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5-Formyltetrahydrofolate Is an Inhibitory but Well Tolerated Metabolite in *Arabidopsis* Leaves*

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5-Formyltetrahydrofolate (5-CHO-THF) is formed via a second catalytic activity of serine hydroxymethyltransferase (SHMT) and strongly inhibits SHMT and other folate-dependent enzymes *in vitro*. The only enzyme known to metabolize 5-CHO-THF is 5-CHO-THF cycloligase (5-FCL), which catalyzes its conversion to 5,10-methenyltetrahydrofolate. Because 5-FCL is mitochondrial in plants and mitochondrial SHMT is central to photorespiration, we examined the impact of an insertional mutation in the *Arabidopsis* 5-FCL gene (At5g13050) under photorespiratory (30 and 370 μmol of CO_2 mol^{-1}) and non-photorespiratory (3200 μmol of CO_2 mol^{-1}) conditions. The mutation had only mild visible effects at 370 μmol of CO_2 mol^{-1}, reducing growth rate by ~20% and delaying flowering by 1 week. However, the mutation doubled leaf 5-CHO-THF level under all conditions and, under photorespiratory conditions, quadrupled the pool of 10-formyl-/5,10-methenyltetrahydrofolates (which could not be distinguished analytically). At 370 μmol of CO_2 mol^{-1}, the mitochondrial 5-CHO-THF pool was 8-fold larger in the mutant and contained most of the 5-CHO-THF in the leaf. In contrast, the buildup of 10-formyl-/5,10-methenyltetrahydrofolates was extramitochondrial. In photorespiratory conditions, leaf glycine levels were up to 46-fold higher in the mutant than in the wild type. Furthermore, when leaves were supplied with 5-CHO-THF, glycine accumulated in both wild type and mutant. These data establish that 5-CHO-THF can inhibit SHMT *in vivo* and thereby influence glycine pool size. However, the near-normal growth of the mutant shows that even exceptionally high 5-CHO-THF levels do not much affect fluxes through SHMT or any other folate-dependent reaction, *i.e.* that 5-CHO-THF is well tolerated in plants.

5-Formyltetrahydrofolate (5-CHO-THF)[1] is formed from 5,10-methenyltetrahydrofolate (5,10-CH=THF) by a hydrolytic reaction catalyzed by serine hydroxymethyltransferase (SHMT) in the presence of glycine (1, 2). Spontaneous chemical hydrolysis of 5,10-CH=THF may be a minor additional source (3). 5-CHO-THF is the most stable natural folate and the most enigmatic, for it is the only one that does not serve as a cofactor in one-carbon metabolism. Instead, 5-CHO-THF is a potent inhibitor of SHMT and most other folate-dependent enzymes *in vitro* (4, 5). 5-CHO-THF probably acts as a stable storage form of folate in seeds and fungal spores (5–7), but it is not clear what role, if any, it plays in metabolically active tissues (8). This question is particularly pertinent for leaves. Leaf mitochondria have very high levels of SHMT and, during photorespiration, receive a massive influx of glycine (which leads to a matching SHMT-mediated glycine → serine flux) (9). Conditions in leaf mitochondria therefore favor 5-CHO-THF formation (Fig. 1). Indeed, 5-CHO-THF can comprise 50% of the folate pool in leaf mitochondria (10, 11), which is far more than in mammalian mitochondria (12–14). Furthermore, 5-CHO-THF is reported to make up 14–40% of the folate pool in leaves and other metabolically active plant organs (10, 15), a much higher proportion than the 3–10% typical of mammals and yeast (2, 16).

5-Formyltetrahydrofolate cycloligase, EC 6.3.3.2 (5-FCL, also known as 5,10-methenyltetrahydrofolate synthetase), is the only enzyme known to recycle 5-CHO-THF to a metabolically active form, which it achieves by catalyzing irreversible, ATP-dependent conversion to 5,10-CH=THF (2, 5). This enzyme is also something of an enigma. For one thing, despite the inhibitory effects of its substrate, 5-FCL is not essential in yeast: 5-FCL disruptants had 4-fold more 5-CHO-THF but no other new phenotype (2). For another, phylogenomic profiling (17, 18) indicates that some bacteria lack 5-FCL even though they have SHMT. Lastly, 5-FCL overexpression in human cells lowered the folate level and raised folate turnover rate, suggesting that 5-FCL may have a second function as a folate-degrading enzyme (19).

