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Cytosolic Hydroxymethyl-dihydropterin Pyrophosphokinase/Dihydropteroate Synthase from *Arabidopsis thaliana*

A SPECIFIC ROLE IN EARLY DEVELOPMENT AND STRESS RESPONSE^{*§}

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In plants, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase (mitHPPK/DHPS) is a bifunctional mitochondrial enzyme, which catalyzes the first two consecutive steps of tetrahydrofolate biosynthesis. Mining the *Arabidopsis* genome data base has revealed a second gene encoding a protein that lacks a potential transit peptide, suggesting a cytosolic localization of the isoenzyme (cytHPPK/DHPS). When the N-terminal part of the cytHPPK/DHPS was fused to green fluorescent protein, the fusion protein appeared only in the cytosol, confirming the above prediction. Functionality of cytHPPK/DHPS was addressed by two parallel approaches: first, the cytHPPK/DHPS was able to rescue yeast mutants lacking corresponding activities; second, recombinant cytHPPK/DHPS expressed and purified from *Escherichia coli* displayed both HPPK and DHPS activities *in vitro*. In contrast to mitHPPK/DHPS, which was ubiquitously expressed, the cytHPPK/DHPS gene was exclusively expressed in reproductive tissue, more precisely in developing seeds as revealed by histochemical analysis of a transgenic cytHPPK/DHPS promoter-GUS line. In addition, it was observed that expression of cytHPPK/DHPS mRNA was induced by salt stress, suggesting a potential role of the enzyme in stress response. This was supported by the phenotype of a T-DNA insertion mutant in the cytHPPK/DHPS gene, resulting in lower germination rates as compared with the wild-type upon application of oxidative and osmotic stress.

Folates play a crucial role as cofactors in a number of one-carbon transfer reactions. These reactions are essential for DNA synthesis and the methylation cycle occurring in all organisms (for review see Refs. 1–3).

Plants and micro-organisms are able to synthesize tetrahydrofolate (H_4FGLu_n)² *de novo* from dihydropterin, *p*-aminobenzoic acid (*p*-ABA), and glutamate, through the consecutive action of five enzymatic activities (Fig. 1). During this process, dihydropterin is first activated by an ATP-dependent enzyme, hydroxymethyl-dihydropterin pyrophosphokinase (HPPK) and then combined to *p*-ABA by dihydropteroate synthase (DHPS) to produce dihydropteroate. The latter compound condenses with a molecule of glutamate, to yield dihydrofolate (H_2FGLu). This step is catalyzed by dihydrofolate synthase (DHFS). H_2FGLu is thereafter reduced to H_4FGLu by dihydrofolate reductase (DHFR), and this is followed by the addition of a polyglutamate chain by folylpolyglutamate synthetase (2, 4). Animals cannot *de novo* synthesize folates because of the lack of the first three essential enzymatic activities, HPPK, DHPS, and DHFS. Therefore, they have to rely on dietary folate intake, which makes plants one of the main sources of folates in human diet.

A unique feature of plant folate biosynthesis is its fairly complex compartmentalization. The pathway itself has been shown to reside in the mitochondria (5), whereas dihydropterin and *p*-ABA, the initial substrates for the pathway, are respectively synthesized in the cytosol (6, 7) and the chloroplasts (8, 9). Both dihydropterin and *p*-ABA must be imported in the mitochondria to be combined (Fig. 1) through the first two steps of H_4FGLu_n biosynthesis. There is a great extent of variability among the enzymes supporting these initial reactions. In bacteria, HPPK and DHPS are separate proteins, whereas they are always part of a multifunctional enzyme in eukaryotes. In pea plants, these two activities are supported by a bifunctional enzyme only detected in mitochondria (5, 10), and genomic experiments strongly suggested a single copy gene (10). A single copy gene coding for the mitochondrial DHFS was also found in *Arabidopsis* (11), indicating that mitochondria comprise the most probable unique site for H_2FGLu synthesis in plants. The mito-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ866732.

§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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² The abbreviations used are: H_4FGLu_n , tetrahydrofolate; mitHPPK/DHPS, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase; cytHPPK/DHPS, cytosolic HPPK/DHPS; *p*-ABA, *p*-aminobenzoic acid; *p*-ABAG, *p*-ABA-glutamate; H_2FGLu , dihydrofolate; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; DHNA, dihydropterin aldolase; RACE, rapid amplification of cDNA ends; GFP, green fluorescent protein; 5-CHO- H_4FGLu , 5-formyltetrahydrofolate; GUS, β -glucuronidase; RT, reverse transcription; T-DNA, transfer DNA.

TABLE 1
Sequences of the primers used in this study

| Primer name | Sequence |
|-------------|-----------------------------------------------------|
| stoser 18 | 5'-TGGGAAGCAATGTTGGAAACAGA-3' |
| stoser 19 | 5'-CTGGAATCCCAGAGAGTTCTTGCT-3' |
| stoser 20 | 5'-ATTTTCAGAGAGGCTTTGCGATTG-3' |
| stoser 21 | 5'-GGATCTCCTCTCATGTGCATGGC-3' |
| stoser 36 | 5'-CCATTTTCTTCACAGCCCATCTCTT-3' |
| stoser 37 | 5'-CGGTATGGCAGAGATGAAGGAAAG-3' |
| stoser 46 | 5'-AAAAAGCAGGCTACCATGCATAGTCACTGGAGACCACG GCT-3' |
| stoser 47 | 5'-AGAAAGCTGGGTTTCAAATGGAGAGCTTGACTCTGT-3' |
| stoser 54 | 5'-AAAAAGCAGGCTCTAAAAATGGATTTCACATCTTTGGAA-3' |
| stoser 55 | 5'-AGAAAGCTGGGTACTAATCAACATTTTGAACCTTTTCG-3' |
| stoser 77 | 5'-AAAAAGCAGGCTGCAAAGGGACCTGGGGAATG-3' |
| stoser 78 | 5'-AGAAAGCTGGGTACATTGCTTCCCAAAGCTAT-3' |
| stoser 117 | 5'-TCAGTCTGAGGTGTGACTGTTGAGACTCC-3' |
| stoser 118 | 5'-TTGACTCTCGGGCTGTATGAACC-3' |
| UBI5-F | 5'-ACCAGCAGGATTGATTTTC-3' |
| UBI5-R | 5'-ACTCCTTCCCAACGCTCA-3' |
| GFP-SEQ | 5'-GCCGGTGGTCAGATGAACCTT-3' |

RACE and Molecular Cloning—For determination of the cytHPPK/DHPS cDNA ends, 5'- and 3'-RACE were performed using a SMARTTM PCR cDNA Synthesis Kit (BD Biosciences Clontech). The following gene-specific nested primer sets (Table 1) were used in combination with the universal primer mix provided in the kit: 1) 5'-RACE: stoser 19 and stoser 36; 3'-RACE: stoser 18 and stoser 37. Poly(A) RNA isolated from leaves of 3-week-old *Arabidopsis* plants was used as a template for cDNA synthesis. Total RNA was isolated using TRIzol reagent (Invitrogen) followed by mRNA extraction with PolyATtract[®] mRNA Isolation System (Promega, Madison, WI). Advantage[®] 2 cDNA Polymerase Mix was used for PCR. The longest PCR fragments representing the cDNA ends were cloned with help of the pGEM[®]-T Easy Vector System (Promega) and sequenced.

For cloning of the full-length cDNA Gateway[®] Technology (Invitrogen) was used. First, the cDNA was amplified with stoser 54 and stoser 55 primers, which contained 12-bp attB recombination sequence extensions, using the same template as in RACE. Then, the PCR product was re-amplified with attB adaptor primers (Invitrogen) and cloned by the BP recombination reaction into pDONR221 vector (Invitrogen). The resulting plasmid was used as the entry clone. Similarly, the full-length cDNA of AtFOLB1 was amplified with stoser 46 and stoser 47 primers. The destination vectors were: 1) pYES-DEST52 (Invitrogen) and pADH1 for expression in yeast and 2) pDEST17 for expression in *E. coli*.

Likewise, a 672-bp-long N-terminal fragment of cytHPPK/DHPS was amplified with stoser 54 and stoser 75 primers and cloned into pDONR221. Further it was recombined by the LR recombination reaction with the pK7FWG2 binary vector (16) to produce a cytHPPK/DHPS-GFP fusion construct.

Stoser 77 and stoser 78 primers were used for amplification of the cytHPPK/DHPS gene promoter from *Arabidopsis* Col-0 genomic DNA (isolated with NucleoSpin[®] Plant Kit, Macherey-Nagel, Düren, Germany). A forward primer (stoser 77) was located 1.2 kb upstream of the 5' untranslated region, and a reverse primer (stoser 78) was located in the second exon of gene to produce an in-frame translational fusion with the reporter. The amplified fragment was cloned in pDONR221

and sequenced. The binary vector pKGWFS7 (16) bearing a chimeric GFP-GUS reporter was used as a destination vector. The LR recombination reaction resulted in a plant transformation construct, Pr-GFP-GUS.

