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# Folate Metabolism in Plants

## AN ARABIDOPSIS HOMOLOG OF THE MAMMALIAN MITOCHONDRIAL FOLATE TRANSPORTER MEDIATES FOLATE IMPORT INTO CHLOROPLASTS\*

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The distribution of folates in plant cells suggests a complex traffic of the vitamin between the organelles and the cytosol. The *Arabidopsis thaliana* protein AtFOLT1 encoded by the *At5g66380* gene is the closest homolog of the mitochondrial folate transporters (MFTs) characterized in mammalian cells. AtFOLT1 belongs to the mitochondrial carrier family, but GFP-tagging experiments and Western blot analyses indicated that it is targeted to the envelope of chloroplasts. By using the glycine auxotroph Chinese hamster ovary *glyB* cell line, which lacks a functional MFT and is deficient in folates transport into mitochondria, we showed by complementation that AtFOLT1 functions as a folate transporter in a hamster background. Indeed, stable transfectants bearing the *AtFOLT1* cDNA have enhanced levels of folates in mitochondria and can support growth in glycine-free medium. Also, the expression of AtFOLT1 in *Escherichia coli* allows bacterial cells to uptake exogenous folate. Disruption of the *AtFOLT1* gene in *Arabidopsis* does not lead to phenotypic alterations in folate-sufficient or folate-deficient plants. Also, the *atfolt1* null mutant contains wild-type levels of folates in chloroplasts and preserves the enzymatic capacity to catalyze folate-dependent reactions in this subcellular compartment. These findings suggest strongly that, despite many common features shared by chloroplasts and mitochondria from mammals regarding folate metabolism, the folate import mechanisms in these organelles are not equivalent: folate uptake by mammalian mitochondria is mediated by a unique transporter, whereas there are alternative routes for folate import into chloroplasts.

Tetrahydrofolate (THF)<sup>5</sup> and its one-carbon-substituted derivatives (collectively termed folates) are involved in key metabolic functions, including the synthesis of methionine, pantothenate, purines, and thymidylate (1). Plants and most microorganisms can synthesize THF *de*

*nov*, whereas mammals cannot and so require a dietary supply of this soluble vitamin. The plant THF biosynthesis has a unique and complex subcellular compartmentation split between three compartments (Fig. 1) (2). Folates are tripartite molecules comprising a pterin moiety, a *p*-aminobenzoate unit, and a  $\gamma$ -linked glutamate chain with one to eight residues. *p*-Aminobenzoate is formed from chorismate in plastids (3, 4), the pterin moiety from GTP in the cytosol (5, 6), and the two are coupled together, glutamylated and reduced in the mitochondria (7–9). The enzyme folylpolyglutamate synthetase that elongates the glutamate chain exists in the cytosol, mitochondria, and plastids (9). This distribution suggests a complex traffic of THF, most probably in a monoglutamate form, and its precursors between the organelles and the cytosol (Fig. 1). In addition to intracellular transports, *in vivo* studies in *Arabidopsis* (10, 11) have shown that plants are able to take up C1 derivatives of THF from the medium (Fig. 1). None of the proteins involved in these multiple transport steps has been characterized so far in plants.

In folate-auxotroph organisms, transport systems involved in the cellular uptake of folates have been cloned and characterized. The reduced folate carriers and the folate receptors mediate separate routes for folates uptake into mammalian cells (for a review, see Ref. 12). In the parasitic protozoa *Leishmania*, the folate-biopterin transporter family includes high affinity folate transporters and a biopterin/folate transporter that are involved in the uptake of folates and the antifolate drug methotrexate (MTX) (13–15). Following their uptake, folates are either converted to polyglutamates to promote their retention in the cytosol or transported to the organelles where they are polyglutamylated to minimize their diffusion (16). The mitochondrial folate transporter (MFT) involved in the traffic of folates from the cytosol to the mitochondria has been cloned and functionally characterized in mammals (17, 18). A point mutation within this protein rendered mitochondria unable to accumulate folates and cells that are auxotrophic for glycine (17, 18). In animals, two multidrug resistance-associated proteins belonging to the ATP-binding cassette transporter superfamily are responsible for antifolate detoxification (19, 20). These transporters catalyze a high capacity and low affinity transport of MTX and physiological folates, as well as a moderate-affinity and low capacity transport of several glutathione and glucuronate conjugates (19, 20). Recently, the plasma membrane ATP-binding cassette transporter AtMRP4 from *Arabidopsis* has been implicated in the regulation of light-induced stomatal opening (21). MTX, but not folates, was found to inhibit this process. The physiological significance of folates efflux across the plasma membrane and the link between this transport and guard cell regulation are still unclear.

In the present work, we used a genomics-based approach to identify an *Arabidopsis* protein, called AtFOLT1, which is a homolog of the mammalian MFTs. Using GFP-tagging and Western blot analyses we

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<sup>5</sup> The abbreviations used are: THF, tetrahydrofolate; MFT, mitochondrial folate transporter; MTX, methotrexate; GFP, green fluorescent protein; IPTG, isopropyl 1-thio- $\beta$ -D-galactopyranoside; T-DNA, transferred DNA; RT, reverse transcription; HsMFT, human MFT; CHO, Chinese hamster ovary.

## Chloroplastic Folate Transporter

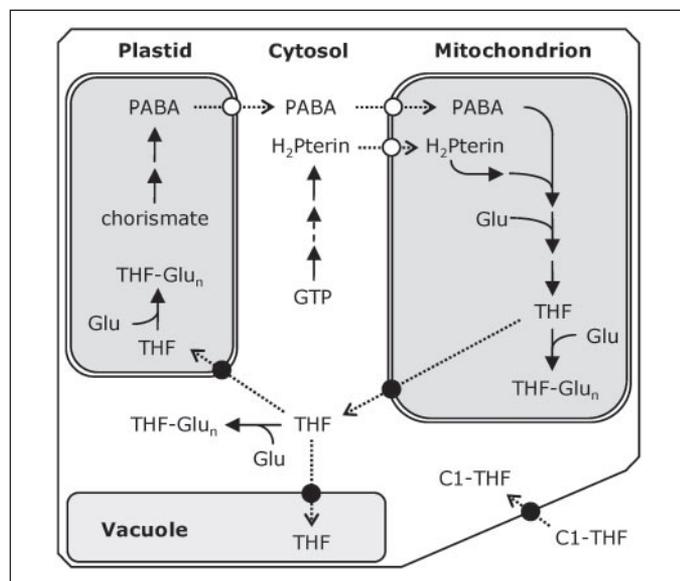


FIGURE 1. Subcellular compartmentation of THF synthesis in plant cells. Enzymes involved in THF synthesis are indicated by solid arrows, and the probable transport steps for THF and precursors are indicated by dotted arrows. PABA, para-aminobenzoate;  $H_2$ Pterin, hydromethylidihydropterin; C1-THF, C1 derivative of THF.

showed that the AtFOLT1 protein is localized in the chloroplast envelope. To address whether AtFOLT1 was able to transport folates, Chinese hamster ovary *glyB* cells lacking a functional MFT were stably transfected with the AtFOLT1 cDNA. The AtFOLT1 protein could partially restore the mitochondrial pool of folates if expressed at sufficiently high levels and thus could complement the glycine auxotrophy demonstrated by *glyB* cells. Also, the expression of AtFOLT1 in *Escherichia coli* allows bacterial cells to uptake exogenous folic acid. These data indicate that AtFOLT1 functions as a folate transporter in two different heterologous expression systems. Disruption of the AtFOLT1 gene in *Arabidopsis* does not lead to any obvious phenotype when plants are grown under standard or folate-depleting conditions, and chloroplasts contain wild-type levels of folates and preserve their enzymatic capacity to catalyze THF-dependent reactions. These findings suggest strongly that, contrary to the unique MFT-mediated folate uptake system existing in mitochondria from mammals, there are alternative routes for import of folates into chloroplasts.

