Filters were probed with the same gene-specific probes and hybridization was carried out at 42°C as above but with 50% formamide in the hybridization solution.

Sequence Analysis. Sequence analyses were carried out using the Wisconsin Genetics Computer Group package (12). Alignment and phylogenetic distances were calculated using CLUSTAL V (13) and PHYLIP 3.5 (14).

RESULTS

Functional Complementation of an *E. coli cysH* Mutant Defective in PAPS Reductase. The *E. coli cysH* mutant JM96 is defective in PAPS reductase activity (refs. 5 and 15; Table 1). When λKC/JM96 lysogens were transfected with the λYES *A. thaliana* cDNA library at a frequency of 10^8 phage per 10^9 cells, 57 colonies were obtained that grew on M9 minimal medium with sulfate as sole sulfur source and supplemented with ampicillin and IPTG. Restriction digestion and partial sequencing sorted the clones into three sequence-specific groups. One cDNA clone member from each group [Prh-19, Prh-26, and Prh-43 (PAPS reductase homolog)] was selected for further study. Plasmids pPRH19, pPRH26, and pPRH43, isolated from colonies PRH19, PRH26, and PRH43, respectively, were each able to restore prototrophy to the *cysH* mutant JM96 upon retransformation (Table 1).

Enzyme Assay. To examine restoration of PAPS reductase activity to the *cysH* mutant JM96, we assayed for PAPS reductase activity in extracts of the *cysH* mutant JM96 and JM96 retransformed with each plasmid. As anticipated (5, 15), the *E. coli cysH* mutant lacked PAPS reductase activity (Table 1). Activity was also absent from extracts of JM96 retransformed with the control plasmid pYES. In contrast, extracts from JM96 that had been retransformed with either pPRH19, pPRH26, or pPRH43 possessed activity but this was very low compared with the activity detected in extracts of the wild-type strain (Table 1). While the assayed PAPS reductase activity was thioredoxin-dependent in the wild-type strain, this appeared not to be so in the case of the very low activity detected in each of the complemented mutant strains (Table 1). This is different from the situation reported for microbial enzymes (7,

Table 1. PAPS reductase activity, APS reductase activity, and growth phenotype of *cysH* mutant, functionally complemented and wild-type *E. coli* strains, and activity of the recombinant PRH19 protein

E. coli strain	Ability to grow on sulfate as sole sulfur source	PAPS reductase activity, pmol·min^{-1}·mg·protein^{-1}		APS reductase activity, pmol·min^{-1}·mg·protein^{-1}	
		+ trx	− trx	+ trx	− trx
TB1 (wild type)	ND	482.7	26.8	0.9	1.0
JM96 (*cysH* mutant)	−	0.0	0.0	0.5	0.4
JM96/pYES	−	0.2	0.0	0.5	0.5
JM96/pPRH19	+	1.3	1.9	601.7	588.0
JM96/pPRH26	+	0.8	0.9	140.0	211.7
JM96/pPRH43	+	1.1	1.4	732.5	766.5
6×His PRH19		36.7	50.3	12,363.0	13,335.0

Cell-free extracts from the *cysH* mutant JM96; JM96 retransformed with either pYES, pPRH19, PRH26, or pPRH43; wild-type strain TB1, and recombinant 6×His PRH19 protein (1 μg) were assayed for PAPS reductase activity and APS reductase activity in the presence or absence of recombinant *E. coli* thioredoxin (trx) (4.5 μg). Ability (+) or inability (−) of the strain to grow on M9 minimal medium with sulfate as sole sulfur source was also scored. ND, not determined.

15, 16), where activity is reported to be thioredoxin-dependent. Independence of the measured PAPS reductase activity from exogenous thioredoxin is not an artefact due to the use of crude extracts; however, since it is also seen with the purified recombinant 6×His PRH19 protein (Table 1). The very low PAPS reductase activity, and the thioredoxin-independence, that we measure in extracts from the complemented strains is unlikely to be due to the poor affinity of the PRH protein for *E. coli* thioredoxin since raising the thioredoxin concentration in the assay 10-fold to ≈40 μM did not affect the measured PAPS reductase activity (data not shown). When [^{35}S]APS was used as substrate, activity, also measured by formation of acid-volatile [^{35}S]sulfite, was absent from the wild-type strain TB1, the *cysH* mutant JM96 and JM96 retransformed with the control plasmid pYES (Table 1). In contrast, extracts from JM96 retransformed with either plasmid pPRH19, pPRH26, or pPRH43 had substantial activity that was not dependent on exogenous thioredoxin, as did the 6×His PRH19 protein (Table 1). Activity with PAPS as substrate was ≈0.5% of the activity measured with APS as substrate. The very low level of activity seen toward PAPS might be a property of the PRH proteins. Alternatively, it might be due to APS contamination of the PAPS or, in the case of the cell-free extracts, to APS produced as a consequence of phosphatase action on PAPS during the assay despite the presence of the phosphatase inhibitor, fluoride.