Another intriguing feature of 5-FCL is that its subcellular location differs among eukaryotes. Whereas the enzyme is largely if not solely cytosolic in yeast and mammals (2, 16, 20, 21), it is exclusively mitochondrial in plants (4). Taken with the key role of mitochondrial SHMT in photorespiration, with the inhibition of SHMT by 5-CHO-THF, and with the presence of 5-CHO-THF in leaf mitochondria, the location of plant 5-FCL

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[1] The abbreviations used are: 5-CHO-THF, 5-formyltetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-FCL, 5-formyltetrahydrofolate cycloligase; SHMT, serine hydroxymethyltransferase; GC-MS, gas chromatography-mass spectrometry; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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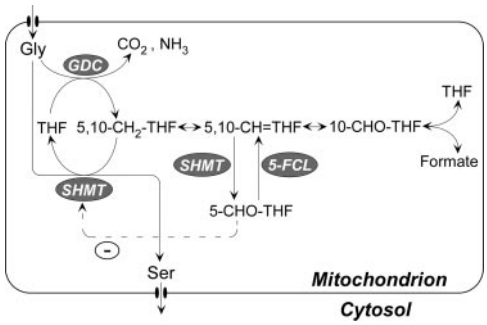

FIG. 1. **Photorespiratory metabolism and folate interconversions in plant mitochondria.** In photorespiration, glycine coming from peroxisomes is converted to serine in the mitochondria by the concerted action of the glycine decarboxylase complex (*GDC*) and SHMT. SHMT also mediates formation of 5-CHO-THF from 5,10-CH=THF; the reverse reaction is catalyzed by 5-FCL, which is solely mitochondrial in plants (4). Possible SHMT inhibition by 5-CHO-THF is shown by a *dashed line*. Plant mitochondria also contain an isoform of the bifunctional 5,10-methylene-THF dehydrogenase/5,10-CH=THF cyclohydrolase that interconverts 5,10-CH_2-THF, 5,10-CH=THF, and 10-CHO-THF and an isoform of 10-formyl-THF synthetase; there are also extramitochondrial isoforms of both enzymes (15).

implies (i) that this enzyme governs mitochondrial 5-CHO-THF levels and (ii) that, via its effect on 5-CHO-THF levels, 5-FCL could regulate the *in vivo* activities of SHMT and other folate-linked mitochondrial enzymes (Fig. 1).

To investigate such regulatory functions for 5-FCL and 5-CHO-THF, we identified and characterized an *Arabidopsis* 5-FCL insertional mutant. This mutant had greatly elevated mitochondrial 5-CHO-THF levels and hyperaccumulated glycine under photorespiratory conditions. However, the mutant grew almost normally in ambient air, indicating that folate-dependent metabolic reactions in plants are substantially tolerant to 5-CHO-THF.

EXPERIMENTAL PROCEDURES

Chemical and Reagents—Folates were from Schircks Laboratories (Jona, Switzerland). 10-Formyldihydrofolate was prepared from (6*R*,6*S*)-5-CHO-THF as described (22). [α-^{32}P]dCTP (3000 Ci mmol^{-1}) was from PerkinElmer Life Sciences.

Plants and Growth Conditions—*Arabidopsis thaliana* plants were grown at 22–28 °C in 12-h days (photosynthetic photon flux density 80 μE m^{-2} s^{-1}) in potting soil irrigated with water. Material was lyophilized to determine dry weight. In experiments at various CO_2 levels, plants were grown for 4 weeks in ambient air (~370 μmol of CO_2 mol^{-1}), then either kept in ambient air or transferred to high CO_2 (3200 ± 40 μmol mol^{-1}) or low CO_2 (30 ± 20 μmol mol^{-1}) conditions for 5 days. CO_2 levels were monitored with a Vernier CO_2 sensor (Vernier Software and Technology, Beaverton, OR).

Arabidopsis Mutant—A 5-FCL mutant (28D07) was identified in the Syngenta T-DNA insertion collection (ecotype Columbia) (23). Segregants, wild type or homozygous for the mutation, were identified by PCR using gene-specific primers located 5′ or 3′ of the T-DNA insertion (5′-CTGAAGTGAGTGGCAACTACA-3′ and 5′-GTCTCACTTCTTCTCTTACCTT-3′, respectively) and the T-DNA-specific primer 5′-GCATCTGAATTTCATAACCAATC-3′. DNA was extracted by the "Shorty" protocol available on the website of the University of Wisconsin Biotechnology Center. The insertion site was confirmed by sequencing.