### EXPERIMENTAL PROCEDURES

**Plant Growth Conditions**—Wild-type and mutant *Arabidopsis thaliana* (ecotype Columbia, Col-0) plants were grown on soil in a growth chamber (22 °C, 60% air humidity, light intensity of 150  $\mu E m^{-2} s^{-1}$ , 16 h light/8 h dark). Seeds of the T-DNA insertion line SALK\_005280 generated at the Salk Institute Genome Analysis Laboratory (22) were obtained from the Arabidopsis Biological Resource Center. For selection of transformed plants and growth studies in controlled conditions, seeds were surface-sterilized and plated on the agar-solidified medium described in Ref. 23 containing either kanamycin (50  $\mu g/ml$ ) or folate analogs (sulfanilamide or MTX,  $10^{-9}$  to  $2 \times 10^{-4}$  M). Plates were kept at 4 °C in the dark for 4 days before being transferred to the growth chamber. *A. thaliana* (ecotype Columbia) cell suspension cultures were grown under continuous white light (40  $\mu E m^{-2} s^{-1}$ ) at 23 °C in Gamborg's B5 medium supplemented with 1  $\mu M$  2-naphthalene acetic acid and 1.5% (w/v) sucrose.

**cDNAs Cloning**—The full-length cDNAs coding At5g66380 (AtFOLT1), At2g47490, and At1g25380 were obtained by PCR using a cDNA amplification library prepared from above-ground parts of

3-week-old *A. thaliana* (ecotype Wassilewskija) plants (9). PCR was done with the proofreading *Pfu* DNA polymerase (Promega) using the following primers: FT1-1, AGGATTCGTTGATGGCGGCG; FT1-2, GTGACAAGTCAACCGCGTGC; At2g47490-1, GGTGACGCGTTTCCTCAAGG; At2g47490-2, CCGGATTTAAAGTATAGAGCTTTG; At1g25380-1, CCCCCAATTGAGAGATTCTAGG; and At1g25380-2, ACGTAGTTGGAACATGGCATCG. The PCR products were subcloned into the pBluescriptII KS vector digested with SmaI and sequenced (GenomeExpress, Meylan, France).

**Localization of AtFOLT1:GFP**—Plasmid p35 $\Omega$ -sGFP(S65T) expressing an engineered version of the green fluorescent protein (GFP) under the control of the cauliflower mosaic virus 35S promoter (24) was used for transient expression experiments in *Arabidopsis* protoplasts. The full-length AtFOLT1 sequence was amplified from pKS-AtFOLT1 by high fidelity PCR using *Pfu* DNA polymerase and primers FT1-GFP5' (GTCGACTGATGGCGCGTCTGTGGC) and FT1-GFP3' (CCATGGAATCTTTTGTGTTGGATGCTG). The resulting fragment was digested with Sall and NcoI and inserted between the corresponding sites of p35 $\Omega$ -sGFP(S65T). The resulting plasmid pGFP-AtFOLT1 and p35 $\Omega$ -sGFP(S65T) were used to transform protoplasts prepared from a 4-day-old *Arabidopsis* cell suspension culture essentially as described in Ref. 25. GFP chimera bearing the transit peptide sequences of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Arabidopsis* and dihydropterin pyrophosphokinase/dihydropteroyl synthase from *Pisum sativum* were used as controls for the targeting of GFP to plastids and mitochondria, respectively (9). Samples were analyzed by confocal laser scanning microscopy using a Leica TCS-SP2 operating system as described (9).

**CaPO<sub>4</sub>-mediated Transfection of AtFOLT1 into *glyB* Cells**—The full-length AtFOLT1 sequence was excised from pKS-AtFOLT1 with XbaI and HindIII and subcloned into the same sites of pcDNA3.1+ (Invitrogen). The resulting plasmid pcDNA-AtFOLT1 allows the expression of AtFOLT1 under the control of the human cytomegalovirus immediate-early promoter. CHO *glyB* cells maintained in  $\alpha$  minimal essential medium (Invitrogen) were plated at a density of  $2 \times 10^5$  cells/100-mm plate. The following day, plasmid (5  $\mu g/plate$ ) was mixed with 1 M CaCl<sub>2</sub> and HEB5 (19.2 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.45 mM Na<sub>2</sub>PO<sub>4</sub>, and 10 mM dextrose) in a total volume of 1 ml/plate and added to the cells for 30 min at room temperature. Nine milliliters of media was added to each plate, and the cultures were incubated at 37 °C. Twenty-four h later, the cells were briefly shocked with Me<sub>2</sub>SO, the medium was changed, and the cells were allowed to recover for 48 h. Transfectants were selected in  $\alpha$  minimal essential medium supplemented with 10% dialyzed fetal calf serum and 1 mg/ml of G418 with or without glycine in the medium. Cells were grown for 10 days and colonies were fixed, stained, and counted.

**Subcellular Distribution of Folates in *glyB* Transformants**—Several colonies of *glyB* cells stably transfected with the AtFOLT1 construct were selected and expanded into cultures. Cultures were seeded at  $6 \times 10^6/175$  cm<sup>2</sup> in  $\alpha$  minimal essential medium supplemented with dialyzed fetal calf serum and 0.3  $\mu Ci/ml$  [<sup>3</sup>H]folic acid (Moravek) for 48 h. Cells were then harvested by trypsinization and pelleted. The pellets were resuspended in 10 ml of phosphate-buffered saline (5% fetal calf serum), counted, and re-pelleted. The cells were homogenized in 0.25 M sucrose and 1 mM EDTA using a hand-held Dounce homogenizer, and the various subcellular fractions (nuclei and unbroken cells, mitochondria, and cytosol) were isolated by centrifugation as described previously (17). Radioactivity in the various fractions was determined by scintillation counting.

**Expression of AtFOLT1 in *E. coli* JM105 Cells**—The AtFOLT1 coding sequence lacking the first six residues located before the first predicted transmembrane  $\alpha$ -helix was amplified from pcDNA-AtFOLT1 by high fidelity PCR using *Pfu* DNA polymerase and primers FT1-pTrc5' (GGAATTCCATATGGAAAATGCCACCGCCGGC) and FT1-pTrc3' (ACGAGTCTCTAATCTTTTGTGTGGATGC). The resulting fragment was digested with EcoRI and SacI, and inserted between the corresponding sites of pTrc99A (Amersham Biosciences). The resulting plasmid pTrc-AtFOLT1 was introduced first in *E. coli* DH5 $\alpha$  strain, the sequence was verified, and then the plasmid was added into *E. coli* JM105 strain. Independent transformants harboring pTrc-AtFOLT1 or the empty pTrc99A vector were grown in LB containing 100  $\mu$ g/ml carbenicillin until  $A_{600}$  reached 0.3. Cells were collected by centrifugation, washed three times in 0.9% (w/v) NaCl, and diluted to  $A_{600}$  = 0.0015 in M9 minimal medium (26) containing 0.4% (w/v) glucose, 10  $\mu$ g/ml thiamine, 100  $\mu$ g/ml carbenicillin, and 30  $\mu$ M asulam. Folic acid (50  $\mu$ M) and/or IPTG (1 mM) were added to this medium, and cells were allowed to grow at 37 °C. Bacterial growth was monitored by measuring  $A_{600}$ . To analyze the expression of AtFOLT1 in JM105 cells, pTrc-AtFOLT1 and pTrc99A transformants were grown in LB medium until  $A_{600}$  was 0.6, at which point 1 mM IPTG was added. Incubation was continued at 37 °C, and cells were collected by centrifugation at different time intervals and then suspended in SDS-PAGE loading buffer. Total proteins from  $\sim 2 \times 10^8$  cells were separated by SDS-PAGE and analyzed by Western blot using purified AtFOLT1 antibodies.

**Nucleic Acid Manipulations**—For PCR analysis *Arabidopsis* genomic DNA was isolated as described in Ref. 27 and amplified using the Titanium<sup>TM</sup> TaqDNA polymerase (Clontech) and primer pairs specific for the AtFOLT1 gene (FT1-2 and FT1-5, CGACCTAGTACCAACGGAATCC) or the T-DNA insertion (RB1, GCTCATGATCAGATTGTCGTTTCCCGCCTT and LB1, GGCAATCAGCTGTTGCCGCTCTACTGGTG). Amplicons were separated by 1.4% (w/v) agarose gel electrophoresis, purified, and sequenced. For Southern analysis, genomic DNA was digested, separated by 0.7% (w/v) agarose gel, and transferred to a Hybond-N<sup>+</sup> membrane (Amersham Biosciences). Hybridization was at 42 °C in 6 $\times$  SSC, 5 $\times$  Denhardt's solution, 50% (v/v) formamide, 0.5% (w/v) SDS, and 100  $\mu$ g/ml herring sperm DNA. The 4.9-kb BglII fragment of the pROK2 vector corresponding to the entire T-DNA region (28) and the full-length AtFOLT1 cDNA were labeled with <sup>32</sup>P using random primers and used as probes.