Sequence Analysis. The three cDNA sequences, Prh-19, Prh-26, and Prh-43, encode deduced polypeptides PRH-19, PRH-26, and PRH-43, of 465, 458, and 453 amino acid residues, respectively, and molecular mass of 51,300, 50,500, and 50,453 Da. The deduced amino acid sequence of PRH-19 is 91.9% and 86.7% identical to those of PRH-26 and PRH-43, respectively. Amino acid sequence comparisons demonstrated not only that the central region of these PRH proteins (for example amino acid residues 89–353 of PRH-19) possessed 48–58% amino acid identity with fungal, yeast, and bacterial PAPS reductase proteins but also that each plant protein possessed both an N-terminal and a C-terminal extension with respect to the other sequences examined (Fig. 1*A*). Over the central homologous sequence each plant protein possesses four regions that are highly conserved between the PAPS reductase proteins. These conserved regions include a motif located toward the N-terminus (DTGxxxPETY) (residues 163–172 of the PRH-19 sequence), a motif located toward the C terminus (ECGLH) (residues 347–351 of the PRH-19 sequence) as well as two other conserved internal motifs (KVxxxxRAL and GYxxV/IG) (residues 231–240 and 315–320, respectively, of the PRH-19 sequence) indicated by the shaded boxed areas in Fig. 1*A*). Site-directed mutagenesis studies of the *E. coli* PAPS reductase demonstrated the functional significance of the conserved Cys residue in the motif ECGLH and the conserved Tyr residue in the motif GYxxxG (16).

The N-terminal extension seen in each of the three PRH proteins, with respect to the microbial sequences, has the characteristics of a chloroplast transit peptide. Such transit peptides are rich in positively charged and hydroxylated amino acids and have a very low percentage of acidic residues (27, 28). Over the first 70 amino acid residues the putative transit peptide possessed by PRH-19, PRH-26, and PRH-43 have 18, 24, and 23 hydroxylated amino acids (Ser or Thr) and 12, 12, and 15 positively charged residues (Arg, His, or Lys), respectively. Additionally each putative transit peptide contains a motif (VHVA, VSAA, and VHLA, respectively) similar to the cleavage-site consensus sequence V/A-X-A/C ↓ A deduced from a comparison of chloroplast transit peptides (29). Most of this extension is not required for the expression of activity in *E. coli* since a truncated Prh-43-type cDNA (Prh-20) lacking the first 48 amino acids is able to complement the *E. coli* mutant JM96 to prototrophy (not shown).

A computer search using the PRH-19 amino acid sequence revealed substantial homology in the ≈139 amino acid C-terminal extension (residues 348–493) of PRH-19, absent from the microbial PAPS reductases, with specific domains of protein disulfide isomerase (PDI) (Fig. 1*B*), a protein that catalyses the formation, breakage, and rearrangement of protein disulfide bonds (reviewed in ref. 30). PDI proteins consist of six domains of which two are homologous to thioredoxin and play a major functional role in the catalytic properties of these enzymes (31). The homology between PRH-19 and PDI lies within these thioredoxin-like domains (Fig. 1*B*) and includes the motif CXXC (at residues 385–388 of PRH-19) that carries the redox-active half-cysteine residues present at the active site of PDI, thioredoxin, and other related redox proteins (30). Substantial homology was also detected between the compared PRH-19 sequence and the endoplasmic reticulum proteins ERp72 (21) and ERp60 (N. Hirano, F. Shibasaki, R. Sakai, T. Tanaka, J. Nishida, Y. Yazaki, T. Takenawa, and H. Hirai, personal communication), and P5 (22) (Fig. 1*B*), that also carry thioredoxin-like domains and possess PDI activity (30). Further analysis of the ≈139-amino acid C-terminal extension of PRH-19 confirmed that it possessed the functional features characteristic of thioredoxins (32–34) (Fig. 1*B*). As well as the highly conserved sequence (CXXC), at positions 385–388, that contains the two redox-active half-cysteine residues, several other amino acid residues are conserved between the PRH-19 protein and plant thioredoxins *m*, *f*, and *h* (23–25) compared. These structural features of PRH-19 are shared with PRH-26 and PRH-43 (data not shown).