Gel Blot Analyses—Total RNA was extracted from 0.1-g samples of rosette leaves using RNeasy kits (Qiagen, Valencia, CA) and treated with DNase (DNA-*free*™ kit, Ambion, Austin, TX). RNA samples were separated by formaldehyde-1.5% agarose gel (25 μg/lane) and blotted to Protran® nitrocellulose membrane (Schleicher and Schuell, Keene, NH), hybridized for 4 h at 65 °C in 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 100 μg ml^{-1} sonicated salmon sperm DNA, and washed in 0.1× SSC, 0.5% SDS at 65 °C. The 5-FCL probe was a 373-bp NdeI-XhoI fragment of pET28b-At5FCL (4) corresponding to the 3′-half of the 5-FCL open reading frame (Fig. 2*A*). The rRNA probe was a 0.9-kb SmaI fragment of a *Zamia pumila* clone (24). Genomic DNA was isolated as described (25) from 2 g of leaves pooled from 20 plants, digested, separated by 0.8% agarose gel (5 μg/lane), and blotted to Protran® membrane. Blots were hybridized as above and washed in 0.1× SSC, 0.5% SDS at 37 °C. The probe was a 436-bp fragment of the *bar* gene amplified using the primers 5′-CATCGTCAACCACTACATCG-3′ (forward) and 5′-GAAGTCCAGCTGCCAGAAAC-3′ (reverse). Probes were labeled with [α-^{32}P]dCTP by the random primer method. Hybridization was detected by autoradiography.

Folate Analysis—Folates were extracted from leaf tissue (0.5 g) by Polytron homogenization in 10 ml of 50 mM Na-HEPES, 50 mM CHES, adjusted to pH 7.9 with HCl, containing 2% (w/v) sodium ascorbate, and 10 mM β-mercaptoethanol (buffer 1), followed by boiling for 10 min and then centrifuging (13,000 × *g*, 10 min). The pellet was re-extracted the same way, and the extracts were combined. Mitochondria were extracted once with 5 ml of buffer 1. Extracts were treated with 1 ml (leaves) or 0.5 ml (mitochondria) of dialyzed rat plasma at 37 °C for 2 h to deglutamylate folates. Samples were then boiled for 15 min, centrifuged, filtered, and applied to folate affinity columns prepared as described (26). Column volume was reduced to 1 ml for mitochondria. After washing columns with 5 ml (2.5 ml for mitochondria) of 25 mM potassium phosphate, pH 7.0, plus 1% sodium ascorbate (buffer 2) containing 1 M NaCl, then with 5 ml (2.5 ml for mitochondria) of buffer 2 alone, they were eluted with 5 ml (1 ml for mitochondria) of HPLC mobile phase A (see below) containing 1% ascorbic acid. Eluate samples (400 μl) were analyzed by HPLC (27) using a Prodigy 5-μm ODS2 column (150 × 3.2 mm, Phenomenex, Torrance, CA) and a four-channel electrochemical detector (CoulArray Model 5600A, ESA, Chelmsford, MA) with potentials set at 0, 300, 500, and 600 mV. The mobile phase was a binary mixture of 28 mM K_2HPO_4 and 0.59 mM H_3PO_4, pH 2.5 (A) and a mixture of 75% (v/v) A and 25% CH_3CN (B) with a 55-min nonlinear elution program from 90% A to 100% B at 1 ml min^{-1}. Detector response was calibrated with authentic tetrahydrofolate (THF), 5-methyl-THF (5-CH_3-THF), 5,10-CH=THF, 5-CHO-THF, 10-formyldihydrofolate, and folic acid.

Amino Acid Analysis—Leaf tissue (~160 mg) was frozen in liquid N_2, lyophilized, weighed, and pulverized. The resulting powder was extracted by shaking with 0.5 ml each of water and $CHCl_3$ and was then stored at −20 °C for 24 h before centrifugation. Ribitol and γ-aminobutyric acid were added as internal standards. For HPLC, 20 μl of the aqueous phase was derivatized with AccQ·Fuor™ reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate; Waters, Milford, MA) in a final volume of 100 μl, and a 20-μl aliquot was analyzed by HPLC-fluorescence according to Waters' recommendations. For GC-MS, aqueous phase aliquots equivalent to 0.625 mg dry weight were dried under N_2 and methoximated and trimethylsilylated in pyridine (final volume 50 μl) as described (28). One μl of the derivatized mixture was injected (pulsed splitless injection, Agilent 6890 series autoinjector, Agilent Technologies, Palo Alto, CA) onto a 60-m DB-5MS column (J&W Scientific, Palo Alto, CA). GC-MS analysis was performed with an Agilent 6890 gas chromatograph and a 5973 series Agilent quadrupole mass spectrometer as described (28).