For RT-PCR analysis, total RNA was isolated from leaves by using the RNeasy plant mini extraction kit (Qiagen). RNA (3  $\mu$ g) was treated with DNase I, and first strand cDNA was synthesized with the ThermoScript RT-PCR system (Invitrogen). AtFOLT1 and *actin2* (At5g09810) were amplified by PCR with the Titanium<sup>TM</sup> TaqDNA polymerase using specific primers: FT1-1 and FT1-11, CTGCCTCTAGCGTACCTCTGC; At5g09810-Fwd, ACATCGTTCTCAGTGGTGGTTC; and At5g09810-Rev, ACCTGACTCATCGTACTCACTC. Amplicons were then analyzed by 1.4% (w/v) agarose gel electrophoresis.

**Folates Measurements in Leaves and Chloroplasts**—Folates were determined using the microbiological assay with *Lactobacillus casei* ATCC7469 (American Type Culture Collection) as described in Ref. 29. Leaves were harvested at different stages of development and ground to a fine powder in liquid nitrogen before extraction of folates. Chloroplasts from 3-week-old *Arabidopsis* rosettes were purified on Percoll gradients as described in Ref. 30. Intact chloroplasts were submitted to three freeze/thaw cycles to ensure complete lysis, and membranes were removed by centrifugation at 16,000  $\times$  g for 30 min at 4 °C. The supernatant was used for folates and protein measurements (31).

**Protein Extraction and Immunoblot Analysis**—Soluble proteins from *Arabidopsis* leaves were extracted by grinding powdered samples in 50 mM Tris-HCl, pH 7.5, 10 mM  $\beta$ -mercaptoethanol, 5% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM aminocaproic acid. Samples were centrifuged at 130,000  $\times$  g for 20 min at 4 °C, and the supernatant was used as a source of soluble proteins. Total proteins were extracted in the above buffer containing 1% (w/v) SDS, incubated at 4 °C for 30 min, and centrifuged at 16,000  $\times$  g for 20 min at 4 °C. The supernatant was used as a source of total proteins. Intact Percoll-purified chloroplasts were lysed in the hypotonic protein extraction buffer described above and plastidial subfractions (envelope, thylakoids, and stroma) were separated by ultracentrifugation on a step gradient of sucrose as described in Ref. 32.

Proteins were resolved by SDS-PAGE and electroblotted to nitrocellulose membrane (BA85, Schleicher & Schuell). The blots were probed with primary antibodies raised against AtFOLT1, IEP45 (2-oxoglutarate/malate translocator, At5g12860 (37)), light-harvesting complex proteins (P16 protein from *Chlamydomonas reinhardtii* (32)), chloroplastic folylpolyglutamate synthetase (9), or methionine synthase (33). The synthetic AtFOLT1 peptide RAKQRYARGRDDEK (residues 90–103) coupled to the carrier protein KLH was used to raise a rabbit polyclonal serum (Neosystem SA, Strasbourg, France). IgG were purified from the serum using a HiTrap NHS-activated HP column according to the manufacturer's instructions (Amersham Biosciences). Purified antibodies were further desalted in phosphate-buffered saline and concentrated using a Microsep 3K unit. Primary antibodies were detected by using a chemiluminescent detection system (ECL, Amersham Biosciences).

## RESULTS

**Cloning of Mammalian MFT Homologs in *Arabidopsis***—We searched the *Arabidopsis* genome for genes encoding putative homologs of the human mitochondrial folate transporter (HsMFT) and found that all the candidates were hypothetical members of the mitochondrial carrier family. *In silico* analysis of the complete *Arabidopsis* genome has revealed 57 members in this family, of which only one-fourth have been functionally characterized (34). Among the best hits of our BLASTP search, the product of the At5g66380 gene shared the highest sequence identity with the human and zebrafish MFTs (40.9 and 36.8%, respectively) (Fig. 2). The corresponding cDNA (named AtFOLT1 for *A. thaliana* FOLate Transporter 1) was cloned by PCR using reverse-transcribed mRNAs from the Wassilewskija ecotype. Sequence analysis revealed nine nucleotide changes between the AtFOLT1 and At5g66380 cDNA (ecotype Columbia, GenBank<sup>TM</sup> accession number BT010139). Among these changes, only one leads to an altered amino acid at position 26 (Pro for AtFOLT1, Ser for At5g66380). It is unlikely that this point mutation affects the AtFOLT1 function because: (i) the full-length *Arabidopsis* (ecotype Columbia) cDNA sequenced by the CERES company (CERES:118596) also encodes for a Pro at position 26; (ii) a Pro residue is present at the same position on the human and zebrafish MFTs; and (iii) this Pro residue is included in one of the energy signature motifs characteristic of the mitochondrial carrier family (Fig. 2B), in which it is involved in the three-dimensional organization of the odd-numbered transmembrane helices (35). The cDNAs coding the At2g47490 and At1g25380 proteins, which share 31–36% identity with HsMFT (Fig. 2), were also cloned by PCR. These cDNAs showed two and one polymorphic sites, respectively, compared with the corresponding sequences deposited in the Arabidopsis Information Resource data base (www.arabidopsis.org). Of these SNP, two resulted in silent substitutions for the At2g47490 cDNA, and one in a