Phylogenetic Analysis. The phylogenetic relationship between all available bacterial, fungal, and yeast PAPS reductase amino acid sequences and the central homologous region (residues 89–353 with respect to PRH-19) of the PRH sequences reported here are shown in Fig. 2. The substantial similarity of PAPS reductases from the bacteria *E. coli*, *Salmonella typhimurium*, *Thiocapsa roseopersicina* and the cyanobacterium *Anacystis nidulans* is evident. The addition of the PRH proteins to this family of PAPS reductases reveals that the yeast (*Saccharomyces cerevisiae*) and fungal (*Aspergillus nidulans*) sequences can be considered to be phylogenetically intermediate between the bacterial and plant amino acid sequences.

Hybridization Analysis. Southern analysis of genomic DNA, performed with each full-length Prh cDNA sequence (Fig. 3*A*), indicated in each case the presence of several copies of a Prh gene sequence in the *A. thaliana* genome, suggesting the presence of a small gene family. However, when analysis was performed with gene-specific probes derived from the 5′ end of each of the Prh cDNA sequences, we were able to demonstrate the presence of a single copy gene in the case of Prh-19 and Prh-43, and a possible small multicopy family in the case of Prh-26 (Fig. 3*B*). RNA blot hybridization carried out with the same gene-specific probes (Fig. 4) demonstrated in each case the presence of a ≈1.8-kb transcript in leaf, root, and stem of mature *A. thaliana* plants. The steady-state level of each PRH transcript in whole plants increased in abundance when sulfate limitation was imposed for 48 h (Fig. 4).

DISCUSSION

Functional complementation of an *E. coli cysH* mutant, defective in PAPS reductase, to prototrophy with an *A. thaliana* cDNA expression library in λYES (6) has enabled us to isolate three *Arabidopsis* cDNA classes. Comparison of the deduced amino acid sequence of one member of each cDNA class indicates that they encode very similar proteins that have conserved regions showing striking homology with fungal, yeast, and bacterial PAPS reductases (Fig. 1*A*). Compared with the microbial sequences, each of the deduced plant protein sequences has an N-terminal extension that has the