Isolation of Mitochondria and SHMT Assays—Mitochondria were prepared from 15–30 g of leaves from 4-week-old *Arabidopsis* plants as described (29), with the following modifications. The pellet containing chloroplasts and mitochondria was suspended in 2 ml of a solution containing 20 mM TES-NaOH, pH 7.2, 0.25 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$, 0.1% bovine serum albumin, 14 mM β-mercaptoethanol, applied to a step gradient composed of 2.5 ml of 21%, 5.5 ml of 26%, and 3 ml of 47% (v/v) Percoll, and centrifuged at 65,000 × *g* for 45 min in a swinging bucket rotor. Mitochondria were recovered from the 26–47% Percoll interface, diluted 12-fold in 10 mM Tricine-NaOH, pH 8.0, 1 mM EDTA, 14 mM β-mercaptoethanol, and 0.25 M sucrose, and then centrifuged at 12,500 × *g* for 20 min. This step was repeated twice. The final mitochondrial pellet was suspended in 300 μl of 10 mM Tricine-NaOH, pH 8.0, 1 mM EDTA and stored under N_2 at −80 °C until analysis. SHMT was assayed in mitochondrial extracts as described (4).

5-CHO-THF Feeding—Three wild type and three mutant plants (5 weeks old) were washed free of soil, and their root systems were severed under water, leaving ~0.5 cm of the main root. The plants were placed in Petri dishes containing 0 or 10 mM 5-CHO-THF in 0.5× Hoagland's nutrient solution and incubated in light (75 μE m^{-2} s^{-1}) at 22 °C for 24 h. The plants were then washed, frozen in liquid N_2, and lyophilized. Samples (100–150 mg) were taken for GC-MS analysis as above.

RESULTS

Identification and Characterization of an Arabidopsis 5-FCL Mutant—A potential 5-FCL mutant was identified in the Torrey Mesa Research Institute T-DNA mutant collection (23) via the sequence flanking the insert. Resequencing of this region

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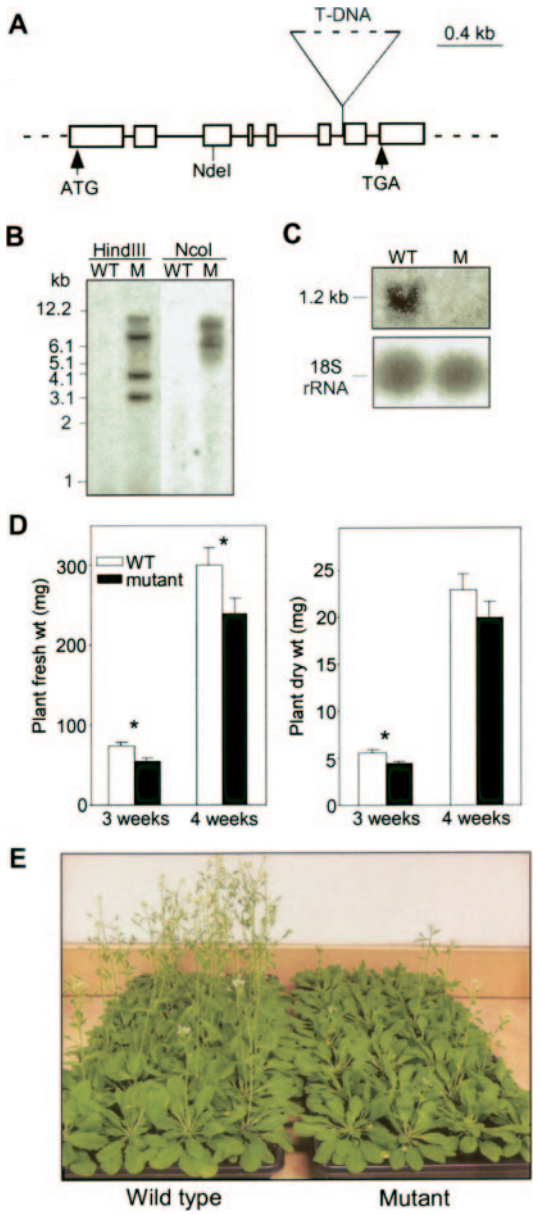

