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Higher Plant Plastids and Cyanobacteria Have Folate Carriers Related to Those of Trypanosomatids^{*[5]}

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Sebastian M. J. Klaus[‡], Edmund R. S. Kunji[§], Gale G. Bozzo[‡], Alexandre Noiriel[‡], Rocío Díaz de la Garza[‡], Gilles J. C. Basset[‡], Stéphane Ravel[¶], Fabrice Rébeillé[¶], Jesse F. Gregory, III^{||}, and Andrew D. Hanson^{†1}

From the [‡]Horticultural Sciences and ^{||}Food Science and Human Nutrition Departments, University of Florida, Gainesville, Florida 32611, the [§]Medical Research Council, Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, United Kingdom, and the [¶]Laboratoire de Physiologie Cellulaire Végétale, CNRS-Commissariat à l'Energie Atomique (Saclay, France) (CEA)-Institut National de la Recherche Agronomique-Université Joseph Fourier, CEA-Grenoble, F-38054 Grenoble Cedex 9, France

Cyanobacterial and plant genomes encode proteins with some similarity to the folate and bipterin transporters of the trypanosomatid parasite *Leishmania*. The *Synechocystis* slr0642 gene product and its closest *Arabidopsis* homolog, the At2g32040 gene product, are representative examples. Both have 12 probable transmembrane domains, and the At2g32040 protein has a predicted chloroplast transit peptide. When expressed in *Escherichia coli* *pabA pabB* or *folE*, mutants, which are unable to produce or take up folates, the slr0642 protein and a modified At2g32040 protein (truncated and fused to the N terminus of slr0642) enabled growth on 5-formyltetrahydrofolate or folic acid but not on 5-formyltetrahydrofolate triglutamate, demonstrating that both proteins mediate folate monoglutamate transport. Both proteins also mediate transport of the antifolate analogs methotrexate and aminopterin, as evidenced by their ability to greatly increase the sensitivity of *E. coli* to these inhibitors. The full-length At2g32040 polypeptide was translocated into isolated pea chloroplasts and, when fused to green fluorescent protein, directed the passenger protein to the envelope of *Arabidopsis* chloroplasts in transient expression experiments. At2g32040 transcripts were present at similar levels in roots and aerial organs, indicating that the protein occurs in non-green plastids as well as chloroplasts. Insertional inactivation of At2g32040 significantly raised the total folate content of chloroplasts and lowered the proportion of 5-methyltetrahydrofolate but did not discernibly affect growth. These findings establish conservation of function among folate and bipterin transporter family proteins from three kingdoms of life.

Tetrahydrofolate (THF)² and its derivatives (collectively termed folates) are essential cofactors for one-carbon transfer reactions in almost all organisms (1, 2). Bacteria and plants synthesize folates *de novo*, but higher animals do not and so need a dietary supply (1, 3, 4). Folates consist of a pteridine, *p*-aminobenzoate (*p*ABA) and one or

more glutamate residues (Fig. 1A). All these moieties are synthesized and assembled into folates in the cytosol of bacteria, but in plants the pathway is split among three subcellular compartments. The pteridine is made in the cytosol, and the *p*ABA is made in plastids; both then enter mitochondria, where they are coupled together and glutamylated to yield folate (5–12) (Fig. 1B). Much of the folate produced exits the mitochondria to supply folate pools in the cytosol, plastids, and vacuoles (13–17). This compartmentation of the synthesis pathway and its end products entails various transmembrane fluxes of folates and their precursors (Fig. 1B). Although *p*ABA can cross membranes by simple diffusion (18), folate and pteridine fluxes are likely to be carrier-mediated, as in animals (19–22) and bacteria (23–25). Plants, therefore, presumably have several as yet undiscovered organellar folate or pteridine transporters (Fig. 1B).

Multiple transporters that act on folates as well as antifolates such as methotrexate or aminopterin have been characterized in mammals. These transporters include the reduced folate carrier, which catalyzes exchange with organic anions, folate receptors, which mediate folate entry via endocytosis, folate efflux pumps of the ATP binding cassette family, and a protein from the mitochondrial carrier family (19–21).

High affinity folate and pteridine transporters are also known from the trypanosomatid *Leishmania*, a parasitic protist that is auxotrophic for folate and pteridine (26). These carriers are a bipterin transporter (BT1) and 2 folate transporters (FT1, FT5), all 3 being similar proteins with about 12 putative transmembrane helices (27–29). Together, they define a small family (the FBT family) that is a distant member of the major facilitator superfamily (30). BT1 is the only carrier yet known to act on pteridines; it also has low affinity folate transport activity (22).

Besides proteins from trypanosomatids, the FBT family (also known as the BT1 family, Pfam03092) includes a highly conserved protein from Cyanobacteria and nine *Arabidopsis* proteins. In this study we used heterologous expression in *Escherichia coli* mutants to probe the functions of a representative cyanobacterial FBT protein and its closest *Arabidopsis* homolog. Having demonstrated that both have folate and antifolate transport activity, we investigated the subcellular location of the *Arabidopsis* protein, the expression of its gene, and the effect of ablating this gene.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Folates and pteridines were from Schircks Laboratories (Jona, Switzerland). *p*-Aminobenzoylglutamate (*p*ABA-Glu) was HPLC-purified before use. Percoll and [5,6-³H]UTP (35 Ci/mmol) were from Amersham Biosciences. [³H]Leucine (59 Ci/mmol) was from PerkinElmer Life Sciences.

***E. coli* Strains**—Strain BN1163 (*pabA1*, *pabB::Kan*, *rpsL704*, *ilvG-*, *rfb-50*, *rph-1*) was supplied by B. Nichols (University of Chicago, Chi-

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1 and Table 1.

