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Isolation, Characterization, and Functional Expression of cDNAs Encoding NADH-dependent Methylenetetrahydrofolate Reductase from Higher Plants*

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Methylenetetrahydrofolate reductase (MTHFR) is the least understood enzyme of folate-mediated one-carbon metabolism in plants. Genomics-based approaches were used to identify one maize and two *Arabidopsis* cDNAs specifying proteins homologous to MTHFRs from other organisms. These cDNAs encode functional MTHFRs, as evidenced by their ability to complement a yeast *met12 met13* mutant, and by the presence of MTHFR activity in extracts of complemented yeast cells. Deduced sequence analysis shows that the plant MTHFR polypeptides are of similar size (66 kDa) and domain structure to other eukaryotic MTHFRs, and lack obvious targeting sequences. Southern analyses and genomic evidence indicate that *Arabidopsis* has two MTHFR genes and that maize has at least two. A carboxyl-terminal polyhistidine tag was added to one *Arabidopsis* MTHFR, and used to purify the enzyme 640-fold to apparent homogeneity. Size exclusion chromatography and denaturing gel electrophoresis of the recombinant enzyme indicate that it exists as a dimer of ≈66-kDa subunits. Unlike mammalian MTHFR, the plant enzymes strongly prefer NADH to NADPH, and are not inhibited by *S*-adenosylmethionine. An NADH-dependent MTHFR reaction could be reversible in plant cytosol, where the NADH/NAD ratio is 10^{-3}. Consistent with this, leaf tissues metabolized [*methyl*-^{14}C]methyltetrahydrofolate to serine, sugars, and starch. A reversible MTHFR reaction would obviate the need for inhibition by *S*-adenosylmethionine to prevent excessive conversion of methylene- to methyltetrahydrofolate.

Methylenetetrahydrofolate reductase (MTHFR)[1] catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH_2-THF) to 5-methyltetrahydrofolate (CH_3-THF), which then serves as a methyl donor for methionine synthesis from homocysteine. The MTHFR proteins and genes of *Escherichia coli* and mammalian liver have been characterized (1–4), and MTHFR genes have been identified in *Saccharomyces cerevisiae* (5) and other organisms. The MTHFR of *E. coli* (MetF) is a homotetramer of 33-kDa subunits that prefers NADH as reductant (1), whereas mammalian MTHFRs are homodimers of 77-kDa subunits that prefer NADPH and are allosterically inhibited by *S*-adenosylmethionine (AdoMet) (2, 3). Two domains have been identified in mammalian MTHFR polypeptides. The NH_2-terminal catalytic domain (about 40 kDa) shows 30% sequence identity to *E. coli* MetF and, like MetF, contains FAD as a noncovalently bound prosthetic group (2). The COOH-terminal domain contains the AdoMet binding site; [*methyl*-^{3}H]AdoMet photoaffinity labeling located this site about 50 residues from the junction between the domains (2, 3). Yeast and other eukaryotic MTHFRs have a two-domain structure similar to the mammalian enzyme (5, 6).

The MTHFR reaction in liver is physiologically irreversible, due to a combination of the large standard free energy change for the reduction of CH_2-THF by NADPH ($\Delta G^{0'}$ = -5.2 kcal mol^{-1}) and the high NADPH/NADP ratio in the cytoplasm (7, 8). A corollary of this irreversibility is that MTHFR has the potential to deplete the pool of CH_2-THF, reducing its availability for synthesis of thymidylate and purines (9, 10). The AdoMet sensitivity of the liver enzyme functions to check such depletion, leaving CH_2-THF available for other metabolic demands (9, 10). Thus, mammalian MTHFR commits one-carbon units to methyl group synthesis and is considered to have a key regulatory role in one-carbon metabolism.

In contrast to the detailed information about MTHFR from mammals and *E. coli*, there are few data on plant MTHFR and no genes have been identified (11, 12), making it the least understood enzyme of folate-mediated one-carbon metabolism in plants. MTHFR activity has been detected in crude extracts of pea tissues using a CH_3-THF-menadione oxidoreductase (*i.e.* reverse direction) assay, and found to be insensitive to methionine (13). The reductant has not been identified. No plant MTHFRs have been purified, and the size and number of their subunits remain unknown. This dearth of information on plant MTHFRs and their regulatory properties has become critical

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF174486 (Zea mays, ZmMTHFR-1), AF181966 (Arabidopsis thaliana AtMTHFR-1), and AF181967 (A. thaliana AtMTHFR-2).

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[1] The abbreviations used are: MTHFR, methylenetetrahydrofolate reductase (EC 1.5.1.20 and 1.7.99.5); CH_2-THF, 5,10-methylenetetrahydrofolate; CH_3-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolic acid; AdoMet, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; EST, expressed sequence tag; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; SMM, L-*S*-methylmethionine; PPFD, photosynthetic photon flux density; NTA, nitrilotriacetic acid; E, einstein(s); TLC, thin layer chromatography.

with the start of work on plant metabolic engineering, because success in many current projects may depend upon understanding and modifying the mechanisms whereby plants balance the demands for methyl groups and other one-carbon moieties. Such projects include engineering the accumulation of betaines or methylated polyols, modifying lignins, and enhancing the synthesis of pharmaceutical alkaloids (14–16).

In this study, we used genomics-based approaches to identify plant MTHFR cDNAs, and expressed them in yeast. The recombinant enzymes were partially characterized, providing a foundation for more detailed study of their catalytic and regulatory properties. We identified cDNAs from plants with the C_3 and C_4 pathways of photosynthesis (*Arabidopsis* and maize, respectively) because C_3 and C_4 species differ in one-carbon metabolism, the former having a large photorespiratory carbon flux through glycine and serine (17). In addition, we developed a sensitive and specific NAD(P)H-CH_2-THF oxidoreductase (*i.e.* forward direction) radioassay that can be used with crude extracts. The results indicate that, in contrast to the mammalian enzymes, the MTHFRs from *Arabidopsis* and maize use NADH as the reductant, and that AdoMet does not feedback-inhibit their activity.