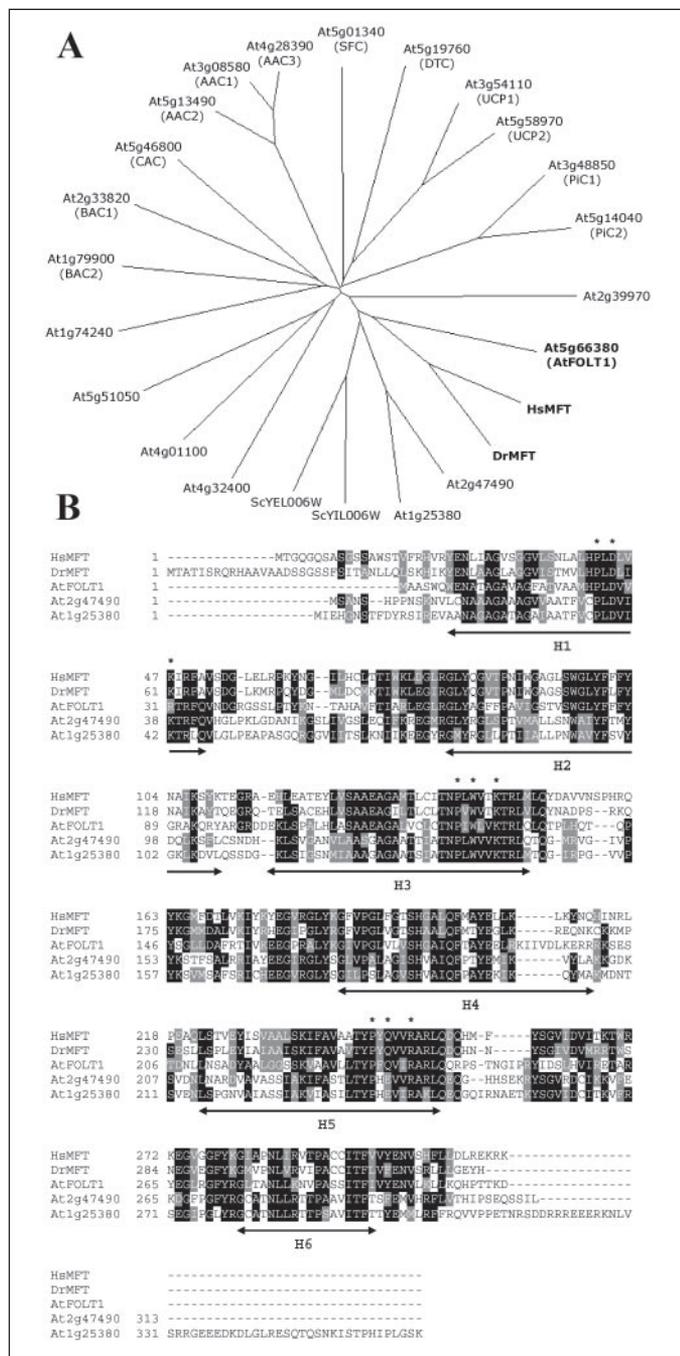


FIGURE 2. Genomic-based identification of MFT homologs in *Arabidopsis*. *A*, phylogenetic tree constructed using a multiple sequence alignment with ClustalW. The following members of the Mitochondrial Carrier family have been included in the analysis: functionally assigned MFTs from human (HsMFT, GenBank™ accession number AF283645) and zebrafish (DrMFT, BC048057); two MFT homologs found in the *Saccharomyces cerevisiae* genome (ScYEL006W, NP\_010910; ScYIL006W, NP\_012260 (17)); the eight best hits from our BLASTP search (AGI numbers); and 12 *Arabidopsis* mitochondrial carriers for which the function has been characterized (AGI numbers plus function in parenthesis; AAC, ADP/ATP carrier; BAC, basic amino acid carrier; CAC, carnitine/acylcarnitine carrier; DTC, di/tricarboxylate carrier; PIC, phosphate carrier; SFC, succinate/fumarate carrier; UCP, uncoupling protein; for a recent review, see Ref. 34). *B*, sequence comparison of eukaryotic MFTs and their *Arabidopsis* homologs. Protein sequences were aligned using ClustalW. The six putative transmembrane domains ( $\alpha$ -helices H1–H6) have been predicted from a multiple sequence alignment including the bovine mitochondrial ATP/ADP carrier for which the three-dimensional structure has been solved (35). According to these structural data, *odd-numbered helices* are kinked by the presence of proline residues located in the energy signature motifs characteristic of mitochondrial carriers (asterisks). Also, the connections within pairs of *odd- and even-numbered helices* are oriented toward the matrix of mitochondria.

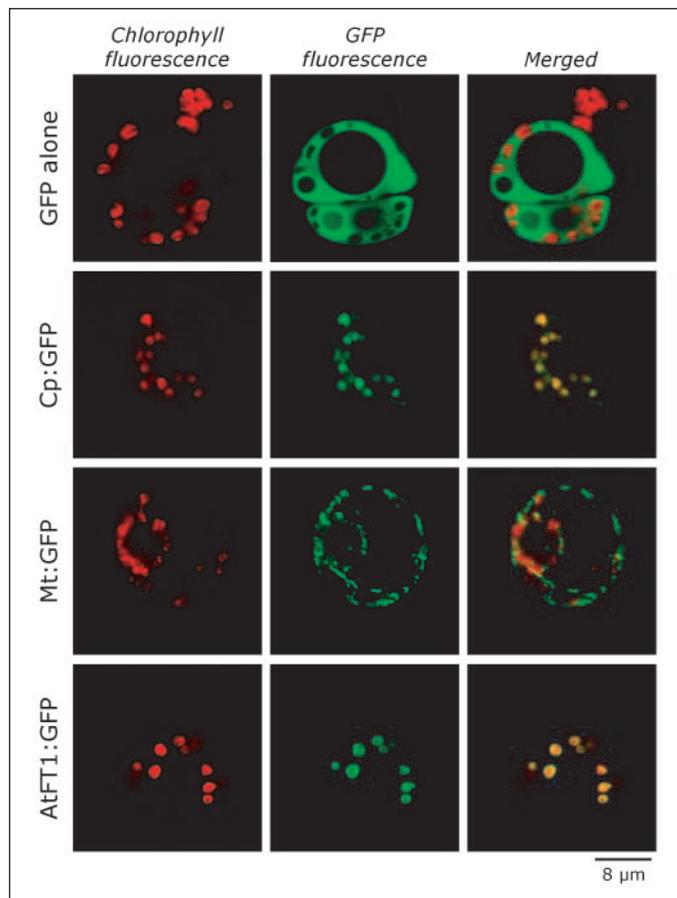
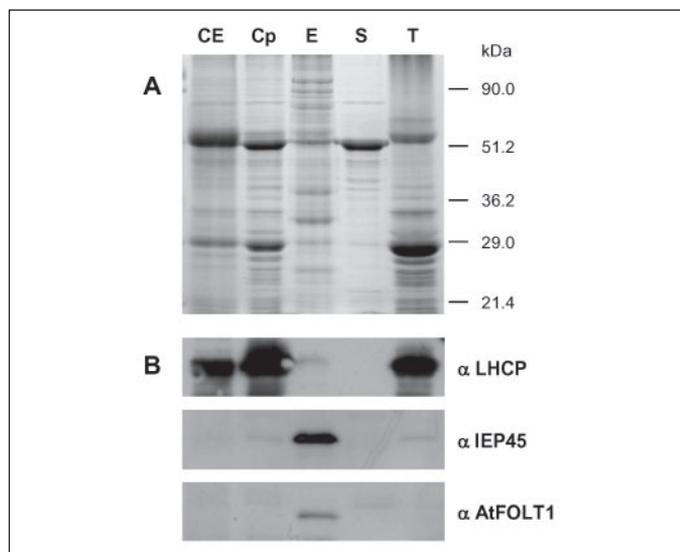


FIGURE 3. **AtFOLT1:GFP is targeted to chloroplasts.** Transient expression in *Arabidopsis* protoplasts of GFP fused in-frame with the full-length AtFOLT1 protein. GFP alone and chimera between GFP and the transit peptides of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*Cp:GFP*) or dihydropterin pyrophosphokinase/dihydropterate synthase (*Mt:GFP*) were used as controls for the targeting of the reporter protein to the cytosol, plastids, and mitochondria, respectively (9). The different constructs were introduced into *Arabidopsis* protoplasts as described in Ref. 25. The localization of green and red fluorescence signals, indicating GFP and chloroplasts, respectively, was examined by confocal microscopy.

change from a Leu (position 84, Wassilewskija sequence) to Ser (Col-0 sequence) in the At1g25380 protein.

AtFOLT1 is a 34-kDa protein that exhibits a hydrophobic profile with six putative transmembrane domains, a tripartite structure made up of related tandem domains of about 100 residues in length, and sequence motifs that are characteristic of the mitochondrial carrier family (34). Sequence homology between AtFOLT1 and HsMFT extends throughout the entire proteins and several highly conserved blocks are preserved (Fig. 2*B*). They particularly correspond to the six putative transmembrane  $\alpha$ -helices and the three domains located within pairs of odd- and even-numbered helices, which are predicted to extrude into the matrix side of the membrane (18, 35).

**Subcellular Localization of AtFOLT1**—Not all of the members of the plant mitochondrial carrier family are actually targeted to mitochondria because the plastid envelope membrane harbors several transporters of this family (for a review see Ref. 36). Analyses of the AtFOLT1 sequence using different programs failed to predict a subcellular location for this protein with a high probability. However, the prediction results from the ChloroP version 1.1 program (chloroplastic transit peptide: score, 0.505; length, 11 residues (37)) and the identification of At5g66380 during the *in silico* analysis of the *Arabidopsis* genome using features typical for the chloroplast envelope integral membrane proteins (38) suggested that AtFOLT1 is a chloroplastic protein.



**FIGURE 4. AtFOLT1 is located in the envelope of chloroplasts.** Total proteins were prepared from *Arabidopsis* leaves (crude extract, CE) and purified chloroplasts (Cp) as described under "Experimental Procedures." Chloroplasts were also fractionated on sucrose gradients to obtain the envelope (E), stroma (S), and thylakoid (T) subfractions. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes and probed with different antibodies (B). Polyclonal antibodies against IEP45 (envelope marker (38)) and light-harvesting complex proteins (thylakoid marker) were used to analyze cross-contaminations between membrane subfractions. The polypeptide detected with the anti-AtFOLT1 purified antibodies was  $33 \pm 1$  kDa. Each fraction contained 15  $\mu$ g of proteins.