¹ To whom correspondence should be addressed: Horticultural Sciences Dept., University of Florida, Gainesville, FL 32611. Tel.: 352-392-1928 (ext. 334); Fax: 352-392-5653; E-mail: adha@mail.ifas.ufl.edu.

² The abbreviations used are: THF, tetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; *p*ABA, *p*-aminobenzoic acid; *p*ABA-Glu, *p*-aminobenzoylglutamate; FBT, folate-bipterin transporter; GFP, green fluorescent protein; RT, reverse transcription; IPTG, isopropyl 1-thio- β -D-galactopyranoside; HPLC, high performance liquid chromatography.

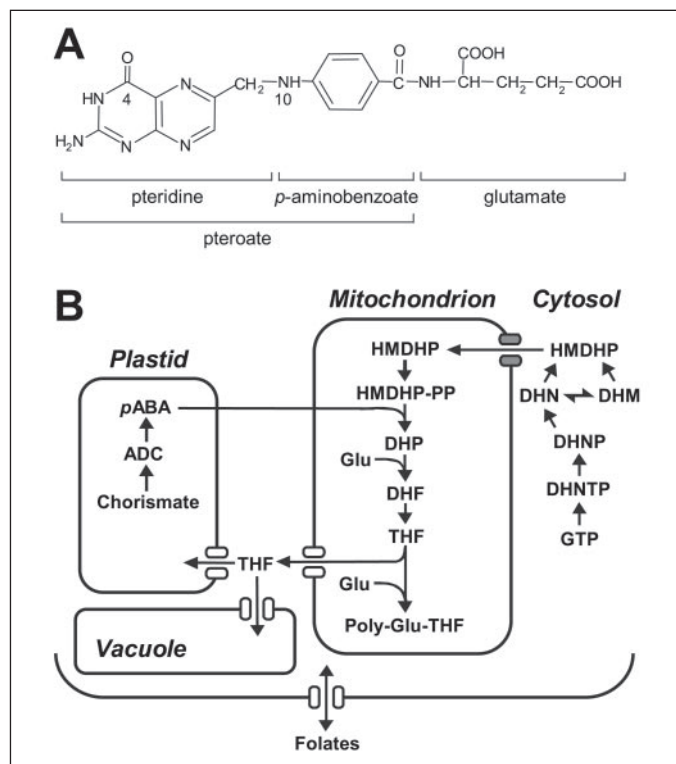


FIGURE 1. Structure of folates and compartmentation of folate biosynthesis in plants. A, folic acid. Aminopterin and methotrexate differ from folic acid in having an amino instead of an oxo group at C⁴, and N¹⁰ is methylated in methotrexate. The pteridine ring of folic acid is fully oxidized; in natural folates it is reduced to dihydro or tetrahydro forms. A γ -linked polyglutamyl tail of up to about six residues is usually attached to the first glutamate. B, distribution of folate biosynthesis steps between cytosol, mitochondria, and plastids and inferred carrier-mediated transport steps for folates (*open symbols*) and pteridines (*gray symbols*). Note that polyglutamylation of folates also occurs in the cytosol and plastids (7). DHN, dihydrooneopterin; DHNTP, dihydrooneopterin triphosphate; DHM, dihydromonapterin; HMDHP, hydroxymethyldihydropterin; DHP, dihydropterate; DHF, dihydrofolate; ADC, aminodeoxychorismate; PP, pyrophosphate.

icago, IL), and strain NM1200 was supplied by N. Majdalani (NCBI, Bethesda, MD). The NM1200 strain was constructed by P1 transduction of the Mini- λ Cm^R prophage carrying the *Red gam*, *beta*, and *exo* genes under the P_L promoter (31) in MG1655 (*E. coli* K12, *E. coli* Genetic Stock Center). The *folE* gene was deleted by targeted homologous recombination using electroporated linear DNA and strain NM1200 as described (32). Briefly, the kanamycin cassette was amplified from plasmid pUC4K with primers FolE-5' and FolE-3' (see Supplemental Table I), which respectively included the start and stop codon of *folE* and 47-bp 5'- and 3'-flanking sequences. After inducing the recombination system at 42 °C for 15 min, strain NM1200 was transformed with the PCR product. Clones with the desired replacement of the *folE* gene were selected on LB plates containing 300 μ M thymidine and 30 μ g/ml kanamycin. The kanamycin resistance locus was then transferred by P1 transduction to strain K12, to give strain P1-7B. Deletion of the *folE* gene was confirmed by PCR and sequencing. P1-7B was maintained on LB medium containing 300 μ M thymidine and 30 μ g/ml kanamycin.

Plants and Growth Conditions—*Arabidopsis* (ecotype Columbia) plants were grown at 23–28 °C in 12-h days (photosynthetic photon flux density 80 microeinsteins m⁻² s⁻¹) in potting soil irrigated with water. When roots were required, plants were grown hydroponically (33).

Expression Constructs—*Synechocystis* slr0642 and *Arabidopsis* At2g32040 amplicons preceded by a Shine-Dalgarno sequence were cloned between the NotI and SstI sites of pLOI707HE (34), which contains the *tac* promoter, the *lacI^R* repressor gene, and the *tet* marker.