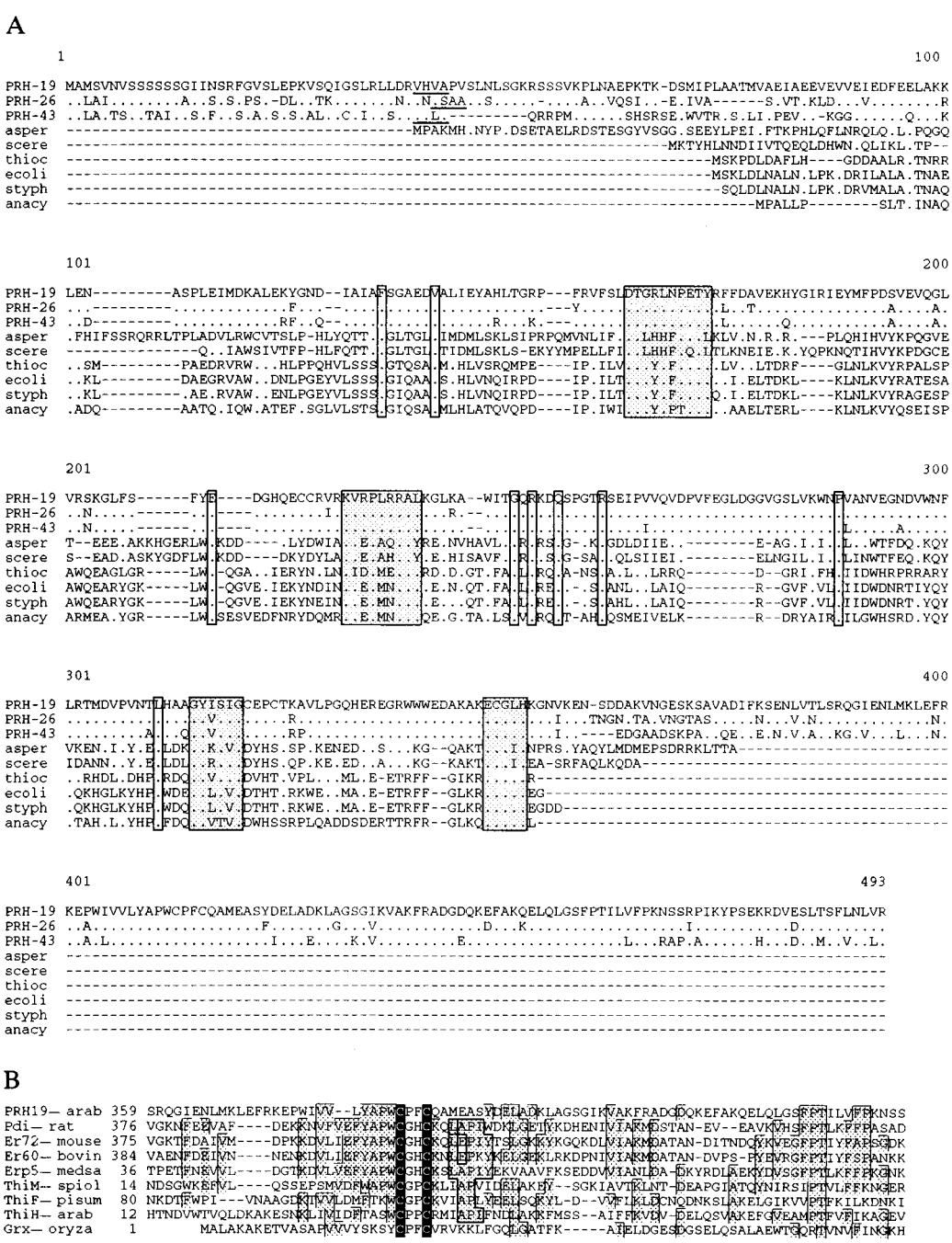

FIG. 1. Comparisons of *A. thaliana* PRH-19, PRH-26, and PRH-43 deduced amino acid sequences. (*A*) Sequence comparison with microbial PAPS reductases. Amino acid residues identical to PRH-19 are indicated by a dot. Breaks in the alignment are indicated by a dash. Putative transit peptide cleavage sites in the PRH sequences are underlined. Conserved amino acid regions are indicated by shaded boxes, and other conserved single amino acid residues are indicated by open boxes. Sequences were aligned with the PILEUP program. Compared microbial PAPS reductase sequences are: asper, *Aspergillus nidulans* SA gene (ref. 17; GenBank accession no. X82555); scere, *Saccharomyces cerevisiae* strain 10197 MET16 gene (ref. 16; GenBank accession no. J05591); thioc, *Thiocapsa reseopersicina cysH* gene (T. Haverkamp, G. Gisselman, and J. D. Schwenn, personal communication; GenBank accession no. Z23169); ecoli, *E. coli* K12 *cysH* gene (ref. 15; GenBank accession no. Y07525); styph, *Salmonella typhimurium cysH* gene (ref. 18; GenBank accession no. J05025); anacy, *Anacystis nidulans* R2 *par* gene (ref. 19; GenBank accession no. M844476). (*B*) Comparison of the C-terminal sequence of the deduced PRH-19 amino acid sequence with other deduced homologous amino acid sequences. Numbers refer to the amino acid residues in the deduced PRH-19 protein and in the proteins being compared. Conserved amino acid residues are indicated by shaded boxes; highly conserved redox-active cysteine residues are indicated by black boxes. Compared sequences are as follows: PRH19-arab, *Arabidopsis* PRH19 gene; Pdi-rat, rat protein disulfide isomerase gene (ref. 20; GenBank accession no. X02918); ERp72-mouse, mouse endoplasmic reticulum protein gene ERp72 (ref. 21; GenBank accession no. J05186); ERp60-bovin, bovine endoplasmic reticulum protein gene ERp60 (Hirano *et al*., personal communication; GenBank accession no. D16235); ERp5-medsau, golden hamster endoplasmic reticulum protein gene ERp5 (ref. 22; GenBank accession no. X62678); ThiM-spiol, spinach thioredoxin *m* gene (ref. 23; GenBank accession no. P07591); ThiF-Pisum, pea thioredoxin *f* gene (ref. 24; GenBank accession no. U35830); ThiH-arab, *Arabidopsis* thioredoxin *h* gene (ref. 25; GenBank accession no. Z35473); Grx-oryza, rice glutaredoxin gene (ref. 26; GenBank accession no. X77150).