FIG. 2. **Characterization of an *Arabidopsis* T-DNA mutant of 5-FCL.** *A*, schematic of the structure of the 5-FCL gene, with the introns as *solid lines* and the exons as *boxes*. The positions of the T-DNA insertion and of the start and stop codons are indicated. *B*, Southern blot analysis of T-DNA mutants (*M*) and their wild type siblings (*WT*). The ^{32}P-labeled probe was specific for the *bar* gene within the T-DNA. Genomic DNA (5 μg/lane) was digested with HindIII (which cuts at the 5′ end of the probe) or NcoI (which does not cut in the probe). Positions of size markers are shown on the *left*. *C*, Northern blot analysis of T-DNA mutants and their wild type siblings for 5-FCL expression (*upper frame*). Lanes contained 25 μg of total RNA. The 5-FCL probe was a 373-bp NdeI-XhoI fragment of pET28b-At5FCL (4). NdeI cuts in the third exon of the 5-FCL gene, and XhoI cuts in the vector immediately after the stop codon of the 5-FCL open reading frame (*A*). The blot was stripped and rehybridized to an 18 S rRNA probe as a loading control (*lower frame*). *D*, fresh and dry weights of the aerial parts of wild type and mutant plants after 3 and 4 weeks growth at 22 °C in ambient air; data are means ± S.E. for 28–55 replicate plants. *Asterisks* mark significant differences ($p < 0.05$) between mutant and wild type. *E*, appearance of wild type and mutant plants at 7 weeks after sowing. Note the delayed flowering in the mutant.

confirmed the presence of an insert close to the 3′ end of the sixth intron, which is located within the protein-coding part of the gene (Fig. 2*A*). Plants homozygous for the mutation and their wild type siblings were identified by PCR and subjected to Southern analysis using a T-DNA sequence (a fragment of the *bar* gene) as probe (Fig. 2*B*). Only the mutant plants gave hybridizing bands, establishing that the T-DNA is inserted only at the 5-FCL locus. The multiple banding pattern in Fig. 2*B* indicates that several concatenated T-DNA copies are present at this locus. Northern analysis of leaf RNA showed no detectable 5-FCL transcript in the mutant (Fig. 2*C*), indicating a knock-out mutation. The homozygous mutants and their wild type siblings were therefore propagated for further work. When grown in soil at ambient levels of CO_2 (~370 μmol of CO_2 mol^{-1}), visible differences between mutant plants and their wild type siblings were modest. The growth rate of mutant plants was ~20% lower (Fig. 2*D*), and they showed a flowering delay of about 1 week (Fig. 2*E*). There was no difference in leaf color or form (Fig. 2*E*).

Folate Metabolic Profiling and Its Interpretation—Folates were treated with conjugase to convert them to monoglutamyl form, purified by affinity chromatography (26), and then separated by HPLC with electrochemical detection (27), which was chosen for its selectivity and sensitivity. These procedures do not distinguish between 10-CHO-THF and 5,10-CH=THF, which are interconverted during sample processing and finally both measured as 5,10-CH=THF (27). (Efforts were made to preserve 5,10-CH=THF by extraction in maleate buffer (30) and to estimate it as an increase in 5-CH_3-THF following $NaBH_4$ reduction (31), but this maneuver gave poor results with plant samples.) Other HPLC-based procedures likewise fail to distinguish 10-CHO-THF from 5,10-CH=THF, measuring both as 10-CHO-THF (30). It is thus currently not feasible to determine the individual amounts of 10-CHO-THF and 5,10-CH=THF present *in vivo*, but only their sum (henceforth termed 10-CHO-/5,10-CH=THF). Similarly, it should be noted that 5,10-methylene-THF (5,10-CH_2-THF) dissociates completely to THF during processing so that THF measurements are the sum of THF and 5,10-CH_2-THF (henceforth termed THF/5,10-CH_2-THF).

Folates in Leaves Exposed to Various CO_2 Concentrations—Plants grown for 4 weeks in ambient air were transferred for 5 days to air containing 30 or 3200 μmol of CO_2 mol^{-1} or kept in ambient air. The lower CO_2 concentration, which is beneath the CO_2 compensation point, stimulates photorespiration (and hence the glycine → serine flux rate in mitochondria) whereas the higher one suppresses it (32). CO_2 concentration had rather little effect on folate profiles so that the divergences between wild type and mutant leaves were generally similar in all three atmospheres (Fig. 3). As might be expected, 5-CHO-THF levels were higher in the mutant (2.1- to 2.6-fold, significant at $p < 0.05$). Less expectedly, 10-CHO-/5,10-CH=THF levels were also much higher in mutant leaves exposed to 370 and 30 μmol of CO_2 mol^{-1}. Added together, these differences made the total folate content of the mutant significantly higher at the two lower CO_2 levels.