Templates were *Synechocystis* sp. PCC 6803 genomic DNA (from W. Vermaas, Arizona State University, Tempe, AZ) and an At2g32040 cDNA (GenBankTM BT003363, from the *Arabidopsis* Biological Resource Center). The following PCR primers were used (see Supplemental Table I): for *Synechocystis* slr0642, Slr1-5' and Slr1-3'; for the mature At2g32040 construct, At-t1-5' and At-t1-3'; for the chimeric At2g32040 construct (with codons 1–128 replaced by codons 1–37 of slr0642), Slr1-5' and Slr-c1-3' to amplify the slr0642 fragment, At-c1-5' and At-t1-3' to amplify the At2g32040 fragment, and Slr1-5' and At-t1-3' to splice the fragments. The complete slr0642 amplicon was cloned into pGEM[®]-T Easy (Promega), excised with NotI and SstI, and then cloned into pLOI707HE; other amplicons were cut with NotI and SstI and cloned straight into pLOI707HE. For the vector minus insert control, pLOI707HE was digested with NotI and SstI, end-polished with T4 polymerase, and religated. Constructs were made in *E. coli* strain DH5 α , sequence-verified, then introduced into *E. coli* strains BN1163 and P1-7B, both harboring pACYC-RIL (Stratagene), which contains three rare *E. coli* tRNA genes and the *cat* marker. Transformants were grown on LB plates containing tetracycline 10 μ g/ml, kanamycin 30–50 μ g/ml, chloramphenicol 20 μ g/ml and (for P1-7B only) thymidine 300 μ M.

Folate Uptake Tests—BN1163 cells harboring pACYC-RIL and a pLOI707HE construct were streaked on minimal medium plates containing M9 salts (35), 0.1 mM CaCl₂, 0.5 mM MgSO₄, trace elements (36), and 0.4% (w/v) glucose supplemented with antibiotics as above and 0.5 mM IPTG. To this medium was added 3.6 μ M *pABA* or 11 μ M (6R,6S)-5-formyl-THF (5-CHO-THF), folic acid, or (6R,6S)-5-formyl-THF triglutamate. P1-7B cells harboring pACYC-RIL and a pLOI707HE construct were streaked on LB medium containing 0.1% (w/v) sodium ascorbate and 1 mM dithiothreitol, appropriate antibiotics and IPTG minus or plus 11 μ M 5-CHO-THF, dihydropterate, hydroxymethyl-dihydropterin, or dihydropterin-6-aldehyde. Tests with the two latter compounds were made in a 10-l vessel flushed with N₂ at 25 ml/h.

Antifolate Uptake Tests—Solid or liquid minimal media were supplemented with antibiotics, IPTG, and *pABA* as described above. For tests on solid medium, cells were streaked on plates containing a central 1-cm disk of Whatman No. 1 paper soaked with 10 μ l of dimethyl sulfoxide alone or containing 100 mM methotrexate or 80 mM aminopterin. For tests in liquid medium, cells from an overnight culture were inoculated to give an A₆₀₀ reading of 0.05 in 1.5 ml of fresh medium supplemented with various amounts of methotrexate or aminopterin. Growth was evaluated by measuring A₆₀₀ after incubating at 37 °C for 9.5 h with shaking at 220 rpm.

Transient Expression of Green Fluorescent Fusion Protein in Arabidopsis Protoplasts—The entire At2g32040 sequence was amplified using primers At-gfp-5' and At-gfp-3', digested with Sall and NcoI, and cloned in-frame upstream of the green fluorescent protein (GFP) gene in pTH2 (37). Protoplasts were prepared from cell cultures, transformed, and cultured as described at 22 °C for up to 48 h (7, 38). Transformation efficiencies were typically 5–20%. Samples were analyzed by epifluorescence microscopy as described (11).

Chloroplast Import Assays—The full-length At2g32040 sequence and a version lacking the first 88 residues were, respectively, amplified using forward primer At-*ifl*-5' or At-*it*-5' and reverse primer At-*i*-3'. The amplicons were cloned as EcoRI-HindIII fragments into pGEM[®]-4Z (Promega). Coupled *in vitro* transcription-translation was performed using a wheat germ TNT[®] kit (Promega) and [³H]leucine according to the manufacturer's protocol. Translation product import into pea chloroplasts was assayed as described (39). Briefly, translation product (50 μ l) was added to 50 μ l of 2 \times import buffer (39) containing 100 mM

unlabeled leucine; 100 μ l of 1 \times import buffer containing 30 mM Mg²⁺-ATP and 200 μ l of purified chloroplasts (0.2 mg chlorophyll) were added, and samples were incubated for 15 min at 25 °C in the light. Chloroplasts were re-purified as described (39), resuspended in SDS loading buffer containing 4 M urea, and held for 10 min at 37 °C before loading.

Real-time Quantitative RT-PCR—Total RNA was extracted using RNeasy plant mini kits (Qiagen) and treated with DNase (DNA-free™ kit, Ambion, Austin, TX). RT-PCR was performed on 250 ng of RNA in 25- μ l reactions using Taq-Man One-Step RT-PCR Master Mix reagents and a GeneAmp 5700 system (Applied Biosystems, Foster City, CA). The primers (At-rt-5', At-rt-3') and Taq-Man probe (At-rt-probe) were designed with Applied Biosystems Primer Express software. The amplicon length was 74 bp. Primer At-rt-5' spanned two exons to avoid amplifying contaminating genomic DNA, and controls without reverse transcriptase were run to verify that no such amplification occurred. RT-PCR conditions were 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An At2g32040 RNA standard was synthesized from a cDNA template using primers At-SP6std-5' and At-std-3' and labeled using [5,6-³H]UTP as described (10). An internal standard RNA was added to each sample before RT-PCR to estimate recoveries, which were 34–100%. Data were corrected for recovery.