properties of a chloroplast transit peptide. However, the encoded proteins are not like "conventional" microbial PAPS reductase proteins since they also possess a ca 139-amino acid C-terminal domain that has striking homology with thioredoxin with respect to the conserved residues required for catalytic function and the maintenance of three-dimensional structure (32–34). While the residues XX in the motif CXXC of the PRH proteins are more characteristic of the redox center

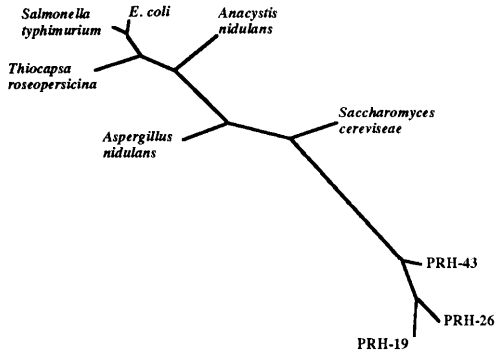

FIG. 2. Phylogenetic relationship between PRH proteins and PAPS reductase proteins. Microbial PAPS reductase sequences and the homologous central sequence of PRH-19, PRH-26, and PRH-43 (residues 89–353 with respect to PRH-19) were aligned using PILEUP, and the phylogenetic distances were identified with PHYLIP and plotted as a phylogenetic tree.

of another redox protein glutaredoxin (CPFC) (reviewed in ref. 34) (Fig. 1*B*), we note that the flanking conserved amino acid residues are characteristic of thioredoxin (Fig. 1*B*). Thus the proteins encoded by the Prh cDNAs reported here appear to encode new members of the thioredoxin superfamily.‡

Despite several structural similarities with microbial PAPS reductases, the PRH proteins prefer APS over PAPS as an *in vitro* substrate for the formation of acid-volatile [^{35}S]sulfite, suggesting that APS is the *in vivo* substrate for these enzymes. The ability to use APS as substrate *in vivo* is supported by our observation that each of the Prh cDNAs are able functionally to complement the *E. coli* APS kinase mutant JM81A to prototrophy (data not shown). Thus our ability to recover these Prh cDNAs by functional complementation may have been due either to the low PAPS reductase activity of the encoded proteins or to this ability of the PRH proteins to convert APS to sulfite, thus bypassing the defective PAPS reductase step in the JM96 mutant.

The activity of the PRH proteins resembles that of APS sulfotransferase activity in that it is specific for APS over PAPS and is also thiol-dependent (data not shown). Further, the presence of a chloroplast transit peptide in each of the deduced PRH amino acid sequences suggests that, like APS sulfotransferase activity (37), the PRH proteins are chloroplast-localized. This raises the question of whether published data on APS sulfotransferase activity is a function of the PRH proteins. The value of $M_r \approx 43{,}000$ deduced for the mature PRH proteins is consistent with the subunit size reported for the APS sulfotransferase enzyme from spinach§ *Porphyra yezoensis* (38), and, by extrapolation, that from *Chlorella* (39). Where a molecular size discrepancy is seen this may be related to the observation that the enzyme APS kinase (subunit size of $M_r \approx$ 26,000) exhibits APS sulfotransferase activity as a side reaction (3). There is no overall similarity in deduced amino acid sequence between APS kinase and the PRH proteins but the motif GYxxxG, conserved between PRH proteins and PAPS reductases, has its counterpart in APS kinase proteins (40). A similar motif is seen in many ATP-binding proteins (41) and may perhaps be involved in APS binding in the case of the PRH proteins.