Sequence Analysis—For the sequence analysis GCG (Genetic Computer Group Inc., Madison, WI) software was used. Pairwise amino acid sequence similarities were calculated using the GAP program. Multiple sequence alignments were carried out with a Multiple Sequence Alignment program at the web BCM Search Launcher server. For the homology searches a BLAST program was used, whereas cellular localization predictions were made with the PSORT, PREDOTAR, and TargetP programs at the PSORT, PREDOTAR, and TargetP web servers, respectively.

Phylogenetic Analysis—A multiple sequence alignment was created with ClustalW (17). Phylogenetic trees were constructed using the Protdist and Neighbor programs from the PHYLIP package (18) and the Dayhoff PAM250 substitution matrix (19). Bootstrap support was estimated using 100 replicates.

Transient Expression Assay in Arabidopsis Protoplasts—*Arabidopsis* mesophyll protoplasts were isolated from rosette leaves of 3-week-old plants grown in soil. Protoplast isolation and transient expression assay were essentially as described previously.³

Microscopic Analysis of GFP and GUS Reporters—For the protoplast GFP fluorescence study, transfected protoplasts were transferred to W5 (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES (pH 5.7)) medium 16 h after transformation and microscopically examined with an epifluorescence microscope. Prior to microscopic analysis, the protoplasts were stained for 15 min with 50 nM MitoTracker Orange fluorescence dye (Invitrogen). An Olympus BX-61 epifluorescence microscope (Olympus Optical, Hamburg, Germany) was used, equipped with a UPlanApo 60×/0.9 objective. The following filter combinations have been used to detect the fluorescence: 1) excitation 460–480 nm, emission 495–540 nm, and dichromatic filter 485 nm for GFP fluorescence detection; 2) excitation 480–550 nm, emission >590 nm, and dichromatic filter 570 nm for MitoTracker and chloroplast autofluorescence detection. The images were processed using a deconvolution plug-in to CellF imaging software (Olympus).

For histochemical analysis of GUS expression, different plant tissues were submerged in 90% ice-cold acetone for 30 min, washed twice with 1 M sodium phosphate buffer (pH 7.2) for 15 min at room temperature under gentle shaking, and incubated for 16 h at 37 °C in the GUS-staining solution (0.1 M sodium phosphate buffer (pH 7.2), 0.5 mM Fe(CN)₂, 0.5 mM Fe(CN)₃, 0.1% Tween-20, 2 mM X-Gluc (Duchefa, Haarlem, The Netherlands). Subsequently, plant tissue samples were incubated overnight in 70% ethanol at 4 °C followed by clearing overnight in CLP solution (21) at room temperature. After CLP (chloral hydrate/phenol/lactic acid, 2:1:1 (v/v/v)) treatment the samples were incubated over-

³ Sheen, J. (2002) *A Transient Expression Assay Using Arabidopsis Mesophyll Protoplasts*, Department of Genetics, Harvard University, Cambridge, MA.

Cytosolic HPPK/DHPS from Arabidopsis

A

genomic DNA ATACATAAGTATTGTTACTTCCTTGGTTCTTAAAATATTAGAACCCATTTTTGTGTTT
 cDNA -----AGAACCCATTTTTGTGTTT
 protein

genomic DNA CTAATAATTGTTTCGTTGTTATCTAAGACTAAACTCATAGTTTGTATACTTTAAGGTCA
 cDNA CTAATAATTGTTTCGTTGTTATCTAAGACTAAACTCATAGTTTGTATACTTTAAGGTCA
 protein

genomic DNA GATCGAAGCAGACAATACTGATATTTTGGAGGAACCTATTCTAAAATGGSTTCGTATCTT
 cDNA GATCGAAGCAGACAATACTGATATTTTGGAGGAACCTATTCTAAAATGG-----
 protein M D

genomic DNA TTTTCATCCGTTTCATTTTCGTTGAAAAAACAGCGTTTACGTTTTGATACACTAATTTCT
 cDNA -----
 protein

genomic DNA GTTAAGTATTGAATCATATTTTCACCTTCGAAACATGTTTTGTATATGACTCAGATTTCAC
 cDNA -----ATTTCAC
 protein F T

genomic DNA ATCTTTGGAAACAACAACCTTTCGAAAGAGTCGTGATAGCTTTGGGAAGCAATGTTGGAAA
 cDNA ATCTTTGGAAACAACAACCTTTCGAAAGAGTCGTGATAGCTTTGGGAAGCAATGTTGGAAA
 protein S L E T T T F E E V V I A L G S N V G N

genomic DNA CAGAATGAATAATTTCAAGAAGCTTTACGATTGATGAA
 cDNA CAGAATGAATAATTTCAAGAAGCTTTACGATTGATGAA
 protein R M N N F K E A L R L M K

B

cytAth -----
 mitAth MAPLLSQTLIHTGRFLLRRLEPPPAVISAVAASRVCFHRYYSKSLSLVSPGLHCSSL
 mitPsat -----MSILKCLGVRGNQLCAA

cytAth -----MDFTSLETTFEEVVIALGNSVGNRMNMFKEALRLMKDYGISVTR
 mitAth FSPPALCNFAFSSSATSTTIEVOSTEHEVVIALGNSIGNRMNMFREALRLMKRGGICVTR
 mitPsat RNYLKVLFSSFHTAPNSSIEIQTODEEVVIALGNSVGNDRLHNFKEALKLRKSGIHTTR

cytAth HSCLYETE PVHVTDQPRFLNAAIRGVTKIKPHELLNLVKKIEKEMGREENGLRYGPRPLD
 mitAth HSCLYETAPVHVTDQPRFLNAAVRGVTKLGPHELLSVLKTIERDMGR-KDGIRYGRPLD
 mitPsat HASLYETAPAVVTDQPRFLNSAVRADTKLGPHELLAALKRIEKDMGR-TDGIRYGRPLD

cytAth LDILFYGKHKTISSDKLIIPHERIWERPFVLAFLVDLLGTEDIDNDKIIVYWHSLSMHSGG
 mitAth LDILFYGKMRISSDKLIIPHERIWERPFVLAFLVDLLGSAYDN--DTVAHWHSLAIHPGG
 mitPsat LDILFYGKFKVRSDFLITVPHERIWERPFVMAFLMDLLGTAIDS-DTVASWHSFSGHSGG

cytAth IFQAWERLGGESLIGKDGITQRVLPIDHLLWDFSKKTYVMGILNLT PDSFSDGGKFQSDV
 mitAth IFQAWERLGGESLIGODG-IQRVLPIDKLLWDFSNKTHVMGILNLT PDSFSDGGKFQSDV
 mitPsat LNALWERLGGESLIGEEG-MYRVMEVANGLLDMSRRITVMGILNLT PDSFSDGGNFQSDV

cytAth TAVSRVRSMISEGVDIIDIGAQSTRPMASRISSEELDRLLIPVVKVVRGMPEMKKCLISV
 mitAth SAVSRVRSMISEGADIIIDIGAQSTRPMASRISSEELDRLLIPVLEAVRGMPEMEKCLISV
 mitPsat SAVSQARLMISEGADIIIDIGAQSTRPMASRISAEELCRLIPVLEAVMSIPEVEGKCLISV

cytAth DTFNSEVALEAIRNGADILNDVSCGSLDENMHKVVADSDVPYMIHMRGDPCTMQNKENL
 mitAth DTFNSEVASEAISNGADILNDVSAGTLDENMHKVVAESGVPYVAMHMRGDPCTMQNKENL
 mitPsat DTFYSEVALEAVRKGAIINDVSAGKLDASMFKVMAEIDVPPYVAMHMRGDPSTMQDSENL

cytAth EYNEICKDVATELYERVREAELSGIPAWRIMIDPGIGFSKGI DHNLDIVMELPKIREEMA
 mitAth QYDDVCKDVASELYLRVRDAELSGIPAWRIMIDPGIGFSKSV DHNLDIIMDLPKIREEMA
 mitPsat KYDNVCKDISSELYSRVREAEISGIPAWRIIMDPGIGFSKKTEDNLAALTGIPDLREEIS

cytAth KKSITGLSHAPILIGPSRKRFLGDI CGRPEASERDAATVACVTAGILKGANIIRVHNVRDN
 mitAth KRSIAMSHAPILVGPSRKRFLGDI CGRPEATDRDAATVASVTAGILKGANIIRVHNVRDN
 mitPsat KRSLAISSHAPILIGPSRKRFLGDI CGSRPSAVDRDPATIASVTAGVILKGANIIRVHNVRDN

cytAth VDAARLCDAMMTKRFKNVD---
 mitAth ADAAKVCDAMLRRRRSKSG---
 mitPsat LDAVKLCDAILKQKSSPIKFKQ

night and mounted in lactic acid. Finally, they were examined using bright field microscopy (Eclipse 80i microscope, Nikon Instech Co., Ltd., Kanagawa, Japan, with CFI Plan Fluor 20×/0.5 or CFI Plan Fluor 40×/0.75 objectives equipped with a DS-5MC digital camera).