Insertional Mutant—An At2g32040 mutant (Salk 109024) was identified in the Salk *Arabidopsis* (ecotype Columbia) T-DNA insertion collection (40). Wild type or homozygous mutant segregants were identified by PCR using gene-specific primers located 5' or 3' of the T-DNA insertion (At-s-5' and At-s-3', respectively) and the T-DNA-specific primer SalkLbB1. Genomic DNA was extracted as described (41). The insertion site was confirmed by sequencing the amplicon from mutant homozygotes. Homozygous mutant and wild type segregants were selfed, and their progeny were used for experiments. Kanamycin resistance was shown to co-segregate with the At2g32040 mutation, indicating the absence of T-DNA insertions at other loci.

Chloroplast Folate Analyses—Chloroplasts were prepared from the leaves of 4-week-old *Arabidopsis* plants as described (42, 43). Foliates were extracted from samples (~1 mg of chlorophyll), deglutamylated, affinity-purified, and determined by HPLC with electrochemical detection as described (41).

RESULTS

FBT Family Genes in Cyanobacteria and Plants—Blast searches of GenBank™ confirmed and extended the delineation of the FBT family given in the Pfam data base. Homologs of the *Leishmania* FT and BT1 transporters were found only in trypanosomatids, apicomplexans, Cyanobacteria, and plants. Cyanobacterial genomes have a single FBT gene specifying a highly conserved (\geq 56% identity) protein. In contrast, plant genomes encode several diverse FBT proteins, there being nine in *Arabidopsis*. Multiple alignment and phylogenetic analysis showed that one of these, At2g32040, clustered with Cyanobacteria, whereas the others fell into two groups of four each (see Supplemental Fig. 1, A and C). The At2g32040 protein shares 52–54% identity with the cyanobacterial proteins but differs in having an ~90-residue N-terminal extension with the features of a plastid targeting peptide (44) (see Supplemental Fig. 1C). The cyanobacterial and plant proteins have 12 predicted transmembrane α -helices, as illustrated in Supplemental Fig. 1B for the *Synechocystis* slr0642 protein.

Because trypanosomatids and apicomplexans have apparently acquired numerous genes from photosynthetic organisms (45, 46) and plants acquired many genes from Cyanobacteria (47), the cyanobacte-

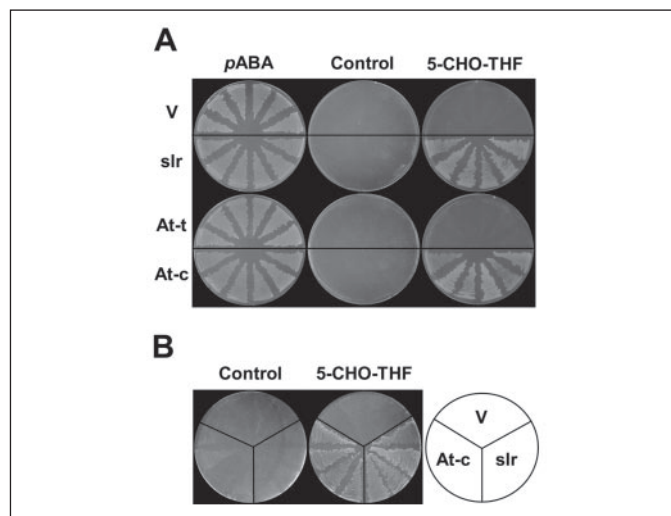


FIGURE 2. Folate uptake tests in *E. coli* expressing *Synechocystis* slr0642 or modified *Arabidopsis* At2g32040. A, strain BN1163 (a *pabA pabB* mutant) harboring pACYC-RIL was transformed with pLOI707HE with no insert (V) or containing slr0642 (*slr*) the truncated At2g32040 protein (At-t) or a chimeric At2g32040 protein with residues 1–128 replaced by residues 1–37 of slr0642 (At-c) (see Supplemental Fig. 1C). Six independent clones of each construct were streaked on minimal medium plus IPTG alone (control) or containing 3.6 μ M pABA or 11 μ M 5-CHO-THF. Clones were streaked once on pABA-supplemented plates and three times in succession on the others. Incubation was at 37 °C. The pABA plates were photographed 1 day after streaking, others after 3 days. Similar results were obtained when 11 μ M folic acid replaced 5-CHO-THF except that growth was slightly slower. B, strain P1-7B (a *folE* deletant) harboring pACYC-RIL was transformed with V, *slr*, and At-c constructs as above. Four independent clones of each construct were streaked on LB medium plus 0.1% (w/v) sodium ascorbate, 1 mM dithiothreitol, and IPTG alone (control) or containing 11 μ M 5-CHO-THF. Plates were incubated for 2 days at 37 °C.

rial protein is potentially the archetype of the FBT family. We accordingly chose a representative cyanobacterial FBT protein, *Synechocystis* slr0642 (henceforth slr0642) for further study along with its close plant relative, *Arabidopsis* At2g32040 (henceforth At2g32040).

Folate Uptake by *E. coli* Expressing slr0642 or At2g32040—Foliates cannot normally cross the inner membrane of *E. coli* cells, preventing rescue of folate synthesis mutants by supplied folates (48). We, therefore, evaluated folate transport activity by expressing FBT constructs in a *pabA pabB* mutant, which is auxotrophic for pABA, and testing for growth on minimal medium with or without 5-CHO-THF or folic acid (the two most stable folates) in place of pABA. The mutant also harbored a plasmid bearing genes for three rare *E. coli* tRNAs (*argU*, *ileY*, *leuW*, corresponding to AT-rich codons); this plasmid was found to be needed to express At2g32040 constructs. The N-terminal extension that contains the predicted targeting peptide was removed from At2g32040 constructs, basing the truncation point (after residue 88) on alignments with slr0642 (see Supplemental Fig. 1C) and with orthologous plant sequences derived from ESTs (not shown). Because the truncated At2g32040 protein had a more hydrophilic N-terminal region than slr0642 and this region may affect membrane targeting or translation in *E. coli* (49), we also constructed a chimeric version in which residues 1–40 of the truncated protein were replaced by residues 1–37 of slr0642.