EXPERIMENTAL PROCEDURES

Chemicals—[^{14}C]Formaldehyde (53 mCi mmol^{-1}) was purchased from NEN Life Science Products, and (6*R*,6*S*)-[*methyl*-^{14}C]CH_3-THF (56 mCi mmol^{-1}) from Amersham Pharmacia Biotech; specific radioactivities were adjusted to the desired values with unlabeled compound. (6*R*,6*S*)-Tetrahydrofolic acid (THF) and (6*R*,6*S*)-CH_3-THF were obtained from Schircks Laboratories (Jona, Switzerland). The purity of THF was 86%, estimated by letting the NADH-CH_2-THF oxidoreductase reaction go to completion. CH_3-THF and $^{14}CH_3$-THF were dissolved in 8 mM sodium ascorbate and stored at −80 °C. THF was dissolved just before use in N_2-gassed 0.25 M triethanolamine-HCl, pH 7, containing 40 mM 2-mercaptoethanol. Glucose-6-phosphate dehydrogenase (recombinant *Leuconostoc mesenteroides* enzyme) and all other biochemicals were from Sigma. Ion exchange resins were from Bio-Rad. Cellulose (0.1-mm) and Silica Gel 60 (0.25-mm) TLC plates were from Merck.

Plant Materials—Arabidopsis plants (ecotype Columbia) were grown in potting soil in a culture room at 26 °C under 14-h days (PPFD = 80 μE m^{-2} s^{-1}). Maize (cv. Florida 32B) for radiotracer experiments and tobacco (cv. Wisconsin 38) were grown in soil in a greenhouse under natural lighting; the maximum temperature was 33 °C. Maize plants (cv. B73) for cDNA library construction were grown in sand in a culture room at 25 °C under 12-h days (PPFD = 300–400 μE m^{-2} s^{-1}) and irrigated with 0.25× Hoagland's nutrients; roots were harvested at 14 days of age. Spinach (cv. Savoy Hybrid 612) was grown in similar conditions and salinized with 200 mM NaCl.

Yeast Strains, Plasmids, and Growth Conditions—The *S. cerevisiae* strains used were DAY4 (*ser1 ura3–52 trp1 leu2 his4*) and RRY3 (*ser1 ura3–52 trp1 leu2 his4* Δ*met12* Δ*met13*) (5). The plasmids were pVT103-U (18), and pVT103-U containing a cDNA encoding normal human MTHFR (5). The synthetic minimal medium and culture conditions were as described (5).

cDNA Generation, Sequencing, and Sequence Analysis—Poly(A)$^+$ mRNA was isolated from maize roots as described (19), and used to construct a λUni-ZAP XR cDNA library according to the manufacturer's protocols (Stratagene). Two amino acid sequences conserved in eukaryote MTHFRs, FEFFPPKT and AVTWGVFP, were used to design the degenerate PCR primers 5′-CGARTTYTTYCCRCCVAARAC-3′ (forward primer) and 5′-GGAAAACWCCCCAMGTKACAGC-3′ (reverse primer), respectively. These were used to amplify a ≈1,500-base pair product by reverse transcription-PCR. The PCR product was cloned into the pGEM T-Easy vector (Promega); sequencing confirmed that it specified a polypeptide homologous to MTHFRs from other organisms. The 1,500-base pair fragment was then used to identify cDNAs from the maize root library. *Arabidopsis* expressed sequence tags (ESTs), GenBank accession numbers W43486 and W43508 (hereafter termed AtMTHFR-1 and -2, respectively), were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). Both strands of cDNAs were sequenced using the ABI Prism dye terminator cycle sequencing Ready Reaction (PE Applied Biosystems) and an ABI model 373 sequencer. Sequence alignments were made using Clustal W 1.7 (20). Homology searches were made using BLAST programs (21). Maize ESTs were sought in GenBank and the data base maintained by Pioneer Hi-Bred International, Inc (hereafter, Pioneer).

cDNA Expression in Yeast—Plant MTHFR coding sequences were amplified from plasmid templates by high fidelity PCR using recombinant *Pfu* DNA polymerase (Stratagene) and primers that included the first or last six codons plus *Bam*HI and *Pst*I site sequences for cloning into pVT103-U. This plasmid contains the *URA3* gene for selection and the *ADH1* promoter to drive gene expression (18). For AtMTHFR-2, the forward primer was used to add 5′-ATGAAG-3′ to restore the missing first two codons (see text). The AtMTHFR-1 coding sequence was amplified in unmodified form, and also with a five-residue histidine tag added to the COOH terminus by inserting 5′-CATCACCATCACCAT-3′ before the stop codon. After ligation into pVT103-U, constructs were introduced into *E. coli* strain DH10B by electroporation. MTHFR constructs were verified by sequencing, and used to transform yeast strain RRY3 as described (5).

Enzyme Isolation, Affinity Purification, and Molecular Mass Determination—All operations were at 0–10 °C. Yeast cultures were grown to an A_{600} of 1–2, washed, and broken by agitation (5 or 10 × 0.5 min) with glass beads in 100 mM potassium phosphate buffer, pH 6.8 or 7.2, containing 1 mM EDTA, 12–25 μM FAD, and 10% (v/v) glycerol (Buffer A) plus 1 mM PMSF (5). Where specified, a protease inhibitor mixture (Sigma P8849) was used (at 3% v/v in the extraction buffer and 1% v/v in the desalting buffer) in place of PMSF. Plant tissues were pulverized in liquid N_2 and extracted with 2 ml per g of buffer A containing 1 mM PMSF. Extracts were cleared by centrifugation (25,000 × *g*, 30 min), desalted on PD-10 columns (Amersham Pharmacia Biotech) equilibrated in buffer A, and concentrated if necessary in Centricon-30 units (Amicon). Extracts were stored at −80 °C after freezing in liquid N_2; this did not affect MTHFR activity. The histidine-tagged AtMTHFR-1 protein was purified by two cycles of affinity chromatography on Ni^{2+}-nitrilotriacetic acid (NTA) superflow resin (Qiagen) as described (22), with the following modifications. Buffers contained 10 μM FAD; binding was carried out at 40 mM imidazole, washing at 60 mM, and elution at 400 mM for the first cycle and 300 mM for the second. Native molecular mass was estimated using a Waters 626 HPLC system equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech); reference proteins were cytochrome *c*, carbonic anhydrase, bovine serum albumin, and β-amylase. SDS-polyacrylamide gel electrophoresis was carried out as described (23). Protein was estimated by Bradford's method (24) using bovine serum albumin as the standard.