Expression and Purification of cytHPPK/DHPS from *E. coli*—Cytosolic HPPK/DHPS was expressed in *E. coli* as an N-terminal His₆ fusion protein. To increase solubility of the fusion protein, it was co-expressed with GroESL chaperones, delivered on the pT-groE plasmid (22).

For the large scale purification, a 1.5-liter bacterial culture was grown. For cell lysis, CellLyticTM BII bacterial cell lysis extraction reagent (Sigma-Aldrich) was used according to the manufacturer's instructions.

After removing cellular debris by centrifugation and addition of NaCl to a final concentration of 0.5 M, the lysate was incubated with 1.5 ml of nickel-nitrilotriacetic acid resin (Qiagen) for 3 h. Subsequently, the resin was washed four times with a solution containing 50 mM sodium phosphate buffer, pH 7.5, and 500 mM NaCl, and the protein was eluted in 6 ml of the elution buffer (50 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl, 250 mM imidazole) and concentrated using Centricon Centrifugal Filter devices (Millipore) to a final volume of 2 ml.

The protein was further purified on NAP-10 columns (Amersham Biosciences) to exchange the elution buffer for storage buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and 10% glycerol, pH 7.5).

In Vitro HPPK/DHPS Activity Determination—These activities were estimated at 30 °C. All the solutions were maintained under a stream of argon to minimize the oxidation of the various dihydropterin substrates.

The HPPK plus DHPS activity was estimated according to Mouillon *et al.* (26). The standard reaction medium (medium A) contained, in a total volume of 120 μl: 40 mM Tris (pH 8.0), 20 mM β-mercaptoethanol, 10 mM MgCl₂, 200 μM ATP, and various amounts of the purified protein. 2 μl of 2 mM [¹⁴C]p-ABA (1.85 GBq·mmol⁻¹, MP Biomedicals, Irvine, CA) were added to the assay medium, and then the reaction was started by the addition of 100 μM dihydropterin. After various times of incubation, the reaction was blocked by the addition of 3 μl of a 10 mM sulfanilamide solution. Then, 100 μl of the assay medium was injected in a reversed-phase high-performance liquid chromatography system (Merck 655A-11 Liquid Chromatograph, equipped with a Shandon, Zorbax ODS Z225 5-μm column) coupled with a Berthold (LB 506D) scintillation counter, as described earlier (10). The high-performance liquid chromatography conditions were: solvent A, 0.1 M sodium acetate, pH 6; solvent B, acetonitrile; B increased linearly of 0.8% every min; flow rate 1 ml·min⁻¹. Within these experimental conditions, excess of [¹⁴C]p-ABA was not retained in the column, whereas

[¹⁴C]dihydropteroate, the final product of the reaction, was eluted after 18 min of chromatography.

Analysis of Gene Expression by RT-PCR—Total RNA was isolated from different organs (with the exception of roots) of 5-week-old *A. thaliana* ecotype Columbia plants grown on soil under a 16-h light/8-h dark regime using TRIzol[®] reagent (Invitrogen). For root total RNA, roots of 3-week-old seedlings grown *in vitro* on ½MS plates were extracted. First strand cDNA was synthesized by a SuperScriptTM First-Strand Synthesis System (Invitrogen) and used as a template for semi-quantitative RT-PCR. To avoid genomic DNA contamination, total RNA was treated with DNase I (Invitrogen) according to the manufacturer's instructions, prior to first strand synthesis. Negative controls lacking RT were included. 19, 21, and 24 cycles of RT-PCR were carried out with JumpStart[®]REDTaq[®]DNA polymerase (Sigma-Aldrich). The amplification products were detected by Southern blotting on Hybond⁺ membranes (Amersham Biosciences) according to the manufacturer's specifications. [α-³²P]dCTP-labeled DNA probes were prepared with the Rediprime DNA labeling system (Amersham Biosciences). Results of the hybridization were visualized and quantified using a Storm 860 Phosphor-Imager (Amersham Biosciences). Quantification of PCR products within the given range of cycles confirmed exponential amplification. Stoser 18 and stoser 19 primers were used for amplification of cytHPPK/DHPS; mitHPPK/DHPS was amplified with stoser 20 and stoser 21. *Ubi-5* gene, which was used as a housekeeping gene control, was amplified with UBI5F and UBI5R primers.

Southern Blotting and Hybridization—Total DNA has been isolated from *Arabidopsis* leaves using Invisorb Spin Plant Mini Kit (Invitex, Invitex Gesellschaft für Biotechnik & Biodesign mbH, Berlin, Germany). One microgram of total plant DNA was digested overnight with 10 units of EcoRV restriction enzyme. The digested DNA was resolved in 0.7% agarose gel, blotted, and hybridized essentially as described above. Primers stoser 54 and stoser 36 were used to amplify a 300-bp fragment of the gene, which was used as a probe.

Salt Stress Treatment—Three-week-old seedlings of *in vitro* grown or the aerial parts of fully developed flowering *Arabidopsis* plants (6 weeks old) were soaked in a 150 mM solution of NaCl or KCl or in water (negative control). Samples were taken after 2 and 4 h, and total RNA was extracted as mentioned above. For RT-PCR of cytHPPK/DHPS (stoser 18 and stoser 19 primers), mitHPPK/DHPS (stoser 20 and stoser 21), and Pr-GFP-GUS (stoser 54 and GFP-SEQ primers) 25, 30, and 35 cycles were run; whereas for ubiquitin (UBI5-F and UBI5-R primers) and *DREB2A* (stoser 117 and stoser 118 primers) 15, 20, and 25 cycles were performed, to assure exponential amplification. The amplification products were resolved by electrophoresis in an agarose gel and detected and quantified by ethidium bromide fluorescence using the Kodak Electrophore-

FIGURE 2. A, sequence of the 5'-end of the cytHPPK/DHPS gene as determined by 5' RACE. Exon sequences are in black boxes. An in-frame stop codon preceding the first ATG codon is marked with an asterisk. Arrowhead points to the methionine start codon of the incorrectly predicted cytHPPK/DHPS from the *Arabidopsis* genome data base. B, multiple sequence alignment of the protein sequences of cytosolic (At1g69190) and mitochondrial (At4g30000) HPPK/DHPS from *A. thaliana* (cytAth and mitAth, respectively) with mitochondrial HPPK/DHPS from *Pisum sativum* (mitPsat). The mitochondrial transit peptide is underlined with a solid line. HPPK and DHPS domains are delineated with dotted and dashed lines, respectively. The asterisk marks the place of T-DNA insertion in the knock-out mutant (described below).