After two passages on minimal medium plus or minus folate, the growth of mutant cells harboring the empty pLOI707HE vector ceased (Fig. 2A). In contrast, cells expressing slr0642 continued to grow when given folates (Fig. 2A), showing that the latter are transported. The truncated At2g32040 did not confer the ability to grow on folates, but the chimeric version was effective (Fig. 2A). Cells expressing slr0642 or chimeric At2g32040 did not grow when 5-CHO-THF triglutamate replaced the monoglutamyl form (not shown), indicating that both

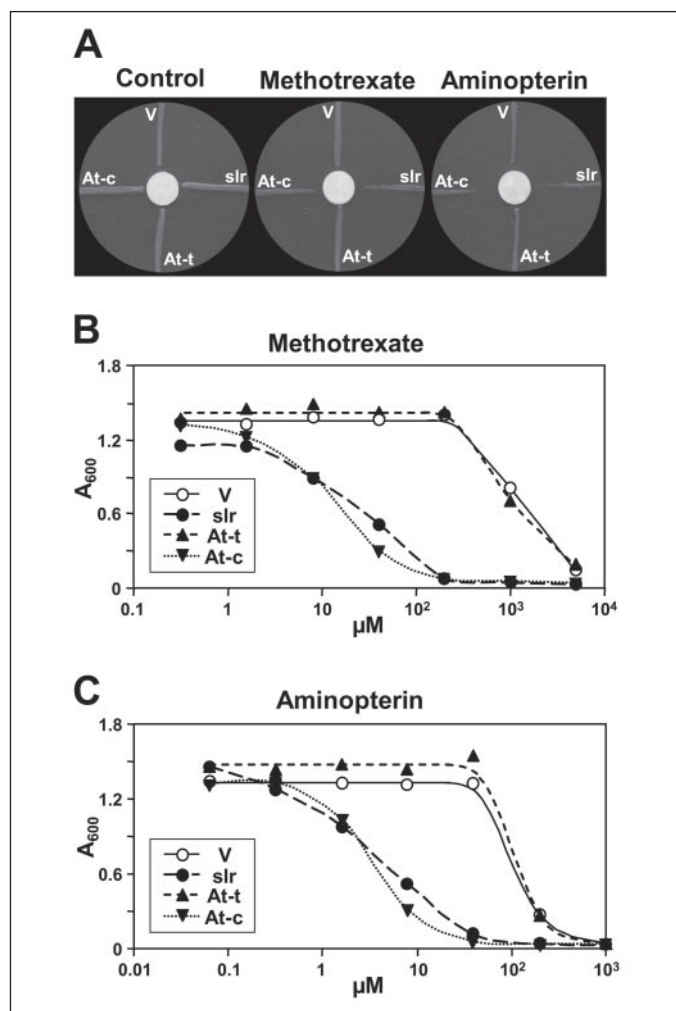


FIGURE 3. Antifolate uptake tests in *E. coli* expressing *Synechocystis* slr0642 or modified *Arabidopsis* At2g32040. Strain BN1163 harboring pACYC-RIL was transformed with pLO1707HE with no insert (V) or containing slr0642 (*slr*), the truncated At2g32040 protein (*At-t*), or the chimeric At2g32040 protein (*At-c*). **A**, clones were streaked radially on minimal medium plates containing IPTG and 3.6 μM pABA, with a central 1-cm filter paper disk impregnated with 10 μl of dimethyl sulfoxide alone (*control*) or containing 100 mM methotrexate or 80 mM aminopterin. Plates were photographed after 2 days. **B** and **C**, growth (absorbance at 600 nm) after 9.5 h in liquid minimal medium containing IPTG, 3.6 μM pABA, and various concentrations of methotrexate (**B**) or aminopterin (**C**). Data are the means of triplicate cultures; S.E. values were $\leq 9\%$ of the means in all cases.

transporters, like most other folate carriers, prefer monoglutamates (note, however, that the triglutamate approaches in size the exclusion limit of the outer membrane porins of *E. coli*, which may also have impeded uptake). Cells cured of their plasmids were not rescued by folates, confirming that the transport trait is plasmid-encoded.

Although 5-CHO-THF and folic acid are relatively stable, some spontaneous breakdown is inevitable, releasing pABA-Glu. To rule out the possibility that the growth seen on folates was due to such release, we confirmed that pABA-Glu did not support growth of mutant cells expressing slr0642 or chimeric At2g32040 (not shown). Because *E. coli* cannot normally take up pABA-Glu but can utilize it once absorbed (50), this result further shows that neither protein is able to transport pABA-Glu.

The results from the *pabA pabB* mutant were corroborated using a second mutant, a *folE* deletant, which lacks the first enzyme of the pteridine branch of folate synthesis (GTP cyclohydrolase I). This deletant could grow on rich medium supplemented with 5-CHO-THF when expressing slr0642 or chimeric At2g32040 but not when harboring the