Assays for MTHFR Activity—Assays were made under conditions in which substrates were saturating, and product formation was proportional to enzyme concentration and time. When imidazole and NaCl were present in enzyme preparations, their final concentrations in the assays were kept ≤45 mM to avoid inhibitory effects. CH_3-THF-menadione oxidoreductase activity was measured by a modification of published methods (25, 26). Assays (final volume 100 μl, in 1.5-ml screw-cap microcentrifuge tubes) contained 100 mM potassium phosphate buffer, pH 6.8 (shown to be the optimal pH), 2 mM EDTA, 180 nmol of sodium ascorbate, 200 nmol of formaldehyde, 2.5 nmol of FAD, 51 nmol (50 nCi) of [*methyl*-^{14}C]CH_3-THF, enzyme extract, and 25 nmol of menadione. Reactions were started by adding a 1 mM menadione solution (in water at 65 °C unless otherwise indicated) to the other components at 0 °C, incubated at 30 °C for 10 or 20 min, and stopped with 50–65 μl of dimedone reagent (26) plus 100 nmol of formaldehyde. After heating at 100 °C for 5 min, 1 ml of toluene was added and the tubes were agitated for 2 min and centrifuged (16,000 × *g*, 2 min). A sample (0.8 ml) of the toluene phase was mixed with 3 ml of scintillation fluid (Beckman Ready Gel) and counted. For assay blanks, enzyme was omitted during incubation and added just before the dimedone reagent. The reaction product was analyzed by TLC on Silica Gel 60 in methanol:acetone:HCl (90:10:4, v/v/v). Product recovery was determined to be 60 ± 3% (mean ± S.E., n = 14) by spiking unlabeled reaction mixtures with [^{14}C]formaldehyde, and experimental data were corrected accordingly.

NAD(P)H-CH_2-THF oxidoreductase activity was measured in reaction mixtures (final volume 20 μl, in 2-ml screw-cap microcentrifuge tubes) containing 100 mM potassium phosphate buffer, pH 7.2, 0.3 mM EDTA, 4 mM 2-mercaptoethanol, 42 nmol (0.1 μCi) of [^{14}C]formaldehyde, 20 nmol of THF, 0.5 nmol of FAD, 4 nmol of NAD(P)H, 20 nmol of glucose 6-phosphate, 0.06 units of glucose-6-phosphate dehydrogenase (1 unit = 1 μmol of NAD reduced min^{-1} at pH 7.2, 24 °C), and enzyme preparation. Blank assays contained no NAD(P)H. The buffer, EDTA, [^{14}C]formaldehyde, THF, and 2-mercaptoethanol were mixed and held for 5 min at 24 °C in hypoxic conditions (to allow $^{14}CH_2$-THF to form) before adding other components. Reactions were incubated at 30 °C for

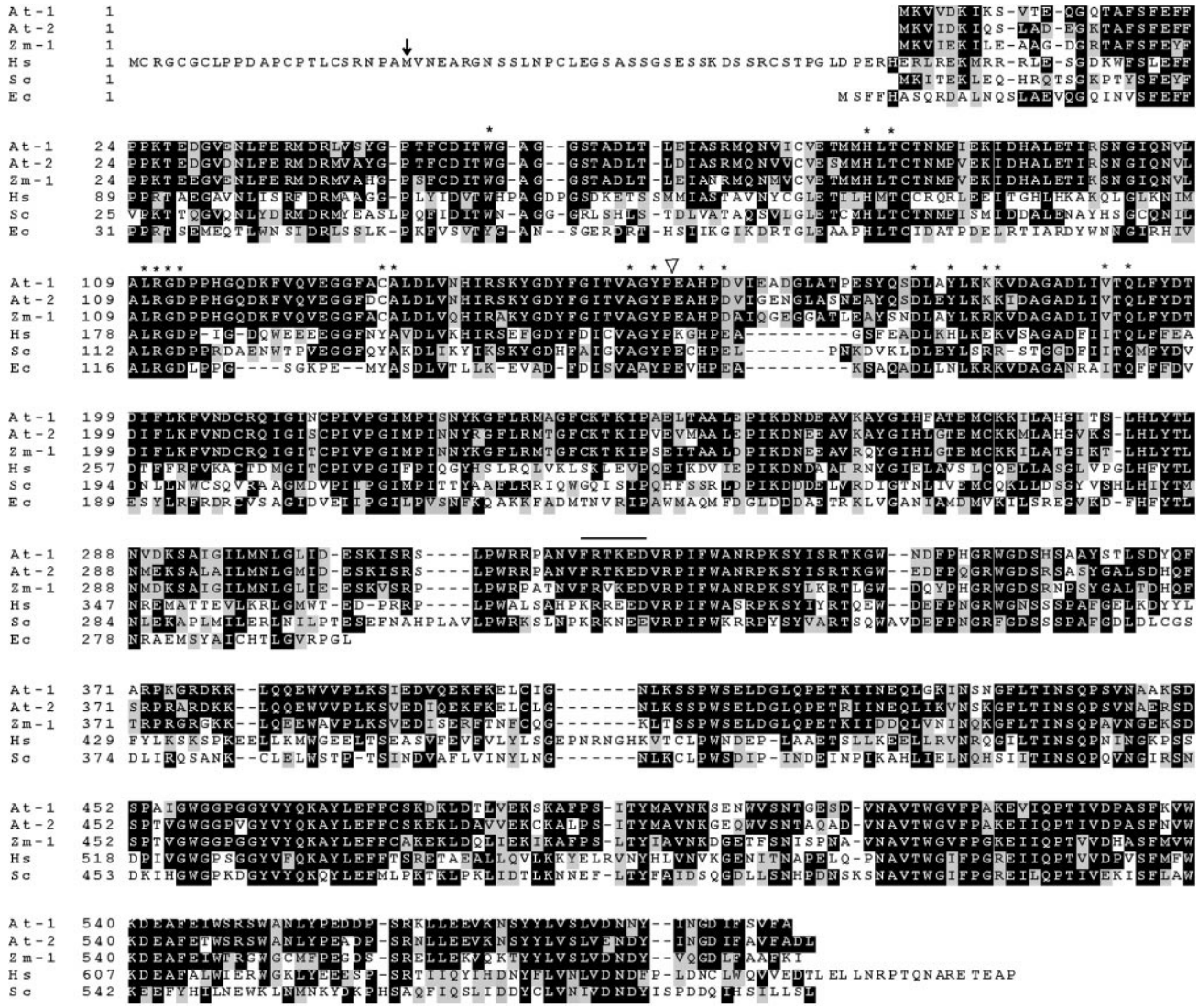

FIG. 1. **Alignment of the deduced amino acid sequences of plant MTHFRs with those from human, *S. cerevisiae*, and *E. coli*.** Identical residues are shaded in *black*, similar residues in *gray*. *Dashes* are gaps introduced to maximize alignment. *Asterisks* mark residues that interact with the FAD prosthetic group in *E. coli* (6). The *bar* indicates the hydrophilic bridge region between the domains (3). The *triangle* shows the position of an artifactual 12-residue insert in the *Arabidopsis* protein (AAC23420) predicted from genomic sequence. The *arrow* near the NH_2 terminus of the human sequence marks an alternative start site (4). *At-1* and *At-2*, AtMTHFR-1 and -2; *Zm-1*, ZmMTHFR-1; *Hs*, human MTHFR (CAB41971); *Sc*, *S. cerevisiae* Met13 (P53128); *Ec*, *E. coli* MetF (P00394). Because the AtMTHFR-2 cDNA lacked the first six nucleotides of the coding sequence, the first two residues were deduced from the genomic sequence.