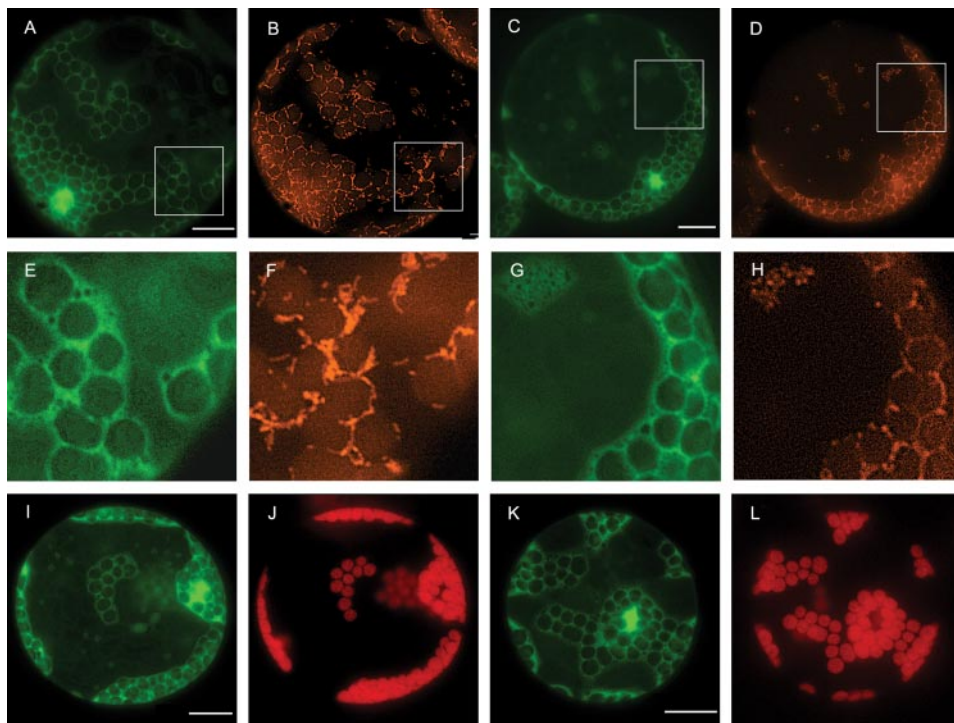


FIGURE 3. Intracellular localization of the cytHPPK/DHPS-GFP fusion protein. The cytHPPK/DHPS-GFP fusion protein was transiently expressed in *Arabidopsis* mesophyll protoplasts, and protoplasts were examined with a fluorescence microscope. *A*, *I*, *C*, and *K*, GFP fluorescence images of the protoplast expressing cytHPPK/DHPS (*A* and *I*) and free GFP (*C* and *K*). *B* and *D*, MitoTracker fluorescence of the same protoplasts as in *A* and *C*, respectively. *J* and *L*, chloroplast autofluorescence of the protoplasts in *I* and *K*. *E*, *F*, *G*, and *H*, magnification of the regions enclosed in rectangles in *A*, *B*, *C*, and *D*, respectively. Scale bar is 20 μm .

sis Documentation and Analysis System (Kodak Digital Science, Eastman Kodak, Rochester, NY).

Determination of Folate Levels—Mature dry seeds or 2-week-old *in vitro* grown plants, treated with NaCl as above, were used for folate level determination by liquid chromatography with tandem mass spectrometry detection essentially as described (23).

Salt Stress Tolerance Test—Salt stress tolerance has been carried out as described (24) with some modifications. Essentially, 2-week-old *in vitro* grown *Arabidopsis* seedlings were transferred to a fresh $\frac{1}{2}$ MS medium, containing 100 mM, 150 mM, and 170 mM NaCl. The survival rate has been monitored for 2 weeks after the transfer.

Seed Germination Rate Assay—*Arabidopsis* seeds were germinated on $\frac{1}{2}$ MS medium (control) and after addition of 10 mM H_2O_2 , 300 mM mannitol, or 150 mM NaCl. This was preceded by stratification on the above mentioned media in darkness at 4 $^\circ\text{C}$ for 4 days. The germinated seeds were scored for radicle emergence after different time intervals. A total of 90 seeds was scored at each time point.

Accession Numbers of the Sequences Used in This Study—cytHPPK/DHPS cDNA sequence has been deposited in the EMBL sequence data base under accession number AJ866732; mitHPPK/DHPS from pea has the accession number CAA69903.

RESULTS

HPPK/DHPS Is Encoded by Two Different Genes in *Arabidopsis*—Mining the *Arabidopsis* genome data base for genes homologous to pea HPPK/DHPS has revealed two

highly similar sequences, At1g69190 and At4g30000, the deduced protein sequences of which shared 80% identical and 87% similar amino acids. Both of them were around 66% identical and 76% similar to HPPK/DHPS from pea. The deduced protein sequence of At4g30000 contained the full-length HPPK and DHPS domains and a long N-terminal extension, predicted to be a mitochondrial transit peptide. This gene is likely an orthologue of the mitochondrial HPPK/DHPS described from pea (10). On the other hand, the At1g69190 gene was predicted to contain a single exon encoding a shorter protein that lacked a part of the N-terminal portion of the mature HPPK domain, rendering it potentially non-functional. To verify the data base predictions, 5'- and 3'-RACE experiments were undertaken. The predicted initial exon was completed, revealing the missing portion of the HPPK domain. Furthermore, the gene had an additional exon at the 5'-end, containing

the 5'-untranslated region and a start codon (Fig. 2A). In the 5'-untranslated region preceding the start codon, the presence of an in-frame stop codon confirmed that the full-length coding sequence was reconstituted. The corresponding cDNA encoded a protein of 484 amino acids with a deduced molecular mass of 54,053 Da. The deduced protein sequence did not show any obvious putative transit peptide (Fig. 2B) suggesting a cytosolic location of the enzyme. In summary, *Arabidopsis* contains two HPPK/DHPS genes, one being a putative orthologue of the earlier characterized mitochondrial HPPK/DHPS (mitHPPK/DHPS) from pea, and a novel gene, whose product possibly is localized in the cytosol. This protein was designated as cytosolic HPPK/DHPS (cytHPPK/DHPS).

Intracellular Localization of cytHPPK/DHPS—To confirm cytosolic localization of the enzyme, a chimeric protein was constructed containing the first 224 amino acids of cytHPPK/DHPS fused to GFP. The fusion protein was transiently expressed in *Arabidopsis* mesophyll protoplasts, and GFP fluorescence was detected with a wide-field epifluorescence microscope. As shown in Fig. 3 (*A*, *E*, and *I*) the signal was spread throughout the cytosol and in the nucleus in a very similar fashion as for free GFP (Fig. 3, *C*, *G*, and *K*). To visualize the mitochondria, the protoplasts were stained with MitoTracker orange, and the fluorescence was recorded in the orange region of the spectrum. As seen in Fig. 3 (*B*, *F*, *D*, and *H*) the pattern of the mitochondrial staining was discrete (*dotted to dashed*) and totally different from the continuous, network-like signal observed for cytHPPK/DHPS-GFP or free GFP. Similarly, the chloroplasts were localized by autofluorescence in the red

range of the spectrum. Clearly, the GFP fluorescence was localized around and between the chloroplasts, which were seen as *dark spots* in the GFP fluorescence image. These data strongly suggest that cytHPPK/DHPS was targeted to the cytosol, as postulated from the *in silico* analysis of the sequence.

Functionality of cytHPPK/DHPS—To demonstrate cytHPPK/DHPS functionality, a *S. cerevisiae* mutant devoid of HPPK/DHPS activity was complemented with the *Arabidopsis* gene. We used in this study the strain Y26466 bearing a complete deletion of the YNL256w open reading frame (*FOL1* gene), thus, lacking DHNA, HPPK, and DHPS activities. Y26466 was initially received from the EuroSCARF collection as a diploid, carrying one intact *FOL1* allele. After initiation of the sporulation and random spore analysis, a number of haploid knockouts were isolated, lacking the *FOL1* gene. All of them

were unable to grow without either simultaneous supplementation of methionine, adenine, histidine and TMP or 5-formyl tetrahydrofolate (5-CHO-H₄FGlu) at concentrations above 1 mg/liter. These strains also showed a “petite” phenotype and were found to have impaired mitochondrial function, because they could not utilize alternative carbon sources, such as glycerol, ethanol, or galactose (25). Although *cytHPPK/DHPS* was placed on a yeast expression vector under the control of the inducible GAL1 promoter, this promoter appeared sufficiently “leaky” to promote HPPK/DHPS expression in the absence of galactose, as shown in Fig. 4. Indeed, when the knock-out mutants were simultaneously transformed with two vectors containing the *Arabidopsis* DHNA gene *AtFolB1* (7) on one hand and the *cytHPPK/DHPS* gene on the other hand, growth was restored in a medium containing glucose as a carbon source, without addition of methionine, adenine, and histidine-TMP or 5-CHO-H₄FGlu. In contrast, no rescue was observed when the two constructs were introduced separately. These results indicate that the *cytHPPK/DHPS* gene is covering functional HPPK and DHPS domains.

To confirm these results, *cytHPPK/DHPS* was overexpressed in *E. coli* as an N-terminal His₆ fusion. The solubility of the protein was enhanced by co-expressing the pT-GroE plasmid bearing *E. coli* GroESL chaperone genes (22). Under these conditions, the major part of the expressed protein remained soluble, and highly purified His₆-*cytHPPK/DHPS* fusion protein could be obtained (Fig. 5A). The purified protein was capable of converting 6-hydroxymethyl dihydropterin into dihydropteroate in the presence of ATP and *p*-ABA. Thus, both activities, HPPK and DHPS, were detected. However, the purified enzyme was not stable at room temperature, which hampered precise kinetic studies, especially in the lower substrate concentration range. Nevertheless, the estimated V_{max} and K_m were very similar to those of the earlier characterized HPPK/DHPS from pea mitochondria (10, 26) (Fig. 5B). The enzyme was also strongly inhibited by sulfonamide drugs such as sulfanilamide, with an I_{50} (the concentration of inhibitor yielding 50% of inhibition) of ~15 μ M when the activity was measured in the presence of 30 μ M *p*-ABA. These data confirm that *cytHPPK/DHPS* is fully functional and has properties very similar to its mitochondrial counterpart.