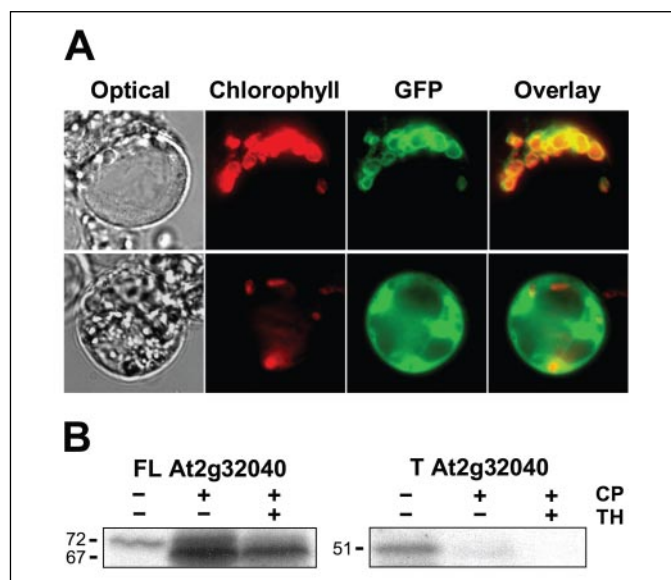


FIGURE 4. Evidence that the At2g23040 protein is targeted to chloroplasts. **A**, transient expression in *Arabidopsis* protoplasts of GFP fused to the C terminus of At2g32040 (upper panels) or GFP alone (lower panels). GFP (green pseudo-color) and chlorophyll (red pseudo-color) fluorescence were observed with an epifluorescence microscope. **B**, protein import into isolated pea chloroplasts. Full-length (FL) and truncated (T) versions of At2g32040 were translated *in vitro* in the presence of [^3H]leucine. The translation products were incubated for 15 min in the light with Mg^{2+} -ATP and chloroplasts (CP), which were then purified, or first treated with thermolysin (TH) to remove adsorbed proteins. Proteins were separated by SDS-PAGE and visualized by fluorography. Samples were loaded on the basis of equal chlorophyll content next to aliquots of the respective translation products. The molecular masses (kDa) shown in the figure were estimated from the positions of standards run on the same gel.

empty vector (Fig. 2B). The *folE* deletant could not be used to test slr0642 or At2g32040 for transport of dihydropteroate or dihydropterins (hydroxymethyl-dihydropterin, dihydropterin-6-aldehyde) as these compounds supported growth of deletant cells harboring the empty vector (not shown).

Antifolate Uptake—The antifolates aminopterin and methotrexate are close structural analogs of folic acid, differing only in one and two groups, respectively (Fig. 1A). *E. coli* normally has little capacity to take up these compounds, and they are inhibitory only at high concentrations (50, 51). Therefore, we tested antifolate transport activity by evaluating growth of cells exposed to antifolate gradients on plates (Fig. 3A) or to various levels of antifolates in liquid medium (Fig. 3, B and C). Expression of slr0642 or the chimeric At2g32040 construct imparted large increases in sensitivity that were qualitatively obvious on plates and were quantified as 30–100-fold in liquid culture. The truncated At2g32040 protein was again ineffective.

Subcellular Localization of At2g32040—This was investigated first by transient expression of an At2g32040::GFP fusion construct in *Arabidopsis* protoplasts. Consistent with the presence of a putative plastid targeting peptide, expression of the fusion protein resulted in green fluorescence that colocalized with the red autofluorescence of chlorophyll; the green fluorescence was strongest at the chloroplast margins, suggesting that the protein is concentrated in the envelope (Fig. 4A). Expression of GFP alone gave green fluorescence throughout the cytoplasm (Fig. 4A).

Import assays with isolated pea chloroplasts substantiated these results (Fig. 4B). After the import reaction with the full-length protein, chloroplasts contained a labeled product that was smaller than the full-length protein and was protected from attack by thermolysin, as expected for a translocated protein. In contrast, a truncated version lacking the predicted targeting peptide was not processed and was ther-