Mitochondrial Folates—Because mitochondria in photorespiring leaves are expected to be the main site of 5-CHO-THF formation and the only site of its removal by 5-FCL (Fig. 1), we investigated the impact of the 5-FCL mutation on mitochondrial folate levels of plants grown in ambient air. Four separate mitochondrial preparations were made from wild type or mutant plants (Fig. 4). Despite some variability among the preparations, mitochondria from the mutant clearly showed massive 5-CHO-THF accumulation relative to wild type (on average 8-fold, significant at $p < 0.01$) and a 120% increase in total folate. The mitochondrial 5-CHO-THF content rose from a mean value of 15% of total mitochondrial folate in the wild type to a mean of 72% in the mutant. The mitochondrial contents of 5-CH_3-THF and 10-CHO-/5,10-CH=THF did not change significantly in the mutant, nor did that of 10-formyldihydrofolate (which forms readily from 10-CHO-THF in isolated mitochon-

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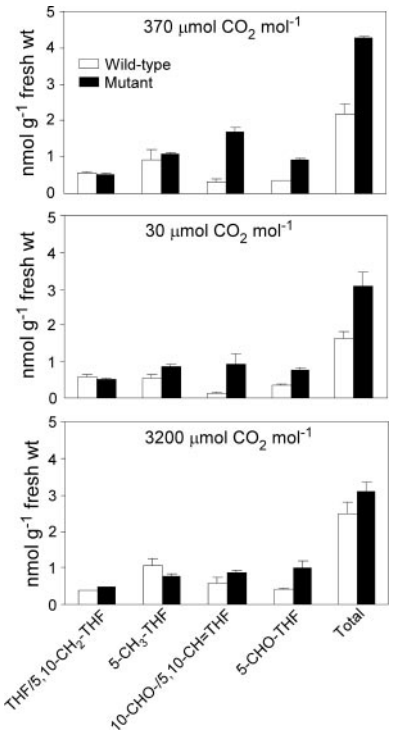

FIG. 3. **Folate profiles of wild type and mutant leaves at three CO_2 levels.** *Arabidopsis* plants were grown in ambient air and transferred for 5 days to 30 or 3200 μmol of CO_2 mol^{-1} or kept in ambient air (370 μmol of CO_2 mol^{-1}) before analysis. Data are means ± S.E. for three independent leaf samples. Folic acid was detected in some samples but never exceeded 1% of total folates; these data have been omitted for clarity.

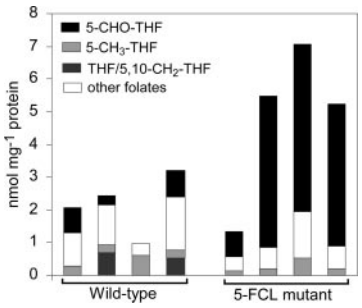

FIG. 4. **Folate profiles of purified wild type and mutant leaf mitochondria.** Four independent preparations of mitochondria were made from wild type or mutant *Arabidopsis* plants grown in ambient air. Folate analysis data are shown for each preparation separately. The category *other folates* comprises 10-CHO-/5,10-CH=THF plus 10-formyldihydrofolate. Mean values for 5-CHO-THF content of wild type and mutant mitochondria were 0.47 and 3.71 nmol mg^{-1} of protein, respectively.

dria, Ref. 33). It is noteworthy that no THF/5,10-CH_2-THF was detected in any of the mutant mitochondrial preparations. As THF and 5,10-CH_2-THF are the folates needed for glycine → serine conversion (Fig. 1), this emphasizes that the inhibitor: substrate ratio (5-CHO-THF:5,10-CH_2-THF) for SHMT is probably very high in leaf mitochondria of the mutant.

SHMT Activity in Mitochondria—The intramitochondrial 5-CHO-THF buildup in mutant plants, coupled with their fairly normal growth, led us to measure mitochondrial SHMT activities to check for a possible compensatory increase in the mutant. No such increase was found; SHMT activities in extracts of wild type and mutant mitochondria were 340 ± 32 and 336 ± 41 nmol min^{-1} mg^{-1} of protein, respectively (means ± S.E., $n = 3$).

Free Amino Acids in Leaves Exposed to Various CO_2 Concentrations—To establish whether the mitochondrial accumulation of 5-CHO-THF inhibits SHMT *in vivo*, we compared the glycine and serine contents of the leaves of wild type and mutant plants exposed for 5 days to 30, 370, or 3200 μmol of CO_2 mol^{-1} (Fig. 5). As CO_2 concentration declined, wild type leaves showed small increases in glycine whereas mutant leaves showed much larger ones, so that the glycine content of mutant leaves was 19-fold higher than the wild type at 370 μmol of CO_2 mol^{-1} and 46-fold higher at 30 μmol mol^{-1}. There was also a small accumulation of serine in the mutant relative to the wild type (1.4-fold at 370 μmol of CO_2 mol^{-1}, 1.8-fold at 30 μmol mol^{-1}, both significant at $p < 0.01$).