20 min, and stopped by adding 1 ml of 100 mM formaldehyde. After standing for 20 min at 24 °C (to allow ^{14}C to exchange out of CH_2-THF), 0.2 ml of a slurry of AG-50(H^+) resin (1:1 with water) was added to bind $^{14}CH_3$-THF. The resin was washed with 3 × 1.5 ml of 100 mM formaldehyde, mixed with 1 ml of scintillation fluid, and counted. The counting efficiency was 40%, determined using assays spiked with $^{14}CH_3$-THF. The identity of the reaction product was verified by reverse-phase HPLC (27). NADP phosphatase activity was measured by incubating extracts with 10 mM NADP in 100 mM potassium phosphate buffer, pH 7.2, at 30 °C for 30 min, followed by enzymatic assay of NAD using yeast alcohol dehydrogenase.

[methyl-^{14}C]CH_3-THF Metabolism—Arabidopsis rosettes (240 ± 30 mg) or sets of three maize leaf discs (11 mm diameter, 70 ± 3 mg/3 discs, cut from a young blade and scarified with eight radial cuts on the abaxial surface) were allowed to absorb 0.5 μCi (9 nmol) of [*methyl*-^{14}C]CH_3-THF dissolved in 20 μl of 8 mM sodium ascorbate, minus or plus 25 mM L-serine. Label was fed to rosettes via the severed root, and to discs via the cuts; after uptake, the feeding solution was replaced by water or 25 mM serine. Incubation was in the light (PPFD = 150 μE m^{-2} s^{-1}) at 28 °C for 3.5 h. Tissues were extracted with 80% acetone, and the extract was separated into amino acid, organic acid/phosphate ester, and sugar fractions using AG-50(H^+) and AG-1 (formate) columns (28). Starch in the insoluble residue was hydrolyzed in 1 M HCl (4 h, 100 °C), and the [^{14}C]glucose formed was purified by ion exchange as above. Amino acids were separated on cellulose TLC plates in *n*-butanol:acetic acid:water (6:2:2, v/v/v) and by electrophoresis in 0.6 M HCOOH, 1.5 M CH_3COOH at 1.8 kV, 4 °C, for 20 min; detection was with ninhydrin. Serine and glycine zones were scraped from electrophoresis plates for ^{14}C assay. Sugars were separated by TLC on cellulose plates in *n*-propanol:ethyl acetate:water (7:1:2, v/v/v) and detected with alkaline $KMnO_4$. Samples spiked with [*methyl*-^{14}C]CH_3-THF were included as controls.

Southern Analyses—Arabidopsis genomic DNA was isolated from leaves as described (29). One-μg samples of the isolated DNA were digested, separated in 0.7% agarose gels, and transferred to supported nitrocellulose membrane (NitroPure, MSI) as described by Sambrook *et al.* (30). The blots were hybridized overnight at 58 °C in 5× SSC, 5× Denhardt's solution, 1% SDS, 1 mM EDTA, and 100 μg ml^{-1} sonicated salmon sperm DNA, and washed at low stringency (1× SSC, 0.1% SDS, 37 °C) (30). The probe was the full-length AtMTHFR-1 cDNA. Maize genomic DNA was prepared from 3-day-old seedlings as described (31); 6.5-μg samples were digested, separated in 0.8% agarose gels, and transferred to Duralon-UV membrane (Stratagene). Hybridization was at 42 °C in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 50% formamide,

and 100 μg ml^{-1} salmon sperm DNA. Washing was at low stringency (0.1× SSC, 0.1% SDS, 25 °C). The probe was the full-length Zm-MTHFR-1 cDNA. Probes were labeled with ^{32}P by the random primer method. Radioactive bands were detected by autoradiography.

RESULTS

Genomics-based Cloning of MTHFR cDNAs from Arabidopsis and Maize—For *Arabidopsis*, the strategy was based on a sequence from chromosome II whose conceptual translation product (unknown protein, GenBank accession no. AAC23420) is homologous to eukaryotic MTHFRs. BLAST searches using the deduced cDNA corresponding to AAC23420 detected 15 *Arabidopsis* ESTs of two types, one essentially identical to the AAC23420 nucleotide sequence, the other differing by ≈15%. Sequencing a nearly full-length representative of each type (both from hypocotyl libraries) confirmed that they encode polypeptides that are 86% identical to each other and ≈43% identical to human and yeast MTHFRs (Fig. 1). The deduced proteins are designated AtMTHFR-1 (592 residues, 66.3 kDa) and AtMTHFR-2 (594 residues, 66.8 kDa). AtMTHFR-2 is identical to the AAC23420 conceptual translation product except that the latter has a 12-residue insert (Fig. 1, *triangle*) attributable to an error made by the gene-prediction algorithm.

For maize, a homology-based PCR strategy was adopted. Two amino acid sequences conserved in eukaryotic MTHFRs were used to design degenerate PCR primers, which amplified a ≈1500-base pair fragment from a root cDNA template. Screening a root library with this fragment yielded 10 apparently full-length cDNAs with the same sequence. They encode a 593-residue (66.4 kDa) protein (ZmMTHFR-1) that is 77% identical to AtMTHFR-1 (Fig. 1). Twelve maize MTHFR ESTs were found in GenBank and Pioneer data bases, all encoding ZmMTHFR-1.

Fig. 1 shows that the deduced plant proteins are homologous to human and yeast MTHFRs throughout their entire length, and appear to lack targeting sequences (*e.g.* chloroplast or mitochondrial transit peptides). In the NH_2-terminal catalytic domain, of the 19 residues shown to interact with the FAD cofactor in the *E. coli* enzyme (Fig. 1, *asterisks*), 17 are identical or conservatively replaced in the plant sequences.