To analyze the subcellular localization of AtFOLT1 we transfected *Arabidopsis* protoplasts with a plasmid encoding a fusion between the full-length AtFOLT1 protein and GFP. Protoplasts expressing GFP alone typically showed green fluorescence throughout the cytosol and nucleoplasm (Fig. 3). In contrast, expression of AtFOLT1:GFP resulted in a punctuate pattern of green fluorescence that colocalized with the red autofluorescence of chlorophyll and that was similar to the one observed for the Cp:GFP plastidial control (Fig. 3). A thorough survey of protoplasts expressing the AtFOLT1:GFP fusion never revealed a fluorescence pattern similar to the one detected for the Mt:GFP mitochondrial control, thus excluding *in vivo* targeting of AtFOLT1:GFP to mitochondria. These data demonstrate that AtFOLT1 is efficiently targeted to chloroplasts, even though it does not exhibit a canonical transit peptide sequence. In addition, the green fluorescence of the AtFOLT1:GFP fusion seems to be brighter at the chloroplast periphery, thus suggesting a localization in the chloroplast envelope (Fig. 3).

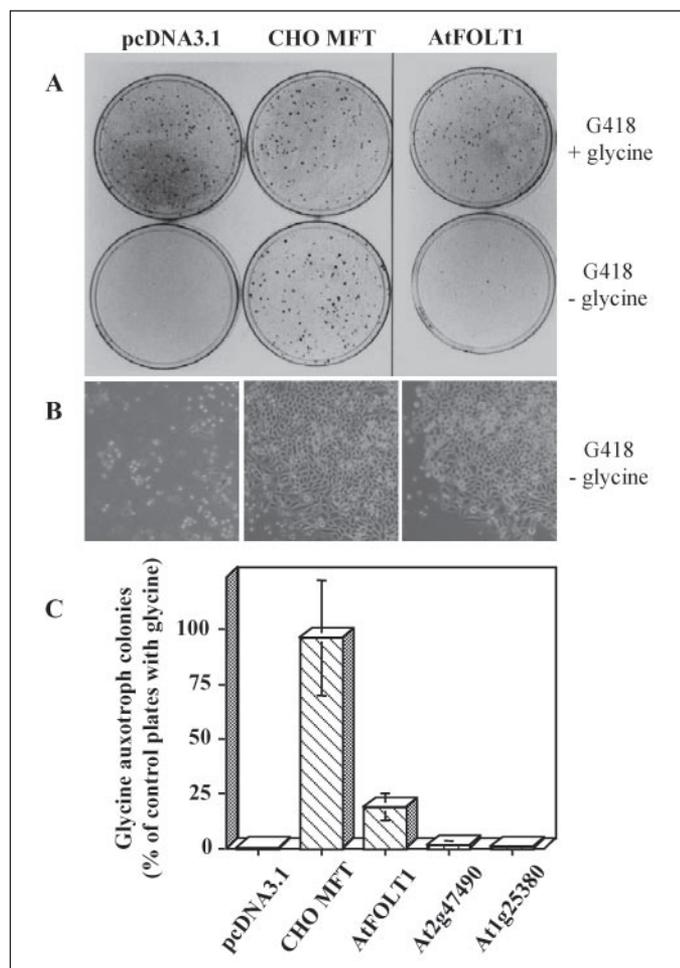
To confirm the subcellular location of AtFOLT1 by an alternative approach we raised a polyclonal serum against a synthetic peptide and prepared subfractions from Percoll-purified *Arabidopsis* leaf chloroplasts. Previous proteomic studies indicated that Percoll-purified *Arabidopsis* chloroplasts isolated using similar procedures contained minute amounts of protein contaminants from other subcellular compartments (33, 38). The envelope, thylakoid, and stromal protein subfractions were analyzed by SDS-PAGE and Western blot to check for cross-contaminations. As shown in Fig. 4B, antibodies against the 2-oxoglutarate/malate translocator (IEP45, envelope marker (38)) gave a strong signal in the envelope fraction and only a very faint labeling in thylakoids. Probing the plastidial subfractions with antibodies against the major thylakoid light-harvesting complex proteins indicated that the envelope preparation contained no more than 1–3% thylakoid proteins (Fig. 4B (33)). These data indicated that the chloroplast subfractions were poorly cross-contaminated and thus suitable to analyze the localization of AtFOLT1 by Western blot. As shown in Fig. 4B, a  $33 \pm$

1-kDa polypeptide was immunodetected in the envelope but not in the stroma or thylakoid subfractions using the purified anti-AtFOLT1 antibodies (this polypeptide was not detected with the pre-immune serum). Also, when membrane proteins from Percoll-purified *Arabidopsis* mitochondria were analyzed using the AtFOLT1 antibodies, no polypeptide having the predicted molecular weight of AtFOLT1 could be detected (data not shown). Together with the GFP-tagging experiments shown in Fig. 3, these results clearly indicate that AtFOLT1 is located in the envelope of chloroplasts.

*AtFOLT1 Can Complement the Auxotrophy Demonstrated by CHO glyB Cells—GlyB*, a CHO-derived cell line (39), was previously characterized to be deficient in accumulation of folates in the mitochondria, an effect that rendered these cells auxotrophic for glycine as a result of a strong restriction in serine hydroxymethyltransferase activity in mitochondria (17, 18, 40). Recently, it was shown that *glyB* cells contain a point mutation (G192E) within the open reading frame of a gene encoding a mitochondrial inner membrane protein responsible for the transport of folates into the matrix of mitochondria (18). cDNAs for the human, mouse, and zebrafish MFT were found to efficiently complement *glyB* cells, indicating their function as folate mitochondrial transporters in a hamster background (18). To address whether AtFOLT1 was able to confer mitochondrial folates transport, we stably transfected pcDNA-AtFOLT1 into *glyB* cells using a calcium phosphate mediated procedure. After 3 days, the cultures were placed under either single selection conditions containing medium supplemented with G418 for determining transfection efficiency or under double selection conditions containing glycine-free medium supplemented with G418. After 10 days of selection, colonies were fixed and stained. In several experiments, transfection of the *AtFOLT1* cDNA consistently allowed the formation of colonies in glycine-free medium with a frequency of 20% (Fig. 5). This value is lower than the frequency seen with a CHO MFT cDNA, for which transfection plates had nearly identical numbers of colonies under single or double selection (Fig. 5). Control transfections using pcDNA3.1 did not result in colony formation under double selection. When cDNAs encoding two other *Arabidopsis* candidate folate transporters (At2g47490 and At1g25380, Fig. 2) were used in transfection assays, no colonies were formed under double selection (Fig. 5C), lending credence to the specificity of the effect seen with AtFOLT1.

To confirm the authenticity of this complementation, 40 colonies were picked from single selection plates, and each colony was expanded in medium containing glycine. Cells from each mass culture were plated in replicate wells in media allowing single and double selection. Of the 40 cultures thus isolated under non-selective conditions, 10 (*i.e.* 25%) showed progressive growth in medium formulated without glycine; growth was followed for two passages. Interestingly, some cultures consistently formed large colonies in glycine-deficient medium when plated at low density, others formed small colonies. This behavior recapitulated the large and small colonies observed under double selection conditions. We concluded that the *Arabidopsis* chloroplastic protein AtFOLT1 can complement the auxotrophy demonstrated by *glyB* cells, albeit less efficiently than mitochondrial folate transporters of mammalian origin.