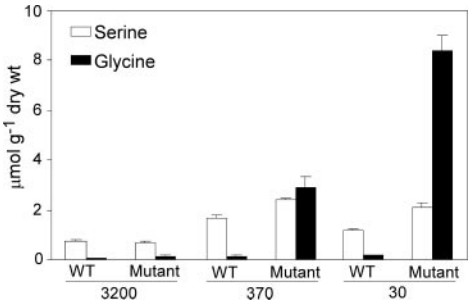

FIG. 5. **Glycine and serine levels of wild type and mutant leaves at three CO_2 levels.** *Arabidopsis* plants were grown in ambient air and transferred for 5 days to 30 or 3200 μmol of CO_2 mol^{-1} or kept in ambient air (370 μmol of CO_2 mol^{-1}) before extraction and AccQ·Fluor™ HPLC amino acid analysis. Data are means ± S.E. for three independent leaf samples, which were from the same type of experiment as Fig. 3.

Besides serine, amino acids such as glutamate and alanine can act as amino donors in the formation of glycine from glyoxylate in the photorespiratory pathway (34). The levels of glutamate, alanine, and also aspartate were substantially reduced in the mutant compared with wild type at 30 μmol of CO_2 mol^{-1} (Table I) but not at the other CO_2 concentrations (not shown).

Effect of Supplied 5-CHO-THF on Glycine and Serine Content—Because exogenous 5-CHO-THF is taken up by *Arabidopsis* and enters mitochondria (35), we examined the effect of feeding 5-CHO-THF via the transpiration stream to illuminated wild type and mutant plants. Leaf glycine and serine levels were measured after 24 h of continuous light (Fig. 6). Consistent with *in vivo* inhibition of SHMT, 5-CHO-THF feeding raised glycine levels in wild type and mutant plants significantly ($p < 0.05$). The increase in glycine was larger in the mutant (9.4 *versus* 2.5 μmol g^{-1} dry weight).

DISCUSSION

Our data demonstrate that 5-CHO-THF can inhibit the activity of mitochondrial SHMT *in vivo* and thus provide support for the view that 5-CHO-THF regulates one-carbon metabolism (5). Despite its important implications, there is rather little evidence for or against this view, and it remains controversial (8). However the most striking aspect of our findings is that 5-FCL ablation and the ensuing 5-CHO-THF buildup in mitochondria had so little impact on plant performance. *A priori*, this impact seemed likely to be devastating, above all to photorespiring leaves (4). We therefore conclude that plants are surprisingly tolerant of 5-CHO-THF, as presaged by earlier reports of remarkably high levels of this folate in leaf mitochondria (10, 11). Several mechanisms could contribute to this tolerance.

The first may be the ability of an expanded glycine pool to offset inhibition of SHMT by 5-CHO-THF. Assuming a matrix volume/protein ratio of 1–2 μl mg^{-1} (36), the concentration of 5-CHO-THF in leaf mitochondria can be estimated from the data in Fig. 4 to be ~0.25–0.5 mM in the wild type and ~2–4

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TABLE I
Levels of alanine, glutamate, and aspartate in leaves exposed to 30 μmol of CO_2 mol^{-1}

Data are means of three replicates ± S.E.

Amino acid	Amino acid level	
	Wild type	Mutant
	μmol g^{-1} dry wt	
Alanine	0.80 ± 0.26	0.32 ± 0.03
Glutamate	2.55 ± 0.12	1.23 ± 0.10[a]
Aspartate	1.15 ± 0.07	0.22 ± 0.04[a]

[a] Differences between mutant and wild type that are significant at $p < 0.05$.

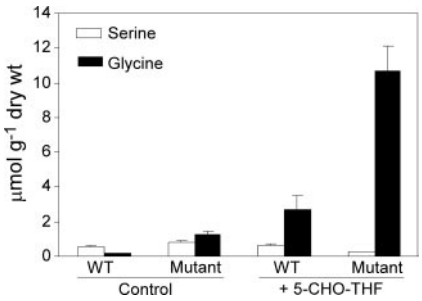

FIG. 6. **Glycine and serine levels in wild type and mutant leaves fed with 5-CHO-THF.** The shoots of wild type and mutant *Arabidopsis* plants were supplied with water or 10 mM 5-CHO-THF via the transpiration stream for 24 h in continuous light in ambient air. Derivatized samples were subjected to GC-MS amino acid analysis. Data are means ± S.E. for the leaves of triplicate plants.

mM in the mutant. Because 5-CHO-THF levels as low as 0.05–0.1 mM significantly reduce mitochondrial SHMT activity *in vitro* (4), the levels reached in the wild type would be expected to be inhibitory and those in the mutant even more so. In line with this prediction, the 5-FCL mutant accumulated more glycine than the wild type. The higher glycine level presumably compensated for the lower SHMT activity and drove a near normal flux through the vital glycine → serine reaction of photorespiration because, had it not done so, the 5-FCL mutation would have been lethal in ambient air, as are glycine decarboxylase or mitochondrial SHMT mutations (37, 38). We were able to rule out the possibility that SHMT activity increased in the 5-FCL mutant.