Complementation of a Yeast met12 met13 Mutant and Detection of CH_3-THF-Menadione Oxidoreductase Activity—The three plant MTHFR cDNAs were subcloned into the expression vector pVT103-U and introduced into yeast strain RRY3, a *met12 met13* double disruptant that totally lacks MTHFR activity and is a methionine auxotroph (5). All three constructs yielded methionine-independent transformants at high frequency; growth of the transformants on plates was comparable to that of the wild-type strain DAY4 (Fig. 2*A*). No complementation was observed with the vector alone (Fig. 2*A*), and retransformation of RRY3 with rescued plasmid containing the AtMTHFR-1 cDNA restored methionine prototrophy, showing that the complementation is due to the encoded plant protein. CH_3-THF-menadione oxidoreductase activity was readily detected in desalted extracts of the complemented strains but not, as expected, in RRY3 (Fig. 2, *panels B* and *C*). To authenticate the observed activity, reactions were allowed to proceed to near completion, and the labeled product was verified by TLC (Fig. 2*C*). Addition of a five-residue histidine tag to the carboxyl terminus of the AtMTHFR-1 polypeptide had no impact on complementation (results not shown) and little effect on enzyme activity (Fig. 2*B*). The specific activities of yeast extracts containing plant MTHFRs (Fig. 2*B*) are at least 150-fold greater than those of wild type yeast (5) and up to 50-fold greater than those of liver (25, 26), indicating that recombinant MTHFR proteins are expressed at a high level in yeast.

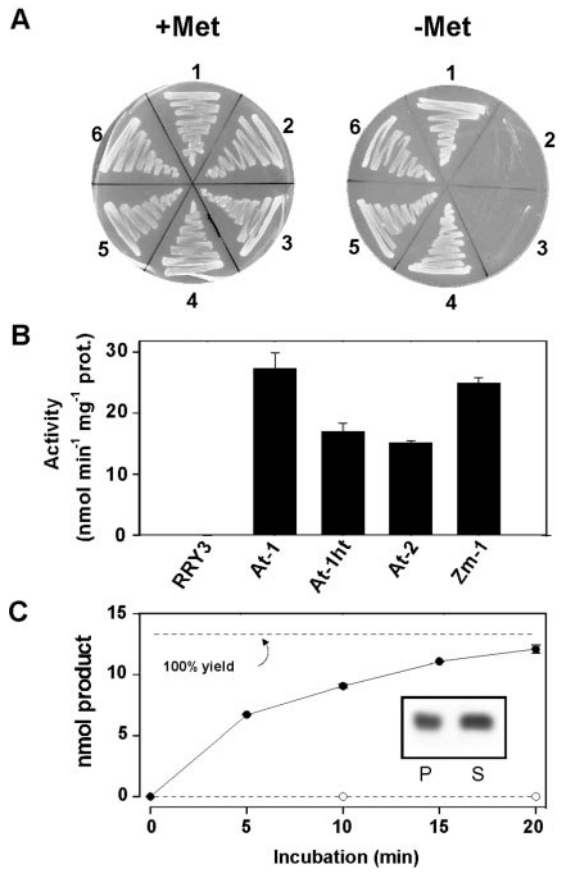

FIG. 2. **Complementation of a yeast *met12 met13* mutant by plant MTHFR cDNAs, and CH_3-THF-menadione oxidoreductase activities in complemented strains.** *A*, similar numbers of cells of DAY4 (wild-type) (*1*), the *met12 met13* mutant RRY3 (*2*), and RRY3 transformed with pVT103-U alone (*3*) or containing AtMTHFR-1 (*4*), AtMTHFR-2 (*5*), or ZmMTHFR-1 (*6*) were plated on synthetic medium with or without methionine. *B*, CH_3-THF-menadione oxidoreductase activities in desalted extracts of RRY3 or RRY3 expressing MTHFR cDNAs; *-ht*, histidine-tagged. Other abbreviations are as in Fig. 1. Data are means ±S.E. ($n \geq 3$). *C*, progress curves of menadione-dependent $^{14}CH_3$-THF oxidation catalyzed by extracts (60 μg of protein) of RRY3 (○) or RRY3 expressing AtMTHFR-1 (●). Assays contained 26.6 nmol of (6*R*,6*S*)$^{14}CH_3$-THF. The *inset* is an autoradiograph of a TLC separation of the reaction product (*P*) and of a [^{14}C]formaldemethone standard (*S*). Product formation at 20 min was 91% of the theoretical maximum (assuming half the $^{14}CH_3$-THF to be in the biologically active 6*S* form).

ular Mass Determinations—The histidine-tagged AtMTHFR-1 enzyme was purified 640-fold by two cycles of affinity chromatography on nickel-NTA resin (Table I). The specific activity of the purified enzyme assayed just after isolation (6.9 μmol min^{-1} mg^{-1} protein at 30 °C) falls between the values reported for human MTHFR and *E. coli* MetF (1, 26). The purified enzyme was found to be unstable, losing about half its activity during 3 h on ice. To investigate the mass and integrity of MTHFR subunits, the purified protein was analyzed by denaturing gel electrophoresis (Fig. 3). A 64-kDa band was evident, consistent with the size of the deduced polypeptide, and no bands of lower molecular mass. This demonstrates that the plant MTHFR protein isolated from yeast is not cleaved at the junction between the domains, a site that is particularly protease-sensitive in mammalian MTHFR and at which cleavage results in loss of AdoMet inhibition (2, 3). In the purification experiment documented in Table I and Fig. 3, a mixture of protease inhibitors (see "Experimental Procedures") was added to the buffers. Because very similar results were obtained when PMSF (1 mM) alone was added (results not shown), for all other

TABLE I

Affinity purification of the histidine-tagged form of AtMTHFR-1

The starting material was 1.5 g (wet weight) of cells from a 0.5-liter culture. Proteins were extracted and desalted in buffers containing a proteinase inhibitor mixture (see "Experimental Procedures"). In cycle 1, enzyme was bound at pH 7.5 to nickel-NTA resin, using 50 mM sodium phosphate buffer containing 300 mM NaCl and 40 mM imidazole; the imidazole concentration was raised to 60 mM for washing, and to 400 mM for elution. After diluting the imidazole concentration to 40 mM, the process was repeated for cycle 2 except that elution was with 300 mM imidazole. Activity was measured at 30 °C using the CH_3-THF-menadione oxidoreductase assay, with 20% methanol as the solvent for menadione. One milliunit equals oxidation of 1 nmol of CH_3-THF min^{-1}.