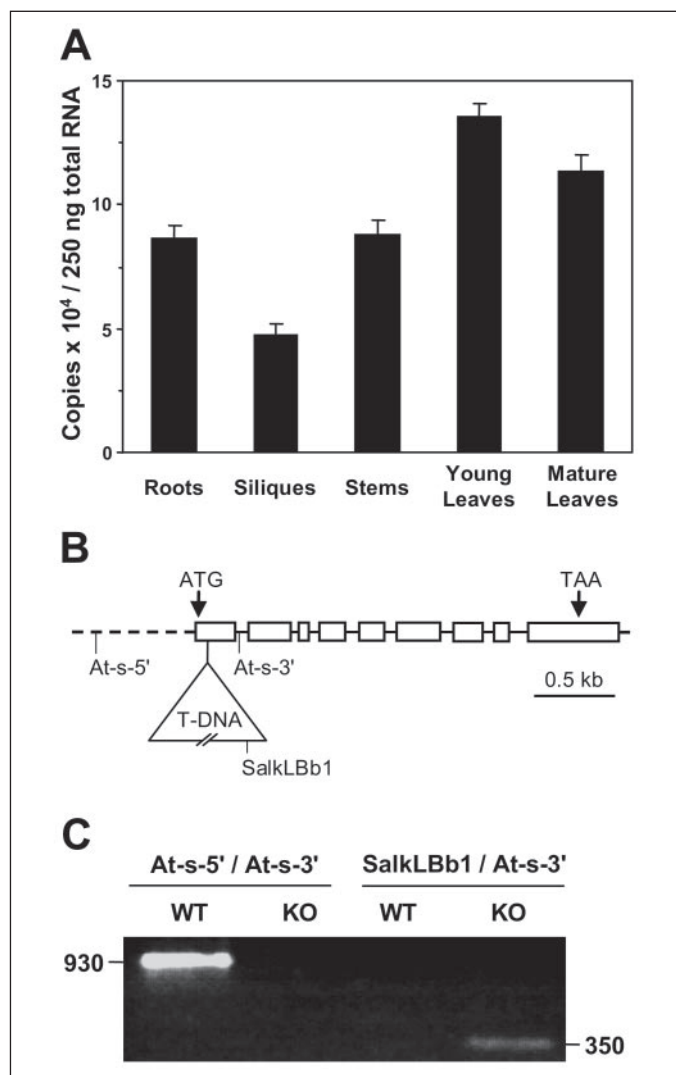


FIGURE 5. Expression of the At2g32040 mRNA in *Arabidopsis* organs and structure of the T-DNA-mutated At2g32040 gene. *A*, levels of At2g32040 mRNA in *Arabidopsis* organs, determined by real-time RT-PCR. Young leaves were harvested at 21 days, and mature leaves, stems, and siliques at 35 days. Roots were from hydroponically grown plants harvested at 35 days. Data are the means of three determinations for independent tissue samples and S.E. *B*, structure of the At2g32040 gene, with introns as solid lines and exons as boxes. The positions of the T-DNA insertion, of the start and stop codons, and of the primers used to identify wild type and mutant segregants are indicated. *C*, agarose gel analysis of the PCR products obtained from pooled genomic DNA from two wild type (WT) or three homozygous mutant plants using the primer pairs indicated. The approximate size of the products (bp) is indicated. *kb*, kilobases. *KO*, knock-out.

molysin-sensitive, indicating that it had not been taken up. The latter protein (calculated molecular mass 51 kDa) behaved in SDS-PAGE as a 51-kDa species as expected, but the full-length protein (calculated mass 61 kDa) migrated as a 72-kDa species (Fig. 4B)). Other FBT family proteins likewise migrate anomalously (52). Thus, although the migration of the imported protein as a 67-kDa band (Fig. 4B) implies that the point we chose to truncate At2g32040 is not identical to the normal processing site, electrophoretic anomalies preclude quantifying the discrepancy.

Expression of At2g32040 in *Arabidopsis* Organs—Quantitative RT-PCR analysis indicated that the At2g32040 gene is constitutively expressed throughout the plant at a low level (sequencing of the RT-PCR product obtained from root and young leaf RNA confirmed that it corresponded to At2g32040 mRNA). Thus, the abundance of At2g32040 transcripts varied rather little among major organs, from 5

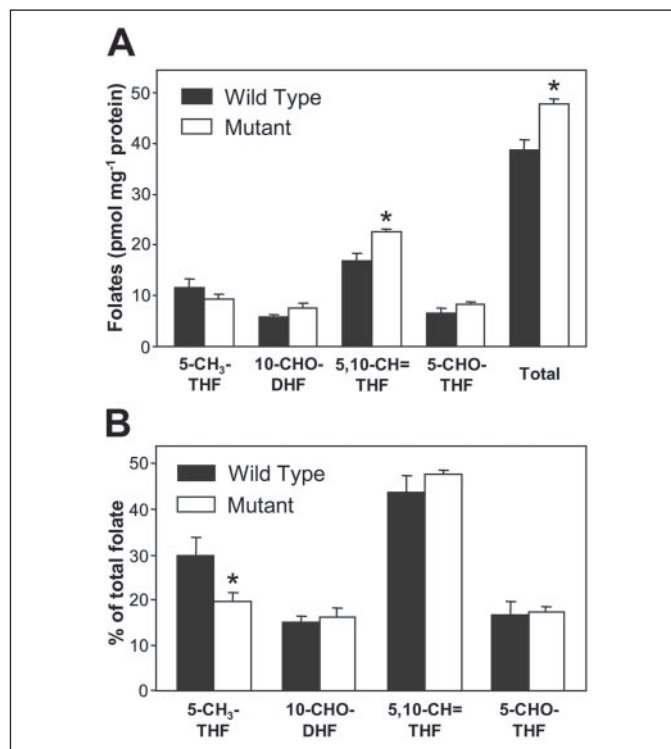


FIGURE 6. Chloroplast folate profiles of wild type and At2g32040 mutant *Arabidopsis*. Chloroplasts were purified by Percoll gradient centrifugation. Foliates were analyzed in deglutamylated samples by HPLC with electrochemical detection. Data are presented per unit protein in *A* and as a percent of total chloroplast folate in *B*. 5-CH₃-THF, 5-methyl-THF; 5-CHO-THF, 5-formyl-THF; 5,10-CH=THF, 5,10-methenyl-THF; 10-CHO-DHF, 10-formyldihydrofolate (formed during isolation from 10-formyl-THF). Data are the means and S.E. from six wild type and five mutant chloroplast preparations. Asterisks indicate differences between mutant and wild type that are significant at $p < 0.05$ by Student's *t* test; percent data were arcsine transformed before the *t* test.

to 14×10^4 copies/250 ng of total RNA (Fig. 5A), which corresponds to a frequency between 1 in 20,000 and 1 in 60,000 mRNAs (assuming that mRNA is 1% of total RNA and has an average size of 1.5 kb). Ubiquitous, low level expression is also reported for At2g32040 in the GENEVESTIGATOR *Arabidopsis* microarray data base (53). The presence of At2g32040 transcripts in roots as well as aerial organs implies that the protein is expressed in non-green plastids as well as chloroplasts.

Identification and Analysis of a T-DNA Insertion Mutant—To explore the function of At2g32040 *in vivo*, an *Arabidopsis* line bearing a T-DNA insertion in the coding region of the gene was identified in the Salk collection (40). This mutant has a single T-DNA insertion in exon 1 at codon 24 (Fig. 5, B and C), as determined from the flanking DNA sequence and co-segregation of kanamycin resistance with the T-DNA. The insertion creates at least two in-frame stop codons and, therefore, is predicted to result in a complete knock-out.