CytHPPK/DHPS Expression Is Confined to Reproductive Tissues—To further characterize the novel HPPK/DHPS gene, its expression was studied in comparison with that of mitHPPK/DHPS, using semi-quantitative RT-PCR. As shown in Fig. 6,

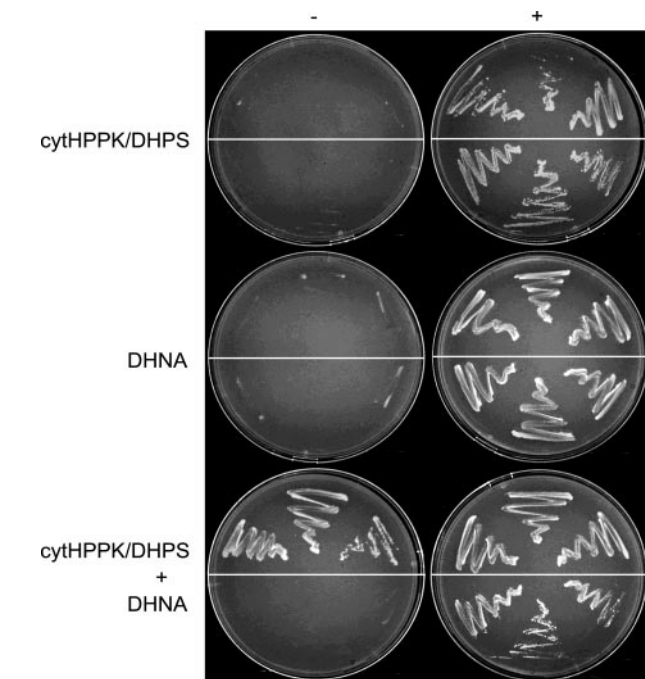


FIGURE 4. Expression of the *Arabidopsis* cytHPPK/DHPS rescues corresponding yeast mutant. Three independent transformed yeast clones were used in the experiment. The *top part* of a plate corresponds to the clones transformed with a cDNA, whereas the *bottom part* contained the clones transformed with a corresponding “empty” vector (negative control). “-” and “+” indicate absence or presence of 5-CHO-H₄FGlu in the growth medium. Yeast DHNA/HPPK/DHPS mutant (Y26466) is rescued by the combination of both the construct expressing plant DHNA and the *cytHPPK/DHPS* construct.

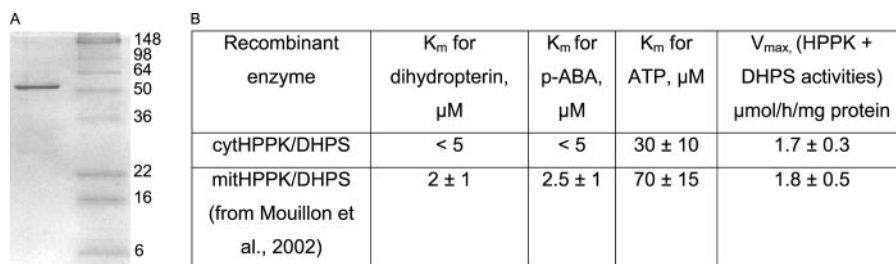


FIGURE 5. *Arabidopsis* cytHPPK/DHPS is active *in vitro*. A, SDS-PAGE of recombinant His₆-*cytHPPK/DHPS* protein overexpressed in *E. coli* and purified. B, comparison of the main kinetic parameters of *cytHPPK/DHPS* from *Arabidopsis* with *mitHPPK/DHPS* from pea. Values are means \pm S.D. from two to four separate determinations. K_m values were estimated by direct fitting to Michaelis-Menten curves using non-linear regression and EasyPlot software (Spiral Software).

with virtually no expression in other plant organs, whereas *mitHPPK/DHPS* was expressed ubiquitously with some quantitative variations. Given the difference in the amplification efficiency for different primer sets as well as different specific activities of labeled probes used for the detection, it was impossible to directly compare expression lev-

Cytosolic HPPK/DHPS from *Arabidopsis*

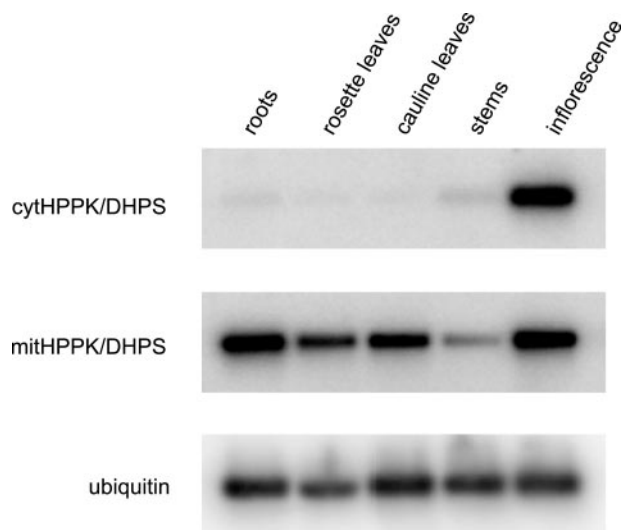


FIGURE 6. Comparison of the tissue-specific expression of cytosolic and mitochondrial HPPK/DHPS genes of *Arabidopsis*. Total RNA was isolated from different *Arabidopsis* tissues and subjected to semi-quantitative RT-PCR. Amplification products were resolved by agarose gel electrophoresis and detected by the Southern blot. The ubiquitin five gene was used to control the amounts of the template in each sample and normalize.

els of *cytHPPK/DHPS* with *mitHPPK/DHPS*. Nevertheless, the expression in flowers of the cytosolic enzyme was considerably lower than that of its mitochondrial counterpart, because it took six more cycles for *cytHPPK/DHPS* to reach signal intensities comparable with those for *mitHPPK/DHPS*.

To determine whether *cytHPPK/DHPS* expression was confined to any particular structure or tissues in reproductive organs, the 5'-upstream sequence of the gene, which contained the potential gene promoter, was cloned and linked to the chimeric GFP-GUS reporter. The fusion, Pr-GFP-GUS, contained a 1.2-kb 5'-upstream fragment, the first and a part of the second exon of the gene fused in-frame with GFP-GUS (Fig. 7A). Thus, the resulting fusion protein contained the 21 N-terminal amino acids of *cytHPPK/DHPS* linked to the GFP-GUS reporter. The construct was stably transformed in *Arabidopsis* and reporter activity was studied *in situ*.

The results of histochemical GUS staining highly correlated with that of the semi-quantitative RT-PCR. The activity was detected in the reproductive tissues. Specifically, all detected activity was found in early stages of seed development. Onset of the expression was observed in gynoeciums of flower buds at approximately stage 8 of flower development (27) (Fig. 7, B and C). The expression is seen as two parallel patches of cells, which supposedly form placenta for further megagametophyte development. Later, the expression is observed in the megagametophytes of mature flowers at stage 13 of flower development, when buds had just opened or were ready to open and anthesis had occurred (Fig. 7D). At this stage the staining was visible in the micropylar and central endosperm region weakening toward the chalazal end (Fig. 7E). Subsequently, at the globular stage of embryo development the expression spread to the entire seed, with the chalazal region being the most heavily stained (Fig. 7, F and G). At the late globular stage, the gene is still expressed in the whole seed, with the staining being concentrated in the developing embryo and in the chalazal region

(Fig. 7, H and I). The expression was not detectable at the heart stage of embryo development and never appears again at later stages (Fig. 7, J and K).

Salt Stress Induces Steady-state Transcription Levels of *cytHPPK/DHPS*—In an attempt to determine the possible function(s) of *cytHPPK/DHPS*, different microarray data from the Affymetrics microarray data base were analyzed. In one of these datasets, the gene was induced upon treatment of *Arabidopsis* roots with KCl (28). To confirm and further study this phenomenon, 3-week-old *Arabidopsis* seedlings were treated with different concentrations of KCl and NaCl, and the expression of *cytHPPK/DHPS* was studied by RT-PCR. The most pronounced induction was observed after 2- to 4-h treatment of the seedlings with 150 mM NaCl (Fig. 8). Seedlings treated with 150 mM KCl also demonstrated elevated levels of steady-state transcripts; however, less pronounced than in the case of NaCl treatment (not shown). In contrast, *mitHPPK/DHPS* was not induced by salt treatment (Fig. 8). Likewise, none of the other folate biosynthesis genes, coding for GTPCHI, DHNA (AtFolB1, AtFolB2, and AtFolB3), ADCL, ADCS, DHFS, and DHER, was induced by salt stress (data not shown). The magnitude of induction of the *cytHPPK/DHPS* gene by NaCl was higher than that of the drought- and salt stress-inducible transcription factor *DREB2A* (29). These data imply a direct or indirect involvement of *cytHPPK/DHPS* in stress response mechanisms, which are probably different from a simple boosting of folate levels, because other folate genes were not induced by salt stress. In support of this hypothesis, determination of folate levels in the seedlings subjected to salt stress has not shown significant difference of the folate level between stressed and non-stressed plants (supplemental Fig. S1).