Step	Protein	Activity	Specific activity	Yield	Purification
	mg	*milliunits*	*milliunits* mg^{-1}	*%*	*-fold*
Desalted extract	21.4	230	10.8	100	1
Cycle 1 eluate	0.042	89.7	2140	39	200
Cycle 2 eluate	0.0044	30.4	6920	13	643

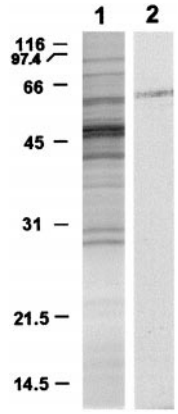

FIG. 3. **Molecular mass of denatured AtMTHFR-1.** The histidine-tagged form of AtMTHFR-1 was affinity-purified by two cycles of nickel-NTA chromatography, separated by SDS-polyacrylamide gel, and stained with Coomassie blue. Proteins were extracted and desalted in buffers containing a proteinase inhibitor mixture (see "Experimental Procedures"). *Lane 1* was loaded with 25 μg of protein from the fraction not bound by the resin, and *lane 2* with 0.22 μg of purified protein. The fractions analyzed were from the experiment summarized in Table I. The positions of molecular mass markers (kDa) are indicated.

work we used PMSF. The molecular mass of the native AtMTHFR-1 enzyme was estimated by size exclusion chromatography (results not shown). The protein migrated as a symmetrical peak with an apparent molecular mass of 141 kDa, which is consistent with a dimer of 66-kDa subunits.

Pyridine Nucleotide Preference—NAD(P)H-CH_2-THF oxidoreductase activity cannot be measured spectrophotometrically in crude extracts due to the presence of NAD(P)H oxidase (26), and spectrophotometric assays are in any case fairly insensitive. We therefore developed a radiometric assay to study the pyridine nucleotide specificity of MTHFRs using desalted crude extracts or small amounts of purified enzyme. In this assay, $^{14}CH_2$-THF (prepared from THF and excess $H^{14}CHO$) is incubated with enzyme, NAD(P)H, and an NAD(P)H recycling system (to prevent any NAD(P) formed from supporting $^{14}CH_2$-THF oxidation by CH_2-THF dehydrogenases). Label remaining in $^{14}CH_2$-THF is then exchanged out into an excess of unlabeled HCHO and the $^{14}CH_3$-THF formed is bound to a cation exchange resin, which is washed and counted. The assay was validated by comparing extracts of RRY3 (MTHFR-deficient) and RRY3 expressing AtMTHFR-1. No activity was detected in RRY3; product formation with the AtMTHFR-1 extract was dependent on pyridine nucleotide and THF, and slightly promoted by FAD (Fig. 4*A*). The reaction product was confirmed to be $^{14}CH_3$-THF by reverse-phase HPLC (Fig. 4*B*).

Using this assay, the three recombinant plant MTHFRs were found to strongly prefer NADH; the activities with 200 μM NADPH were <2% of those with 200 μM NADH, which was a saturating concentration (Table II). Recombinant human enzyme (HsMTHFR) was tested as a control and shown to be NADPH-dependent (Table II), as it is when extracted from liver (2). The NADH-CH_2-THF oxidoreductase/CH_3-THF-menadione oxidoreductase activity ratio for the plant enzymes was 0.9 ± 0.1, similar to the corresponding ratio for mammalian MTHFR (25, 32).

Sensitivity to S-Adenosylmethionine and S-Methylmethionine—Recombinant plant MTHFR activity in desalted extracts was tested for inhibition by high concentrations (1–2 mM) of AdoMet using both NADH-CH_2-THF oxidoreductase and CH_3-THF-menadione oxidoreductase assays. Extracts were preincubated at 24–30 °C with AdoMet (or buffer for controls) before assays, because onset of AdoMet inhibition is slow (25, 26). Recombinant human enzyme (HsMTHFR) was used as a positive control to check that expression in yeast did not desensitize it to AdoMet. In both assays, the activity of the human enzyme was strongly inhibited by AdoMet, whereas that of ZmMTHFR-1 was unaffected, AtMTHFR-1 was stimulated by 10–20%, and AtMTHFR-2 was stimulated by 50–70% (Tables II and III). The effect of *S*-methylmethionine (SMM) was also tested, because SMM is a major plant metabolite whose levels can exceed those of AdoMet (33). Physiological concentrations of SMM (2–5 mM) had no effect on either CH_3-THF-menadione oxidoreductase (Table III) or NADH-CH_2-THF oxidoreductase activities (results not shown). Methionine (5 mM) or *S*-adenosylhomocysteine (2 mM) were also found to have no effect (results not shown).

NADH Preference and S-Adenosylmethionine Insensitivity of Purified AtMTHFR-1—To confirm that the pyridine nucleotide specificity and AdoMet response of the purified recombinant protein are the same as those observed in desalted extracts, the histidine-tagged form of AtMTHFR-1 was tested (Table IV). The instability of the purified enzyme resulted in significant loss of activity during preincubation with AdoMet or buffer alone. The results with purified enzyme nonetheless mirrored those with extracts: the enzyme strongly preferred NADH and was not inhibited by AdoMet. As for crude extracts, there was an apparent stimulation by AdoMet. However, in this case it was shown to be due principally to slower loss of activity during preincubation when AdoMet was present, *i.e.* to a stabilizing effect of AdoMet.

S-Adenosylmethionine-insensitive NADH-CH_2-THF Oxidoreductase Activity in Plant Extracts—To rule out the possibility that the NADH-preference and AdoMet-insensitivity of the recombinant plant enzymes are artifacts of the yeast expression system, enzymes extracted from *Arabidopsis*, maize and two other plants were tested (Table V). In root and leaf extracts of all species, the MTHFR activity showed a strong preference for NADH and was not inhibited by AdoMet; the activities of the extracts were up to ≈50-fold greater than those in liver. That the ratios of NADPH- to NADH-dependent activities were higher for plant extracts than for recombinant enzymes is attributable to conversion of NADP(H) to NAD(H) by phosphatases in the plant extracts. NADP phosphatase activities