*AtFOLT1 Enhances Levels of Folates in the Mitochondria of glyB Cells—Two* of the clonally expanded pcDNA-AtFOLT1 transfectants were selected to determine whether the AtFOLT1 protein could circumvent the folates depletion in mitochondria. These two expanded colonies differed in growth rate, with one (designated *glyB/AtFOLT1-2*) forming exclusively large colonies from single cells, and the other (*glyB/AtFOLT1-32*) forming smaller colonies. Cultures of both transfectant cell lines were incubated with [ $^3$ H]folic acid for 48 h,



**FIGURE 5. Complementation of *glyB* cells glycine auxotrophy by transfection of *AtFOLT1* cDNA.** *A*, *GlyB* cells were transfected with CHO MFT (18), *AtFOLT1*, and pcDNA3.1 alone using a  $\text{CaPO}_4$  procedure. Subsequently, cells were fed for 10 days with medium containing G418 in the presence or absence of glycine, then fixed and stained. *B*, *GlyB* cell transfectants at  $200\times$  magnification after being maintained for 10 days in the absence of glycine. Actively growing colonies of cells were detected in both CHO MFT and *AtFOLT1* transfected *glyB* cells; none were detected in vector alone. *C*, graphical representation of *glyB* cell complementation. Colonies formed on plates of transfected *glyB* cells from both single and double selection were counted. Single selection conditions with 1 mg/ml G418 yielded 50–129 colonies per plate with near-equivalent transfection frequencies with all of the constructs. The bars represent the number of colonies per plate in each double selection condition as a percentage of colonies formed under single selection. Three plates were used for each transfection condition and data shown represent the pooled data from three experiments  $\pm$  S.D.

and the cells were harvested and fractionated into various compartments (TABLE ONE). In each of three experiments, the mitochondrial folate pool in *glyB/AtFOLT1-2* cells was expanded 4-fold relative to the folate content of *glyB* cells, which corresponded to 39% of the mitochondrial pool found in CHO cells (TABLE ONE). The mitochondrial folate content in *glyB/AtFOLT1-32*, which grew much slower than *glyB/AtFOLT1-2*, was consistently higher than that found in *glyB* cells, but it was much lower than that found in *glyB/AtFOLT1-2*. This difference suggested that the substantial variation in colony size seen in the *AtFOLT1* transfection plates may reflect changes in levels of expression of the *AtFOLT1* protein with concomitant differences in folates uptake into mitochondria. Overall, we concluded that the *Arabidopsis* chloroplastic protein *AtFOLT1* can partially restore the mitochondrial folates pool in *glyB* cells if expressed at high enough levels. The level of folates found in the cytosol of *glyB/AtFOLT1-2* cells was also noticeably ( $\sim 2.5$ -fold) higher than those found in either *glyB* or CHO cells (TABLE ONE). We concluded that expression of the *Arabidopsis* protein in

hamster cells probably also affected plasma membrane transport of folate, thus suggesting some degree of incorrect targeting that is not unusual in heterologous expression systems.

*AtFOLT1* Allows *E. coli* Cells to Mediate Folic Acid Uptake—To establish whether *AtFOLT1* can mediate folate transport in another physiological context we used *E. coli* as an alternative heterologous expression system. Many bacteria, including *E. coli*, are folate autotrophs and normally do not have the ability to import and utilize exogenous folates. Thus, when *de novo* THF synthesis is blocked by specific inhibitors such as the *p*-aminobenzoate analogs sulfonamides, bacterial growth cannot be restored by the addition of folates in the culture medium. The cDNA coding a truncated *AtFOLT1* protein without the short N-terminal extension located before the first predicted transmembrane  $\alpha$ -helix was cloned in pTrc99A and introduced in *E. coli* JM105 cells. Transformants harboring pTrc-*AtFOLT1* or the empty pTrc99A vector were tested for growth in liquid minimal medium containing asulam (methyl sulfanylylcarbamate) and with or without folic acid. As shown in Fig. 6A the inhibition of the dihydropyrimidine synthase activity by asulam fully impaired growth of the pTrc99A transformants in the presence of exogenous folic acid. In contrast, the expression of *AtFOLT1* restored growth of the JM105 strain only when folic acid is present in the medium. When IPTG was omitted from the selective medium the growth of cells harboring pTrc-*AtFOLT1* was delayed, thus suggesting that growth kinetics depend on the expression level of the recombinant protein (the IPTG-inducible *trc* promoter located upstream the *AtFOLT1* sequence is strong, and uninduced cells may show low level of protein expression). To verify this hypothesis we analyzed by Western blot the expression of *AtFOLT1* in JM105 cells. As shown in Fig. 6B, only *E. coli* cells bearing the pTrc-*AtFOLT1* plasmid and cultured for several hours in the presence of IPTG expressed a  $33 \pm 1$ -kDa polypeptide detected with the purified anti-*AtFOLT1* antibodies, thus indicating that the recombinant protein accumulated upon *trc* promoter induction. These data indicate that the expression of *AtFOLT1* allows *E. coli* cells to mediate folic acid uptake from the external medium.

*Characterization of an Arabidopsis atfolt1 Null Mutant*—To investigate the function of *AtFOLT1* in planta, we required a mutant that lacked a functional *AtFOLT1* allele and searched the indexed insertion mutant data base from the SIGnAL website (signal.salk.edu). We found a knock-out mutant (SALK\_005280, line *atfolt1-1*) and obtained the corresponding T3 segregating seeds from the Arabidopsis Biological Resource Center. Homozygous plants (*atfolt1-1#6* and *atfolt1-1#10*) were selected by PCR and sequence analysis of the junction fragments obtained with T-DNA and gene-specific primers identified the T-DNA insertion in exon 10 (Fig. 7, A and B). The *AtFOLT1::T-DNA* locus was verified by DNA gel blot analysis using *AtFOLT1* and T-DNA probes. The observed patterns (Fig. 7C) are consistent with PCR analysis and indicate that the homozygous plants do not contain any additional T-DNA insertions elsewhere in the genome. RT-PCR analysis indicated that the *AtFOLT1* transcript was not expressed in homozygous *atfolt1-1#6* and *atfolt1-1#10* plants (Fig. 7D).

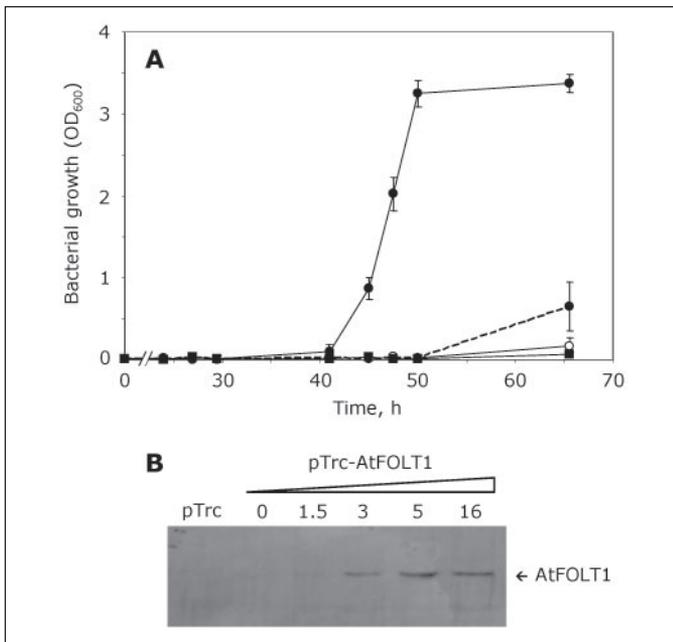
A survey of the expression pattern of the *At5g66380* gene using the *Arabidopsis* microarray data base Genevestigator (41) indicated that *AtFOLT1* is expressed in all the examined organs and tissues at almost similar levels. Also, the use of the Genevestigator “gene chronologer” toolbox and quantitative RT-PCR analysis done with Col-0 plants grown in our greenhouse (data not shown) indicated that the *AtFOLT1* gene is expressed constitutively at all growth stages, from germination to plant senescence. From these data, we hypothesized that a phenotype could be detected for the *atfolt1* null mutant at any stage of plant devel-