Homozygous mutant plants did not differ noticeably from their wild type siblings in growth rate, morphology, leaf color, or fertility. Because At2g32040 is located in the plastid (Fig. 4), we compared the folate profiles of chloroplasts from wild type and mutant plants (Fig. 6). As in pea (15–17), total folate levels in wild type chloroplasts were low, about 50 pmol/mg of protein or 150 pmol/mg of chlorophyll, which is equivalent to a stromal concentration of $\sim 2 \mu\text{M}$ (taking stromal volume as 66 μl /mg chlorophyll (54)). The most prominent folate forms were 5-methyl- and 5,10-methenyl-THF (the acidic HPLC mobile phase used in the analysis converts 10-formyl-THF to 5,10-methenyl-THF, so that the 5,10-methenyl-THF measured includes 10-formyl-THF plus any preexisting 5,10-methenyl-THF). Relative to wild type, mutant chloroplasts contained significantly ($p < 0.05$) more 5,10-methenyl-THF and

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total folate per unit protein (Fig. 6A) and, when data were expressed on a percent basis, a significantly lower proportion of 5-methyl-THF (Fig. 6B).

DISCUSSION

We report here the functional expression of an FBT family protein in *E. coli*, an advance that should help studies of transport mechanisms. Expressing the *Synechocystis* slr0642 protein in *E. coli* was straightforward, but success with *Arabidopsis* At2g32040 required the presence of a plasmid bearing rare AT-rich codons and modification of the protein. Deleting its N-terminal extension did not suffice; it was necessary also to replace the N terminus of the truncated protein with the corresponding sequence from slr0642, a region that may affect membrane insertion or translation in *E. coli*. This modification altered only the initial part (the first third) of the predicted transmembrane helix number 1, which part is not involved in substrate binding in other major facilitator superfamily proteins (55, 56).

As shown in Supplemental Fig. 1C, there is a remarkably high sequence conservation between the cyanobacterial carrier and At2g32040 that extends over the entire protein except the region between transmembrane helices 6 and 7, a large loop that separates the two halves of the molecule. The residues that are conserved both in these proteins and the *Leishmania* FT1, FT5, and BT1 carriers are likewise spread throughout the sequence except for helices 6 and 7 and the region between them (see Supplemental Fig. 1C). In this interstitial region the *Leishmania* sequences are predicted to have two additional transmembrane α -helices, an uncommon configuration in the major facilitator superfamily (57).

Folate transport was evaluated via bacterial growth, which is a sensitive way to detect transport activity but not to assess its rate or substrate specificity. For example, growth on 5-CHO-THF or folic acid lagged behind that on *pABA*, but this does not necessarily imply that the uptake rate of either folate limited growth. Both 5-CHO-THF and folic acid need enzymatic conversion to metabolically active forms (5,10-methenyl-THF and THF, respectively) before use (1, 58) so conversion rates could have been limiting. Moreover, 5-CHO-THF inhibits folate-dependent enzymes (58) and may have slowed growth on this account. Respecting substrate specificity, growth tests could not show whether or not slr0642 and At2g32040 act on dihydropteridines because *E. coli* cells took such compounds up unaided. These proteins, thus, remain unconfirmed candidates for the pteridine transport activities observed or inferred in Cyanobacteria and chloroplasts (59–61).

Although *E. coli* is suspected to have a cryptic gene (*abgT*), not normally expressed, that can allow transport of folate analogs and perhaps folates (51), three arguments exclude activation and selection of this or any other *E. coli* gene as an explanation for our data. First, curing transport-proficient cells of the expression plasmid abolished folate transport capacity. Second, the antifolate uptake results of Fig. 3 could not have involved selection because transport-proficient cells were ipso facto unable to divide. Third, when cells harboring empty expression vector underwent prolonged incubation on folate media, the growth of colonies was far too infrequent to compromise the folate uptake experiments of Fig. 2, where all cells expressing an active FBT carrier grew.

The subcellular localization and expression pattern of At2g32040 imply that it has a housekeeping function in green and non-green plastids alike. That this function is folate transport is supported by the finding that ablating At2g32040 caused a 23% rise in chloroplast total folate and a 34% fall in the proportion of 5-methyl-THF. That these changes were not more marked implies that there are other chloroplast

folate transporters, and there is indeed recent evidence for a second one in *Arabidopsis* (62).

Because At2g32040 acts on 5-CHO-THF, folic acid, and antifolates, it is clearly not highly specific and so can presumably transport the monoglutamyl form of any folate found in plastids. In this connection it is noteworthy that plastid folate transport fluxes include not only net import but also an exchange with the cytosol driven by methionine synthesis. Plastids synthesize homocysteine and contain an isoform of methionine synthase, which converts homocysteine to methionine using 5-methyl-THF as methyl donor (63). However, plastids cannot make 5-methyl-THF due to lack of the enzyme that produces it from 5,10-methylene-THF and so must import it from the cytosol (64) in exchange (not necessarily via the same carrier) for other folates in a shuttle reaction. Because stoichiometric, not catalytic, amounts of 5-methyl-THF are needed for methionine synthesis, this methyl group shuttle must greatly exceed the net import of folates into plastids.

Finally, this work establishes conservation of folate transport function among FBT family proteins from species representing three kingdoms of life, monera (*Synechocystis*), plants (*Arabidopsis*), and protists (*Leishmania*). Given the evidence for lateral transfer of genes from Cyanobacteria to plants (47) and from Cyanobacteria or plants to trypanosomatids and apicomplexans (45, 46), it seems likely that the FBT family arose in Cyanobacteria and passed to the other groups, the evolutionary driver being the selective advantage conferred by folate transport. Whatever the case, these folate carriers that function across kingdom boundaries may offer new options for engineering folate production in bacteria and plants (1, 65). Such carriers might be used, for example, to enhance folate export from bacteria or folate sequestration in storage compartments in plant cells.

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