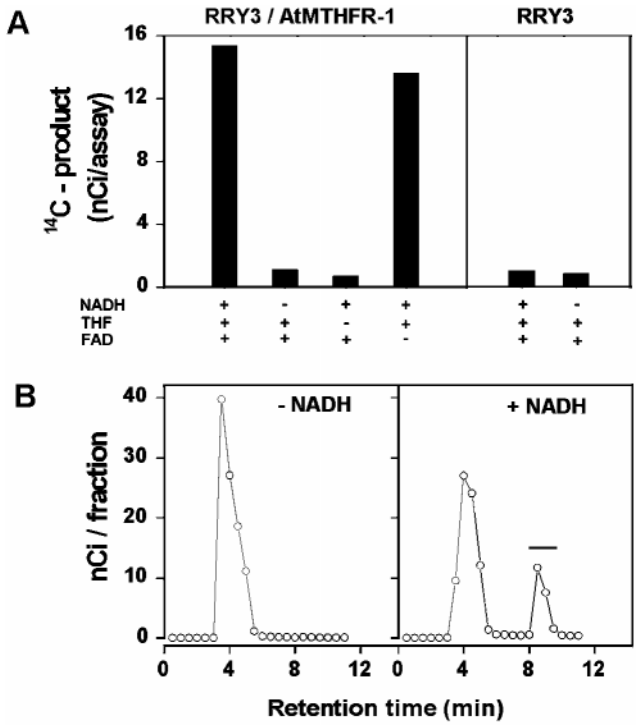

FIG. 4. **Characteristics of the NAD(P)H-CH_2-THF oxidoreductase radioassay.** *A,* effects of omitting assay components. Complete reactions contained extract (7.5 μg of protein) of RRY3 expressing AtMTHFR-1 (*left panel*) or RRY3 alone (*right panel*) and were otherwise as described under "Experimental Procedures" except that 0.2 μCi of H^{14}CHO was used. *B*, reverse-phase HPLC separation of reactions containing extract of RRY3 expressing AtMTHFR-1 (30 μg of protein) minus (*left frame*) or plus (*right frame*) NADH. Reactions were incubated at 30 °C for 45 min to ensure that they went to completion. The peak position of CH_3-THF (retention time ≈8.5 min) is shown with a *horizontal line*. The ^{14}C activity in the CH_3-THF peak represents 86% of the maximum theoretical yield. The large peak at ≈4 min is H^{14}CHO.

TABLE II

Pyridine nucleotide preferences and S-adenosylmethionine sensitivities of NAD(P)H-CH_2-THF oxidoreductase activities in cell-free extracts of transformed yeast

Desalted crude extracts of yeast cells expressing plant or human MTHFRs were assayed for NAD(P)H-CH_2-THF oxidoreductase activity as described under "Experimental Procedures" using NADH or NADPH (200 μM) as reductant, minus or plus 1 mM AdoMet. Extracts were preincubated for 15 min at 24 °C with buffer or AdoMet before the assays. Data are means of 3–8 replicates ± S.E.

cDNA expressed	NAD(P)H-CH_2-THF oxidoreductase activity		
	NADH	NADPH	NAD(P)H[a] + AdoMet
	nmol min^{-1} mg^{-1} protein		
AtMTHFR-1	20.5 ± 0.4	0.3 ± 0.03	22.0 ± 0.9
AtMTHFR-2	11.6 ± 0.2	0.1 ± 0.01	19.8 ± 0.5
ZmMTHFR-1	18.0 ± 0.2	0.1 ± 0.02	18.6 ± 0.1
HsMTHFR	0.6 ± 0.03	11.2 ± 1.0	3.8 ± 0.6

[a] NADPH for HsMTHFR; NADH for all others.

(estimated using an NADP concentration of 10 mM) in *Arabidopsis* and maize tissue extracts were 14–34 nmol min^{-1} mg^{-1} protein, which would allow significant NADH formation during the oxidoreductase assays. Yeast contained no detectable NADP phosphatase activity (<0.3 nmol min^{-1} mg^{-1} protein).

Metabolism of [methyl-^{14}C]CH_3-THF—MTHFRs in yeast and mammals are cytosolic enzymes (9), and the lack of NH_2-terminal transit sequences (Fig. 1) indicates that plant MTHFRs are likewise cytosolic. If they are, the very low NADH/NAD ratios that prevail in plant cytosol (10^{-3}) (34) might allow the MTHFR reaction to proceed in the reverse direction. An exploratory test of this possibility was made by supplying a tracer quantity of [*methyl*-^{14}C]CH_3-THF to illuminated leaf tissue and analyzing labeled metabolites (Fig. 5, *panels A* and *B*). In both *Arabidopsis* and maize, ^{14}C was readily metabolized to serine, sugars, and starch. A simple explanation for this labeling pattern is that $^{14}CH_3$-THF is oxidized to $^{14}CH_2$-THF, allowing ^{14}C to enter serine via the action of glycine hydroxymethyltransferase (11, 12). From serine, label is expected to flow to photosynthetic end products (17, 35). Consistent with this explanation, when a large dose of serine was given together with $^{14}CH_3$-THF, label was trapped in the serine pool (Fig. 5, *panels C* and *D*). That the trapping effect was less marked in the C_3 plant *Arabidopsis* may be explained by its high capacity to metabolize serine; measurements showed that ≈60% of the serine supplied to *Arabidopsis* was metabolized during the experiment.

TABLE III

Sensitivity to S-adenosylmethionine or S-methylmethionine of the CH_3-THF-menadione oxidoreductase activity in cell-free extracts of transformed yeast

Desalted crude extracts of yeast cells expressing plant or human MTHFRs were assayed for CH_3-THF oxidoreductase activity without (control) or with 2 mM AdoMet or SMM as described under "Experimental Procedures." The extracts used were the same as those in Table II. Extracts were preincubated for 10 min at 30 °C with buffer (control), AdoMet, or SMM before the assays. Data are means of three to six replicates ± S.E.

cDNA expressed	CH_3-THF-menadione oxidoreductase activity		
	Control	+AdoMet	+SMM
	nmol min^{-1} mg^{-1} protein		
AtMTHFR-1	26.3 ± 0.3	31.4 ± 0.7	23.7 ± 0.4
AtMTHFR-2	12.2 ± 0.5	18.4 ± 1.0	11.1 ± 0.9
ZmMTHFR-1	21.9 ± 0.6	22.4 ± 0.5	21.6 ± 0.9
HsMTHFR	19.7 ± 0.5	2.5 ± 0.1	19.5 ± 0.8

TABLE IV

Pyridine nucleotide preference and S-adenosylmethionine sensitivity of the affinity-purified histidine-tagged form of AtMTHFR-1

Histidine-tagged AtMTHFR-1 enzyme was purified as described in Table I and assayed for NADPH-CH_2-THF, NADH-CH_2-THF, and CH_3-THF-menadione oxidoreductase activities, preincubating without (control) or with AdoMet as described in Tables II and III. Data are means of 3 replicates ± S.E.