To determine in which tissues NaCl induced *cytHPPK/DHPS* gene expression, 3-week-old seedlings of the transgenic line expressing the *cytHPPK/DHPS* promoter-GFP-GUS fusion were subjected to salt stress as mentioned above and stained for GUS activity. However, no obvious staining was observed, implying that the induction was below GUS-staining sensitivity. The possibility that the promoter fragment fused to GFP-GUS did not completely reproduce the expression pattern of the endogenous promoter has been ruled out by RT-PCR of the *cytHPPK/DHPS*-GFP-GUS fusion mRNA. The induction of the promoter-GFP-GUS fusion by salt stress was very similar to the one observed for the endogenous *cytHPPK/DHPS* gene (data not shown).

Given the failure to detect *cytHPPK/DHPS* expression upon salt treatment by the GUS staining, semi-quantitative RT-PCR was employed as an alternative to study the tissue-specific pattern of the *cytHPPK/DHPS* expression in different organs of salt-stressed mature, flowering *Arabidopsis* plants (6 weeks old). However, the pattern of the expression was indistinguishable from that under non-stressed conditions, with no induction being observed in any of the organs studied, namely, roots, rosette and cauline leaves, stems, and inflorescences (data not shown). This result indicates that *cytHPPK/DHPS* might be involved in stress protection at the seed to seedling developmental stages.

Characterization of *cytHPPK/DHPS* T-DNA Knock-out Line—To elucidate the possible function of *cytHPPK/DHPS*, a



FIGURE 7. Tissue-specific activity of the cytHPPK/DHPS gene promoter. *A*, schematic representation of the cytHPPK/DHPS promoter GFP-GUS fusion construct. The *scheme* illustrates only the construct structure; scale is not preserved. In the construct, 1.2 kb of the gene upstream sequence the promoter, the 1st exon and a part of the 2nd exon coding for 21 amino acids of cytHPPK/DHPS protein are linked in-frame with the reporter. The promoter as well as the first intron are designated by the solid black line. The gene exons are in orange. *B–K*, *in situ* detection of GUS activity in one of the homozygous transgenic lines carrying the Pr-GFP-GUS in-frame construct; *B*, the gynoecium of developing flowers at approximately stage 8 of flower development. All other floral organs are removed. The expression is seen as two parallel patches of cells, which are supposedly form placenta for further megagametophyte development; *C*, same as in *B* but at later developmental stage (stage 9); *D*, later, the expression is found exclusively in megagametophytes of mature pollinated flowers (stage 13); *E*, close up of the same flower as in *D*. The expression is confined to the developing nuclear endosperm; most pronounced in the micropylar region (*mp*) and the central endosperm and fainting toward the chalazal end (*ch*). *F*, flower at a later stage of development. The expression is spread through the whole developing seed. *G*, close up of one of the developing seed (globular stage of embryo development) of the flower in *F*. The chalazal region is the most intensively stained; *H*, young developing silique (stage 17). GUS staining is spread through the developing seeds; *I*, close up of the developing seed (late globular stage of the embryo) of the silique pictured in *H*. The highest expression is observed in the chalazal region and the embryo; *J*, the expression is abolished in almost fully extended siliques stage 17; *K*, seed of the silique in *J* at heart stage of the embryo development. No expression was observed. The scale bars: *B*, *C*, *G*, *I*, and *K*, 100 μ m; *D* and *F*, 200 μ m; *E*, 20 μ m; and *H* and *J*, 1 mm.

T-DNA insertion line, SALK_093782, has been characterized. The mutant was received from the Nottingham *Arabidopsis* Stock Centre as a hemizygous transgenic line as judged from a 3:1 segregation of kanamycin resistance of the seeds. The

sequence flanking the left T-DNA border has been amplified and sequenced to confirm the insertion location. The T-DNA insertion has occurred 372 bases downstream of the start codon, leaving only 124 N-terminal amino acids of the original protein (Fig. 2*B*). Although these 124 amino acid residues represent a large part of the HPPK domain, a substantial stretch of highly conserved amino acids at the C terminus of this domain are missing together with the complete DHPS domain. Hence, it is unlikely that this truncated protein possesses any enzymatic activity. Thus, the T-DNA insertion most probably results in a full knock-out of the gene.

After two self-crosses of the initial hemizygous line, a potentially homozygous line showing 100% resistance to kanamycin, was selected. This was confirmed by PCR amplification of genomic DNA and Southern blotting hybridization (Fig. 9). As seen from Fig. 9*B*, amplification of a wild-type copy of the gene in the mutant line was impossible. Similarly, Southern blotting hybridization of mutant genomic DNA revealed only the mutated gene fragment of the expected size (~4300 bp in the mutant and ~3650 bp in the wild-type) (Fig. 9*D*); while no trace of the wild-type gene fragment was detectable. Moreover, RT-PCR with stoser 54 and 55 primers failed to yield an amplification product in the mutant line (Fig. 9*C*).

The phenotype of the mutant plants was indistinguishable from wild-type plants under normal growth conditions. No differences in the germination rate, rosette development, bolting, and flowering time have been observed. Because cytHPPK/DHPS is expressed exclusively in developing seeds, the seed and embryo morphology were studied using bright field microscopy with differential interference contrast. No obvious

difference was noticed between different stages of development of mutant and wild-type embryos. To investigate whether stress resistance is affected in the mutant, stress tolerance tests were conducted. Mutant seeds displayed reduced germination rates

Cytosolic HPPK/DHPS from *Arabidopsis*

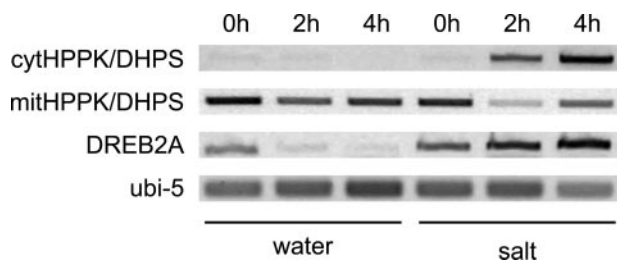


FIGURE 8. Comparison of cytosolic and mitochondrial HPPK/DHPS expression under salt stress. *Arabidopsis* seedlings were soaked in 150 mM NaCl or in water (negative control). At different time points samples were taken for total RNA extraction followed by RT-PCR. The PCR products were resolved by agarose gel electrophoresis and detected by ethidium bromide fluorescence. Three independent biological repeats were performed; a representative gel is shown. The image was inverted to negative for clarity. Ubiquitin-5 was used as a control to monitor equal amounts of template; *DREB2A* was used as a positive control for salt-stress induction.

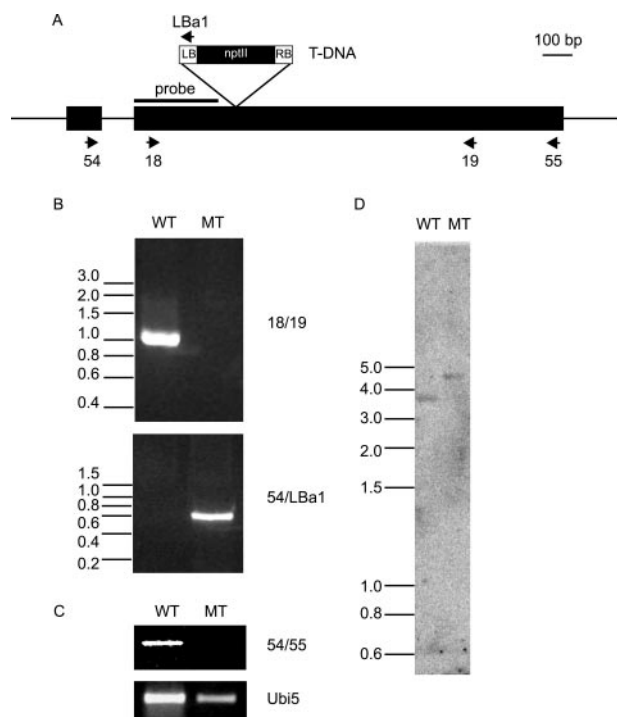


FIGURE 9. Molecular characterization of the *cytHPPK/DHPS* T-DNA insertion mutant. *WT*, wild-type; *MT*, mutant. Molecular masses are in kilobases. *A*, schematic representation of the T-DNA insertion into the *cytHPPK/DHPS* gene. The scale of the T-DNA (~4.5 kb) was not preserved for sake of convenience. *Closed boxes* represent gene exons. *Arrows* depict stoser primers with their corresponding number (Table 1). *LB* and *RB*, left and right T-DNA borders, respectively. *npIII*, neomycin phosphotransferase. *B*, PCR amplification of the *cytHPPK/DHPS* gene and T-DNA flanking sequence in mutant and wild-type. Primer pairs used are at the *right*. *C*, RT-PCR of *cytHPPK/DHPS* transcript in the mutant as compared with wild-type. *D*, Southern blotting of total genomic DNA from mutant and wild-type plants digested with *EcoRV* and hybridized with the radioactive probe depicted in *A*.