TABLE ONE

Folate uptake in *glyB* transfectants stably expressing AtFOLT1

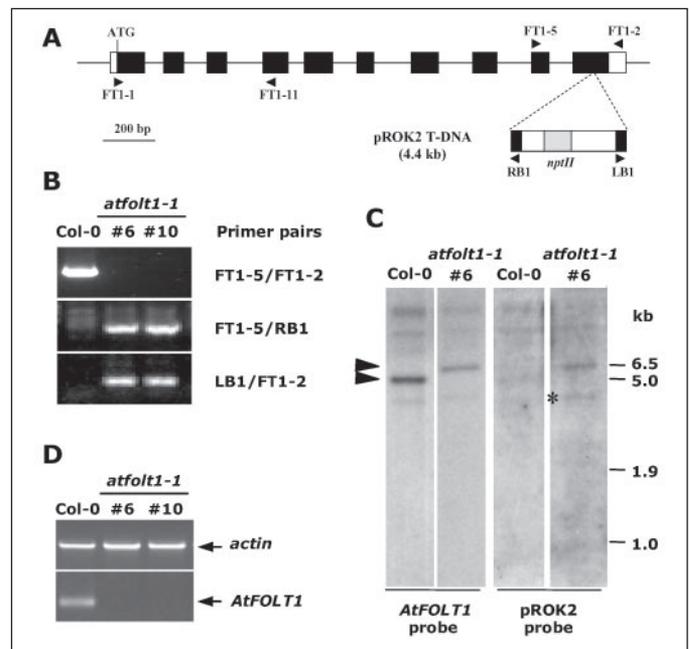
Cell lines were grown in  $\alpha$  minimal essential medium supplemented with  $0.3 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]folic acid for 48 h. The cells were harvested, homogenized, and submitted to differential centrifugation to isolate mitochondria (see "Experimental Procedures"). Each fractionation was performed on triplicate cultures, and data were combined from three experiments. Values in parentheses indicate the percentage of mitochondrial folates in the different *glyB* transfectants as compared to the level found in wild-type CHO mitochondria.

Cellular compartment	Cell line/transfected DNA			
	CHO/none	<i>glyB</i> /none	<i>glyB</i> /AtFOLT1-2	<i>glyB</i> /AtFOLT1-32
	<i>pmol folate/10<sup>6</sup> cells</i>			
Cytosol	2.1 $\pm$ 0.47	2.4 $\pm$ 0.46	5.8 $\pm$ 1.3	3.0 $\pm$ 0.93
Mitochondria	0.31 $\pm$ 0.09 (100%)	0.025 $\pm$ 0.008 (8%)	0.12 $\pm$ 0.067 (39%)	0.047 $\pm$ 0.018 (15%)
Unbroken cells/nucleus	1.8 $\pm$ 0.77	1.0 $\pm$ 0.28	1.2 $\pm$ 0.64	1.3 $\pm$ 0.42
Sum of compartments	4.2 $\pm$ 0.91	3.4 $\pm$ 0.54	7.1 $\pm$ 1.4	4.3 $\pm$ 0.15



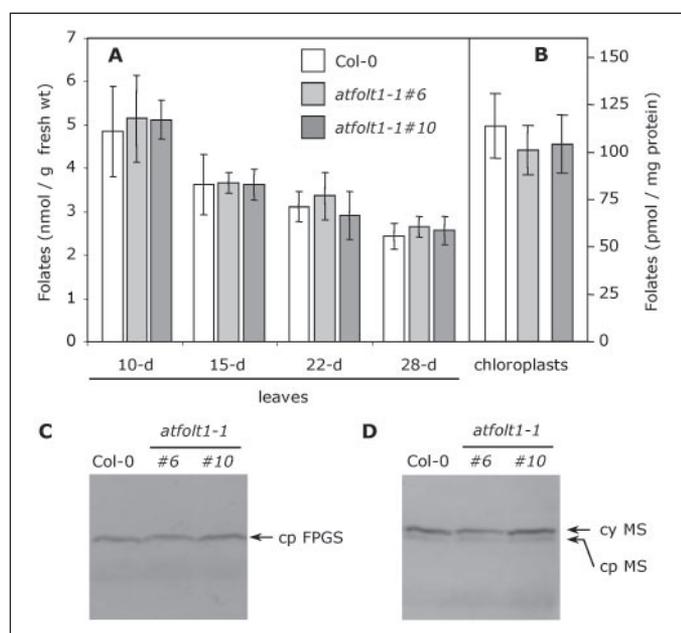
**FIGURE 6. Expression of AtFOLT1 in *E. coli* allows cells to uptake exogenous folic acid.** A, the *E. coli* JM105 strain transformed with either the empty pTrc99A vector (squares) or the pTrc-AtFOLT1 construct (circles) was grown at 37 °C in M9 minimal medium containing 30  $\mu\text{M}$  asulam plus 50  $\mu\text{M}$  folic acid plus 1 mM IPTG (solid symbols, continuous lines) or asulam plus folic acid (solid symbols, dotted lines) or asulam plus IPTG (open symbols, continuous lines). Cell growth was followed by measuring  $A_{600}$ . Curves represent the average  $\pm$  S.D. for three independent transformants. Because the behavior of JM105 transformed with the empty pTrc99A vector is similar in both conditions only the growing curve in medium containing asulam plus folic acid plus IPTG is shown. B, time course analysis of AtFOLT1 production in *E. coli* JM105. Transformants bearing pTrc-AtFOLT1 or the empty expression vector were grown at 37 °C in LB medium and cells were collected at different time points after the addition of IPTG (0–16 h). Total proteins were separated by SDS-PAGE and analyzed by Western blot using anti-AtFOLT1 purified antibodies. The recombinant AtFOLT1 protein was detected at  $33 \pm 1$  kDa. The pTrc99A (pTrc) protein extract corresponded to cells harvested 5 h after the addition of IPTG.

opment. Thus, the *atfolt1-1#6* and *atfolt1-1#10* null mutants and the wild-type Col-0 were cultured side-by-side under standard conditions and their growth characteristics were compared. Throughout the examined growing period, there were no significant differences between the mutant and wild-type plants in terms of germination efficiency, growth rates, morphology, seed production, and fertility. Also, measurements of photosynthesis and respiration rates in rosette leaves at different stages of development did not show significant changes in the mutant as compared with the wild-type. To characterize further the phenotype of the *atfolt1* mutant, we grew plants under conditions leading to a depletion of the cellular pool of folates. For this purpose, plants were allowed to germinate and grow under defined nutrient conditions on agar medium supplemented with sulfanilamide or MTX, two folate antago-



**FIGURE 7. Molecular characterization of an *Arabidopsis atfolt1* null mutant.** A, scheme of the *AtFOLT1::T-DNA* locus. The gene structure shows exons, closed bars, and 5'- and 3'-untranslated regions, open bars. The pROK2 T-DNA bearing the neomycin phosphotransferase *nptII* gene conferring resistance to kanamycin is not described in actual size. Positions of the primers used for genomic-PCR (B) and RT-PCR analysis (D) are indicated with arrowheads. B, characterization of the T-DNA insertion locus by PCR. Genomic DNA from the wild-type (Col-0) and mutants (*atfolt1-1#6* and *atfolt1-1#10*) was amplified using the primer combinations indicated. C, Southern blot analysis of HindIII-digested genomic DNA from the wild-type and mutant (*atfolt1-1#6*). Blots were hybridized successively with the *AtFOLT1* and pROK2 probes. DNA fragments corresponding to the wild-type (~5.1 kb) or mutated (~6.6 kb) copies of the *AtFOLT1* gene are indicated with arrowheads. Hybridization with the pROK2 probe resulted in a second signal (~4 kb, asterisk) with genomic DNA from the mutant, as expected from restriction analysis of the *AtFOLT1::T-DNA* locus. Similar patterns were obtained for *atfolt1-1#6* and *atfolt1-1#10*. D, RT-PCR analysis of the *AtFOLT1* and *actin* mRNAs in wild-type and mutant plants. Total RNA was reverse transcribed and amplified using the primer pair FT1-1/FT1-11 indicated in A. *Actin2* (At5g09810) is a constitutive control.

nists that were found to be useful in manipulating the availability of THF in plants (10). Sulfanilamide is an inhibitor of dihydropteroyl synthase, whereas MTX blocks the reduction of dihydrofolate to THF catalyzed by dihydrofolate reductase (2). Seeds were exposed to a range of concentrations (from  $10^{-9}$  to  $2.10^{-4}$  M) of the drugs added separately and analyzed during a 2-week period. The presence of one of these inhibitors in the medium resulted in a dose-dependent growth retardation with more severe effects of MTX versus sulfanilamide, as previously observed (10). Under these conditions we could not observe any clear phenotypic difference between the wild-type and the *atfolt1* mutant, thus suggesting that the absence of AtFOLT1 did not modify seedling sensitivity to the antifolate drugs. In another set of experiments, seedlings were



**FIGURE 8. Foliates and folate-dependent enzymes levels in leaves and chloroplasts from the wild-type and *atfolt1* null mutant.** A, foliates were determined in leaves collected at different stages of development using a microbiological assay with *L. casei* ATCC7469. B, intact chloroplasts were purified from 22-day-old leaves, and foliates were measured in the soluble fractions. Foliates levels are expressed on a fresh weight basis for leaves and on the basis of soluble proteins for chloroplasts. Values are the means  $\pm$  S.D. of two independent experiments, each analyzed in triplicates of dilution series. C and D, Western blot analysis of chloroplastic folate-dependent enzymes. Total soluble proteins (60  $\mu$ g) from 3-week-old *Arabidopsis* leaves were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the antibodies raised against the chloroplastic folylpolyglutamate synthetase (*cp FP GS*, C) or methionine synthase (D). The high and low molecular weight bands correspond to the cytosolic (*cy MS*) and chloroplastic (*cp MS*) methionine synthase isoforms, respectively, which are detected by the antibodies (33).

grown for 10 days in a standard medium and then transferred to sulfanilamide or MTX-containing medium. Again, the developmental consequences of folate depletion we observed were not significantly different for the wild-type and mutant lines.