Another facet of tolerance to 5-CHO-THF may be its sequestration in mitochondria. Assuming that the soluble protein content of leaves is ~10 mg g^{-1} fresh weight and that mitochondrial protein is ~4% of the total (39), it can be estimated from the data of Figs. 3 and 4 that the mitochondria contain ~50% of the total 5-CHO-THF in wild type leaves and ≥80% in mutant leaves. The basis for intramitochondrial retention of 5-CHO-THF may be that it is polyglutamylated, as are the bulk of mitochondrial folates (40), and that mitochondrial folate transporters prefer monoglutamates, as do most other folate carriers (41). Keeping much of the 5-CHO-THF inside mitochondria clearly reduces its potential to interfere with cytosolic or plastidial isoforms of folate enzymes. However, this is only a partial solution because plant mitochondria themselves contain various folate-dependent enzymes (42, 43) that, in other organisms, are sensitive to 5-CHO-THF (44). These include dihydrofolate reductase and thymidylate synthase and isoforms of methionyl-tRNA transformylase, 5,10-CH=THF cyclohydrolase, 5,10-methylene-THF dehydrogenase, and the purine synthesis enzyme aminoimidazole-4-carboxamide-1-β-D-riboside transformylase.

It is therefore hard to escape the conclusion that another mechanism of plant tolerance to 5-CHO-THF is either relative insensitivity of mitochondrial enzymes to this compound or metabolic plasticity in the form of flux redistribution from mitochondrial to extramitochondrial isoforms, where the latter exist. Some support for flux redistribution comes from the accumulation of 10-CHO-/5,10-CH=THF in photorespiring mutant plants. This must have been outside mitochondria as the mitochondrial 10-CHO-/5,10-CH=THF pool did not change. Perhaps the extramitochondrial pool expands because of interference with mitochondrial one-carbon metabolism and then drives compensating fluxes through non-mitochondrial reactions. Although 10-CHO-THF and 5,10-CH=THF could not be distinguished analytically, it seems probable that 10-CHO-THF predominates *in vivo* because the chemical equilibrium between the two forms lies strongly (~95%) toward 10-CHO-THF at physiological pH (30).

Apparent insensitivity could be due, in part at least, to the sheer abundance of mitochondrial folate-dependent enzymes: SHMT and glycine decarboxylase alone constitute up to 40% of soluble mitochondrial protein (45). At such high abundances, it can be calculated from the data of Fig. 4 that even in mutant mitochondria, containing ~4 nmol of 5-CHO-THF mg^{-1} of protein, folate binding sites probably still outnumber 5-CHO-THF molecules. Furthermore, binding of 5-CHO-THF to SHMT and glycine decarboxylase may decrease the inhibition of other folate-dependent enzymes in mitochondria.

Because 5-CHO-THF is quite chemically stable and 5-FCL is the only enzyme known to metabolize it, the action of SHMT in a 5-FCL mutant would in the long run be expected to convert much of the cellular folate pool to 5-CHO-THF, especially under photorespiratory conditions. That the total leaf 5-CHO-THF pool increased no more than 2.6-fold (to a maximum of 31% of total folate) suggests that there may be another, unknown, way to metabolize 5-CHO-THF, or in effect to detoxify it, and that this contributes to tolerance. The same may hold true of yeast, where the 5-CHO-THF level increased only 4-fold in a 5-FCL knock-out (2), and of those bacteria that apparently lack 5-FCL genes (17, 18). Unlike the 5-FCL reaction, which salvages 5-CHO-THF as an intact folate, any alternative detoxification route seems likely to entail cleavage of the *p*-aminobenzoate-glutamate bond or the pteridine-*p*-aminobenzoate bond (2, 46). In support of the latter possibility, exploratory work in our laboratory has shown a large (~15-fold) accumulation of a novel pteridine in the leaves of 5-FCL mutant plants.

Finally, we saw no evidence that 5-FCL moonlights as a folate-degrading enzyme in plants, as has been suggested for the mammalian enzyme (19). Although total folate content indeed increased in the 5-FCL mutant, it did so only under photorespiratory conditions, suggesting that the increase was not due to the mutation *per se* but rather to its metabolic sequelae.

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