on media supplied with different stress agents, such as H_2O_2 (oxidative stress), mannitol, and NaCl (osmotic stress). The most pronounced reduction of germination rate was detected on a medium containing 10 mM H_2O_2 (Fig 10). A complete reversal of hemizygous mutant to the wild-type phenotype was observed upon a backcross with the Col-0 wild type. These data strongly suggest that *cytHPPK/DHPS* plays a role in seed stress response and survival.

To check whether the mutant seeds have a different content and/or composition of folates, which might be related to their reduced survival, folates were measured both in dry mutant seeds and seeds of the wild-type. A modest but significant decrease of 5- CH_3 -PteGlu_(n) and 5-CHO-PteGlu_(n) (by 11 and 33%, respectively) was observed in dry seeds of the mutant as compared with the wild-type, resulting in a decrease in total folate content by 11% (Fig. 11).

DISCUSSION

The existence of a cytosolic isoform of HPPK/DHPS raises the question as to whether it plays a particular role *in planta*, different from that of its mitochondrial counterpart, and whether the presence of the cytosolic enzyme is common in higher plants. Searches of the rice and poplar genome databases (the only fully sequenced plant genomes besides *Arabidopsis*) revealed only one gene encoding HPPK/DHPS, containing a putative mitochondrial transit peptide. Based on Southern blot analysis, the existence of a single copy gene (*mitHPPK/DHPS*) has also been postulated in pea (10). Single copy *HPPK/DHPS* hits were also found in non-redundant GenBank™ sequence databases for lower eukaryotes, such as fungi and protozoa. Thus, the genomic sequence data available today suggest that the cytosolic HPPK/DHPS found in *Arabidopsis* is a unique feature, at least in the plant kingdom, that might have been created by a relatively recent gene duplication (30). Phylogenetic analysis of the known HPPK/DHPS supports this hypothesis (Fig. 12). Indeed, it can be seen in the group of higher plants that the two *Arabidopsis* genes are very close and belong to the same branch of the tree. In addition, this duplication appeared later than the speciation of the different plant families. Genome duplication is generally followed by sub-neofunctionalization (31). In the latter process, a newly formed gene copy first shares its function with the original sequence (subfunctionalization) but acquires a new function later in evolution (neofunctionalization). Indeed, although the mitochondrial enzyme is expressed in all plant tissues, obviously playing a housekeeping role by ensuring a continuous supply of folate, *cytHPPK/DHPS* presented a specific pattern of gene expression apparently strongly related to seed development, as was clearly shown by our histochemical GUS assays indicating that the expression was confined to the developing seeds. This result might explain why the RT-PCR data showed a considerably lower level of *cytHPPK/DHPS* expression in the inflorescence as compared with the mitochondrial enzyme: *cytHPPK/DHPS* mRNAs were likely “diluted” with non-expressing reproductive tissues. The highest expression is detected on the earliest stages of megagametophyte/seed development, which are accompanied by a substantial cellular division activity. Thus, it is possible that a very high local concentration of folate cofactors is necessary to sustain the high rate of DNA synthesis during this process and that *cytHPPK/DHPS* plays a role to boost folate supplies. Earlier, it has been shown that one of the three cytosolic DHNA isoforms present in *Arabidopsis*, AtFolB3, was expressed exclusively in the developing siliques (7). Although nothing is known yet about the precise pattern of expression and the exact developmental stage, it is tempting to speculate that, in seeds,

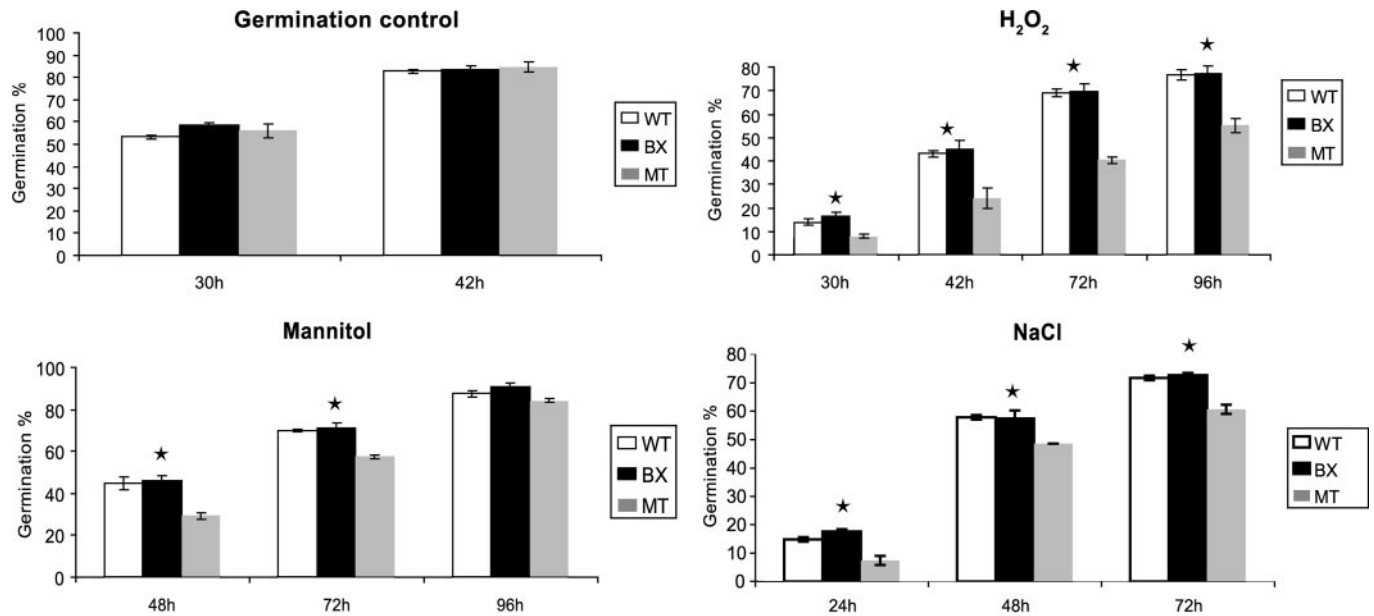


FIGURE 10. Seed germination rates of wild-type *Arabidopsis* and the cytHPPK/DHPS knock-out mutant on media supplemented with 10 mM H_2O_2 , 300 mM mannitol, and 150 mM NaCl. Seeds germinated in the absence of stress agents were used as a control. All seeds were stratified for 4 days at 4 °C in darkness. Germination rates were scored by radicle emergence after different time intervals. Averages of three independent repeats are shown. Bars indicate standard deviation. Asterisks mark datasets, in which a significant difference ($p < 0.005$) of the wild-type and the backcross as compared with the mutant was observed. WT, wild-type Col-0 seeds; MT, homozygous cytHPPK/DHPS mutant seeds; and BX, F2 mutant \times Col-0 backcross seeds.

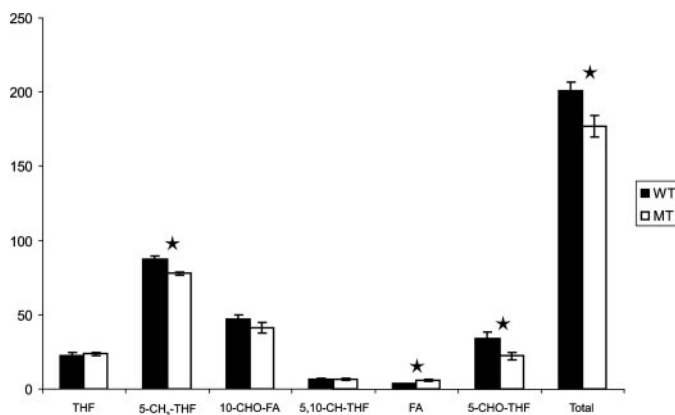


FIGURE 11. Analysis of major folate species in dry seeds of the knock-out cytHPPK/DHPS mutant as compared with wild-type seeds. Two independent biological repeats were analyzed, with similar results; a representative data set is shown. Data are averages of three technical repeats, and error bars indicate standard deviations. Asterisks mark datasets in which a significant difference ($p < 0.05$) between the wild type (WT) and the mutant (MT) were observed. THF, tetrahydrofolate; 5- CH_2 -THF, 5-methyltetrahydrofolate; 10-CHO-FA, 10-formylfolic acid; 5,10-CH-THF, 5,10-methenyltetrahydrofolate; FA, folic acid; 5-CHO-THF, 5-formyltetrahydrofolate; and Total, total folate.