To determine whether the absence of AtFOLT1 affects the folate status of the null mutants, we measured the pools of folates (THF and its C1 derivatives) in leaves. As shown in Fig. 8A, the folates levels in 10-day-old leaves was about 5 nmol per gram of fresh weight and progressively decreased down to 2.5 nmol g<sup>-1</sup> fresh weight in 28-day-old leaves. A similar behavior was found for the *atfolt1* null mutants, and the values obtained at any stage of growth were not significantly different for the wild-type and the mutants (Fig. 8A). Chloroplastic folates have been shown to contribute to only 10% of the cellular pool of the vitamin (42), thus modest changes in folates levels in leaves could have been masked by experimental variations. To deal with this hypothesis, we purified chloroplasts from 22-day-old leaves and measured their folates content (Fig. 8B). The wild-type, the *atfolt1-1*#6, and *atfolt1-1*#10 mutants contained 114  $\pm$  17, 101  $\pm$  15, and 104  $\pm$  17 pmol folates per milligram of chloroplastic soluble proteins, respectively (means  $\pm$  S.D. for two independent chloroplast purifications analyzed in triplicates). These results indicate that the absence of AtFOLT1 reduced neither the chloroplastic nor the cellular pools of folates in *Arabidopsis* leaves.

To assess the effect of the *atfolt1* mutation on one-carbon metabolism in chloroplasts, we analyzed the expression of two folate-dependent enzymes present in this compartment. Western blot analysis indicated that the chloroplastic folylpolyglutamate synthetase was detected at similar levels in the wild-type and mutant plants (Fig. 8C). Also, both the cytosolic and chloroplastic methionine synthase isoforms are pres-

ent in nearly similar amounts in both *Arabidopsis* lines (Fig. 8D). Taken together these data suggest that the *atfolt1* null mutant and the wild-type have the same enzymatic capacity to catalyze both the glutamylation of THF and the methyl-THF-dependent synthesis of methionine in the chloroplasts.

We have selected a second independent *Arabidopsis atfolt1* null mutant (SALK\_059769) in which the T-DNA insertion is located at the junction between intron 7 and exon 8 of the *AtFOLT1* gene. Plants homozygous for the mutation failed to accumulate the *AtFOLT1* mRNA, had no discernable phenotype when grown under folate-sufficient conditions, and contained wild-type levels of folates in leaves (data not shown). These results are consistent with the data presented for the *atfolt1-1* knock-out line.

## DISCUSSION

In this report we describe the characterization of an *Arabidopsis* protein located in the envelope of chloroplasts that is able to mediate folate transport. The AtFOLT1 transporter is able to reinstate folates levels in mitochondria of CHO *glyB* cells lacking a functional endogenous MFT and mediates exogenous folate uptake when expressed in *E. coli* cells. Functional complementation of a mitochondrial default in mammalian cells with a chloroplastic envelope integral protein was reasonably unexpected and may be due to the non canonical features of the AtFOLT1 transit peptide, which allows a quite efficient targeting of the plant carrier to mitochondrial membranes. Despite the evolutionary distance between plants and mammals, there are several points of convergence between C1 metabolism in mitochondria from mammals and chloroplasts that can explain the functional homology between MFT and AtFOLT1. Indeed, both organelles contain folates and the folate-dependent enzymes that are involved in the interconversion of serine and glycine, and the formylation of initiator tRNA. Also, mitochondria from mammals and chloroplasts must import from the cytosol at least one folate species that is subsequently polyglutamylated and charged with a C1 unit within the organelle. Despite these common features, the present work indicates that the physiological importance of MFT/AtFOLT1 in mitochondria/chloroplasts is very different. On the one hand, a point-mutation of MFT in CHO cells prevents the accumulation of folates in mitochondria and cells are auxotroph for glycine (17, 18). On the other hand, the *Arabidopsis atfolt1* null mutant contains wild-type levels of folates in chloroplasts and preserves the enzymatic capacity to glutamylate THF and to synthesize methionine in this subcellular compartment. As a consequence, the absence of AtFOLT1 does not lead to phenotypic alterations in folate-sufficient or folate-deficient plants. Our findings suggest strongly the existence of a second folate transporter located in the chloroplast envelope. Such a duplication of folate transport mechanisms exists in mammalian cells where different systems participate in folate uptake, *i.e.* folate receptors and reduced folate carriers (12) and in *Leishmania*, which has several folate transporters with different characteristics (15). The partial or total functional redundancy of folate carriers in the envelope of chloroplasts may indicate that AtFOLT1 is not able to transport all the folate derivatives that are needed for C1 metabolism in plastids. If AtFOLT1 and MFTs display the same substrate specificity, AtFOLT1 can probably provide chloroplasts with folate derivatives utilized for the interconversion of serine and glycine and for the synthesis of formyl methionyl-tRNA. However, AtFOLT1 may be unable to transport methyl-THF from the cytosol, its unique site of synthesis (43), to chloroplasts where it is required for *de novo* synthesis of methionine (33). The transport of methyl-THF may be attributable to a second chloroplastic folate transporter.

It is unlikely that the alternative route for folates import into chloro-

plasts is achieved by the AtFOLT1 paralogs encoded by the *At2g47490* and *At1g25380* genes, because these proteins are not capable of functioning as folate transporters in a hamster background (Fig. 5) and are not targeted to plastids, as demonstrated by GFP-tagging and/or immunolocalization studies (data not shown). The involvement of additional plastid localized transporters belonging to the *Arabidopsis* Mitochondrial Carrier family cannot be excluded, however. We searched the *Arabidopsis* genome for putative chloroplastic folate carriers belonging to other transporter families. Proteins of the Reduced Folate Carrier family are restricted to animals (12) but homologs of the folate/biopterin transporters characterized in protozoa are found in cyanobacteria and plants (see the PlantsT data base for functional genomics of plant transporters at plantst.sdsc.edu/). In *Arabidopsis*, nine putative integral transporters have been assigned to folate/biopterin transporter homologs in the ARAMEMNON data base (44), although their sequence homology with the proteins from *Leishmania* is quite low (<20% identity). Among these folate/biopterin transporter homologs, the proteins encoded by the *At2g32040* and *At1g04570* genes are predicted to be plastidial with high probability.

It has been shown recently that the *At2g32040* protein can mediate folate and antifolate analogs transport when expressed in *E. coli* cells.<sup>6</sup> Also, T-DNA inactivation of the *At2g32040* gene had no noticeable effect on plant growth and did not reduced chloroplastic folate levels.<sup>6</sup> From these data, it is tempting to postulate that the AtFOLT1 and *At2g32040* proteins participate to alternative routes for folates import into chloroplasts. Because the insertional inactivation of the *AtFOLT1* or *At2g32040* gene does not alter significantly the chloroplastic pool of folates, it is likely that the absence of one of these carriers can be compensated by the second. Additional experiments, including the characterization of the *AtFOLT1/At2g32040* double mutant, are now needed for a better understanding of how chloroplasts regulate their folate homeostasis.

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