If the PRH proteins are functionally equivalent to (at least some of the) previously described APS sulfotransferase activity then the presence of a thioredoxin domain in the PRH proteins shows APS sulfotransferase in an entirely new light. Rather than acting as APS sulfotransferases, the PRH proteins may act as APS reductases, perhaps in a manner analogous to that described for the *E. coli* thioredoxin-dependent PAPS reductase (16) but involving the internal thioredoxin domain rather than exogenous thioredoxin. Berendt *et al.* (16) suggested that exogenous reduced thioredoxin maintains the catalytically functional Cys-239 residue of the PAPS reductase homodimer as a redox-active dithiol. Binding of PAPS then allows its reduction to free sulfite and PAP. We note that this cysteine residue is conserved between PAPS reductases and the PRH proteins, as is the tyrosine residue (Tyr-316 in the PRH-19 sequence) that site-directed mutagenesis suggests is also catalytically functional (16). Our data provide further support for the suggestion that APS can be utilized directly, without activation to PAPS, as an intermediary substrate in reductive sulfate assimilation (reviewed in ref. 1). We cannot exclude the possibility that a reductive sulfate assimilation pathway with PAPS as a direct intermediate also exists in higher plants, especially since the presence of a thioredoxin-dependent PAPS reductase activity has been reported in spinach extracts (2), but we note that none of the cDNAs isolated in the study reported here encode a "conventional" PAPS reductase.

Studies at the enzyme and/or transcript level show up-regulation of a number of steps of the sulfate assimilation pathway on sulfate starvation, including APS sulfotransferase activity (42). The steady-state transcript level of each of the PRH cDNAs described here also increases on sulfate starvation. This does not appear to be a response to stress imposed by transfer from sulfate-replete to sulfate-free medium since control plants were also transferred (to sulfate-replete medium) and the transcript level of chalcone synthase, a gene

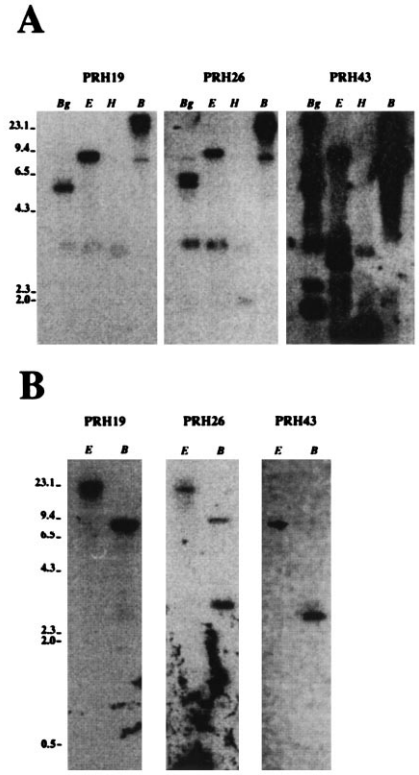

FIG. 3. Southern analysis of *A. thaliana* genomic DNA. (*A*) Hybridization with either Prh-19, Prh-26, or Prh-43 full-length cDNAs respectively. (*B*) Hybridization with gene-specific probes derived from the 5′ end of Prh-19, Prh-26, and Prh-43 cDNAs, respectively. Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI.

‡A report of the cloning of similar cDNA species from *Arabidopsis* was presented recently (Leustek, T., Abstracts of the Third Workshop on Sulfur Metabolism in Higher Plants, April 9–13, 1996, University of Newcastle upon Tyne, U.K.).

§Ara, T. & Sekiya, J., Abstracts of the Third Workshop on Sulfur Metabolism in Higher Plants, April 9–13, 1996, University of Newcastle upon Tyne, U.K.

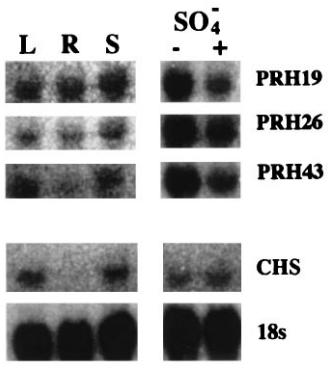

FIG. 4. Northern blot analysis of expression of the PRH genes in *A. thaliana.* (*Upper Left*) Total leaf (L), root (R), and stem (S) RNA was extracted from 21-day-old compost-grown plants grown under normal sulfur nutrition. (*Upper Right*) Total RNA was extracted from *A. thaliana* plants growing in liquid shake culture in MS medium with 1.6 mM sulfate (+ SO_4^-) or deprived of sulfate for 48h (− SO_4^-). RNA was hybridized with gene-specific probes derived from the 5′ end of Prh-19, Prh-23, and Prh-43 cDNAs, respectively. (*Lower*) RNA was hybridized with the chalcone synthase probe (35) and with an 18S rRNA probe (36).

whose expression is known to be affected by stress (35), is unaltered. This suggests to us that the PRH proteins described here are involved *in vivo* in sulfate assimilation and that they are part of the "stimulon" that is activated on sulfate starvation in an attempt to scavenge for available sulfur.

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