AtFolB3 and cytHPPK/DHPS catalyze three consecutive steps of an alternative cytosolic pathway producing dihydropteroate to participate in H_4 F Glu_n synthesis.

Dihydropteroate formed in the cytosol must enter the mitochondria, because the next step of biosynthesis is catalyzed by DHFS, an enzyme encoded by a unique gene and localized exclusively in the mitochondria (11). Although there is no direct evidence for any specific translocator, its existence is supported by results showing that the yeast DHPS knock-out mutant, EHY1, was rescued by external addition of dihydropteroate (32). The advantage of two parallel ways producing dihydropteroate is not clear. It was pre-

viously shown that mitHPPK/DHPS could be a potential point of regulation, feedback inhibited by H_4 F Glu_n (26), with the K_i of the monoglutamate form approaching 10 μ M. The concentration of H_4 F Glu_n in the mitochondria was estimated to be ~ 600 pmol mg^{-1} protein (33). Assuming a volume of 1–2 μ l mg^{-1} protein (5) the H_4 F Glu_n concentration in the matrix could be 300–600 μ M. The concentration of H_4 F Glu_n in the cytosol is not known, but the total concentration of folates is thought to be about three times lower than in the mitochondria (34). Thus, a cytosolic HPPK/DHPS could be less inhibited than its mitochondrial counterpart, providing a way to bypass this point of regulation. Alternatively, H_2 F Glu could be directly formed in the cytosol if p -ABA-glutamate (p -ABAG) is used as substrate instead of p -ABA. This reaction was previously observed in bacteria (35) and plants (36), although p -ABAG was a less efficient substrate for folate synthesis than p -ABA was for pteroyl synthesis. Recently, the first experimental evidence of folate recycling and, particularly, p -ABAG salvage in plant cells has been obtained (37). The data, however, favor further degradation of p -ABAG to free p -ABA and Glu, which subsequently re-enter the biosynthesis pathway after mitochondrial import, rather than the direct conversion of p -ABAG to H_2 F Glu_n . In addition, p -ABAG appears to be a poor substrate for mitHPPK/DHPS, which makes the scenario of a direct conversion of p -ABAG to H_2 F Glu_n by mitHPPK/DHPS highly unlikely. These facts, however, cannot rule out the possibility of such a role for cytHPPK/DHPS in developing seeds. However, whether the cytosolic isoform is able to use p -ABAG as a substrate instead of p -ABA, requires further investigation. If this hypothesis holds true, H_2 F Glu produced in the cytosol has to be reduced into H_4 F Glu . In pea plants there was no evidence of DHFR/TS activity in the

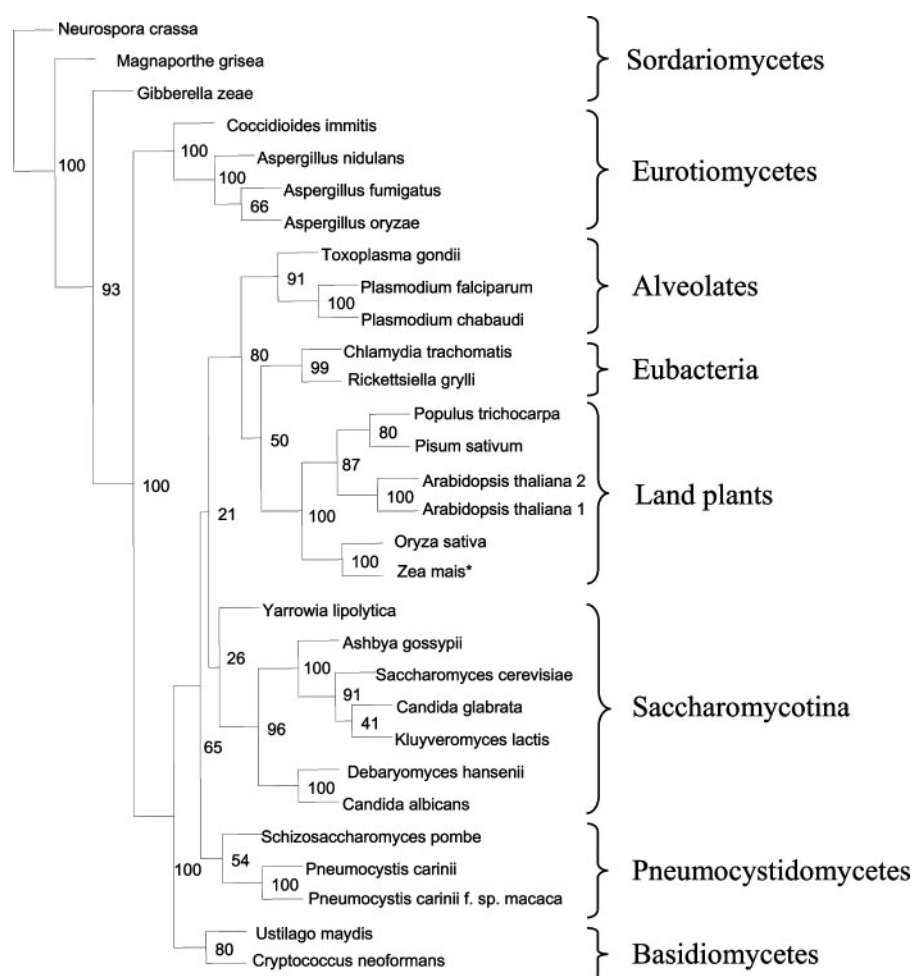


FIGURE 12. **Phylogenetic tree for known HPPK/DHPS.** Phylogenetic inference of enzymes containing the two domains HPPK and DHPS was made using 29 complete coding sequences and a partial (*) sequence of maize.

cytosol (5), but this enzyme is encoded by three genes in *Arabidopsis*, with one isoform potentially residing in the cytosol (38).

Considerably lower germination rates of a loss of function cytHPPK/DHPS mutant upon application of oxidative and osmotic stress strongly suggest involvement of this gene in oxidative stress response. This is supported by mRNA accumulation upon exposure of wild-type seedlings to salt stress, which is known to induce reactive oxygen species (39). These observations might suggest a relation between oxidative stress and folates. However, a protective role of folates due to their antioxidant properties (40, 41), a higher requirement for nucleotide synthesis and DNA repair, or a specific role of dihydropteroate during oxidative stress remain to be demonstrated. It is unknown whether higher folate levels may help plants cope with stress. Our experiments indicate no difference in folate content in *Arabidopsis* seedlings upon salt stress. However, a modest decline in folate content was observed in mature seeds of the cytHPPK/DHPS knock-out mutant as compared with wild-type seeds. It is not clear, however, whether this decrease underlies the reduced seed viability under stress conditions. Remarkably, the amount of folic acid (thought to be a folate oxidation product) was almost doubled in the mutant seeds as compared with the

wild type, which implies potentially lower reducing capacity of the seed cell anti-oxidation systems during seed maturation, desiccation, and/or storage. Recently, dihydroneopterin has been identified as a reporter metabolite (a metabolite for which significant changes in transcript abundance of both its producing and consuming enzymes occur under certain conditions) of oxidative stress in *Arabidopsis* cell suspension cultures (20). Among three isoforms of its consuming enzyme DHNA, the transcript of the seed-specific AtFolB3 enzyme was the only significantly induced (3.4 times), strengthening the above-mentioned idea that AtFolB3 and cytHPPK/DHPS are two consecutive steps of dihydropteroate synthesis in the cytosol in response to stress.

In conclusion, there is in *Arabidopsis* a cytosolic isoform of the HPPK/DHPS enzyme that is specifically expressed in developing seeds and under salt stress at the seedling stage. The enzyme is important for the seed survival under oxidative stress conditions; however, the exact mechanism by which its activity helps to cope with stress remains to be elucidated.

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Cytosolic Hydroxymethyldihydropterin Pyrophosphokinase/Dihydropteroate Synthase from *Arabidopsis thaliana*: A SPECIFIC ROLE IN EARLY DEVELOPMENT AND STRESS RESPONSE

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