Oxidoreductase assay	Oxidoreductase activity	
	Control	+AdoMet
	nmol min^{-1} mg^{-1} protein	
NADPH-CH_2-THF	≤20	
NADH-CH_2-THF	1130 ± 50	1530 ± 40
CH_3-THF-menadione	2100 ± 70	3300 ± 250

Southern Analyses—Southern analyses were carried out in order to estimate the number of MTHFR genes in *Arabidopsis* and maize (Fig. 6). For *Arabidopsis* (Fig. 6, *panel A*), the sizes and intensities of hybridizing bands indicated two genes, corresponding to the AtMTHFR-1 and -2 cDNAs with respect to the predicted restriction sites. For maize (Fig. 6, *panel B*), the banding pattern indicated at least two MTHFR genes. Taken with the evidence from the data bases, the Southern analyses show that the cDNAs that we have identified represent both MTHFR genes of *Arabidopsis*, and what appears to be the most strongly and widely expressed MTHFR gene of maize.

DISCUSSION

The identification of cDNAs encoding MTHFR completes the set of plant genes required for the synthesis of methyl groups from serine and formate (12). This opens the way for systematic application of reverse genetics to investigate folate-mediated one-carbon metabolism in plants. It will also permit comprehensive studies of the expression of one-carbon metabolism

TABLE V

Pyridine nucleotide preferences and S-adenosylmethionine sensitivities of NAD(P)H-CH_2-THF oxidoreductase activities in cell-free extracts of plant tissues

Desalted crude extracts of were assayed for NAD(P)H-CH_2-THF oxidoreductase activity as described under "Experimental Procedures" using NADH or NADPH (200 μM) as reductant, without or with 1 mM AdoMet. Extracts were incubated for 15 min at 24 °C with buffer or AdoMet before the assays. Data are means of 3 replicates ± S.E.

Species	Tissue	NAD(P)H-CH_2-THF oxidoreductase activity		
		NADH	NADPH	NADH + AdoMet
		nmol min^{-1} mg^{-1} protein		
Arabidopsis	Root	14.0 ± 0.2	1.2 ± 0.1	17.7 ± 0.3
	Leaf	11.4 ± 0.1	1.1 ± 0.01	13.2 ± 0.2
Maize	Root	22.8 ± 0.5	1.9 ± 0.1	24.7 ± 0.03
	Leaf	8.1 ± 0.2	1.2 ± 0.02	8.8 ± 0.1
Tobacco	Root	19.9 ± 0.2	1.9 ± 0.2	19.6 ± 0.2
	Leaf	4.8 ± 0.03	1.0 ± 0.02	4.7 ± 0.01
Spinach	Leaf	9.4 ± 0.1	2.2 ± 0.04	11.6 ± 0.1

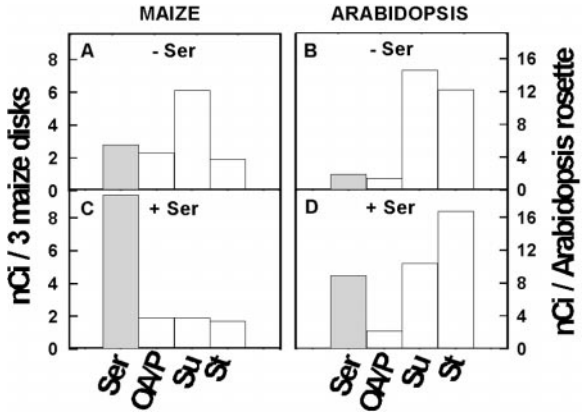

FIG. 5. **Metabolism of [*methyl*-^{14}C]CH_3-THF by illuminated leaf tissues.** *Arabidopsis* rosettes or sets of three maize leaf discs were supplied with 0.5 μCi (9 nmol) of [*methyl*-^{14}C]CH_3-THF, without (*panels A* and *B*) or with (*panels C* and *D*) 25 mM serine (see "Experimental Procedures"). Incubation was for 3.5 h at 28 °C at a PPFD of 150 μE m^{-2} s^{-1}. Serine was the major labeled amino acid in all samples; the only other amino acid that acquired significant ^{14}C (≤18% of that in serine) was glycine. *Ser*, serine; *OA/P*, organic acids and phosphate esters; *Su*, sugars; *St*, starch.

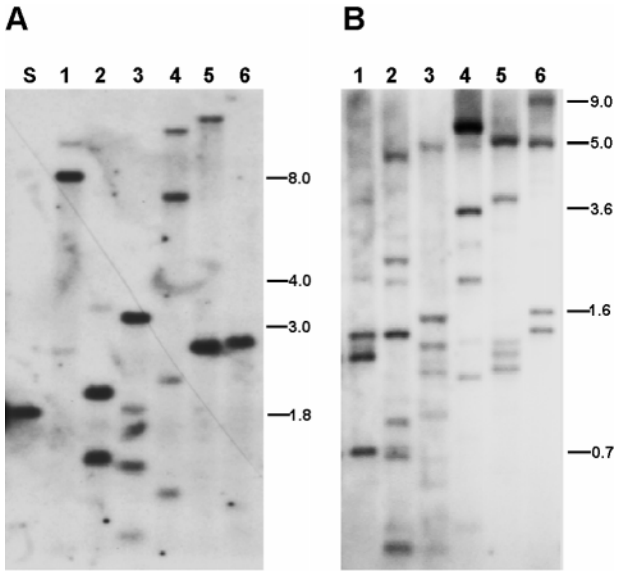

FIG. 6. **Southern analysis of MTHFR genes.** Genomic DNA was isolated from *Arabidopsis* leaves (*panel A*) or maize seedlings (*panel B*), digested with the restriction enzymes indicated, separated on agarose gels, blotted, and probed with the complete AtMTHFR-1 cDNA (*Arabidopsis*) or ZmMTHFR-1 cDNA (maize). Washing was at low stringency. The restriction enzymes used are indicated by *numbers* above the lanes. *Panel A*: *lane 1*, *Nde*I; *lane 2*, *Bgl*I; *lane 3*, *Dra*I; *lane 4*, *Nco*I; *lane 5*, *Bam*HI; *lane 6*, *Xho*I. *Panel B*: *lane 1*, *Eco*RI+*Xho*I; *lane 2*, *Eco*RI+*Bgl*II; *lane 3*, *Eco*RI+*Xmn*I; *lane 4*, *Bgl*II; *lane 5*, *Xmn*I; *lane 6*, *Eco*RI. A genomic reconstruction standard in *panel A*, made with AtMTHFR-1 cDNA equivalent to one copy per haploid genome, is indicated by the *letter S*. The positions of DNA molecular size standards (kb) are indicated beside each panel.

genes. The finding that a histidine-tagged form of AtMTHFR-1 can be expressed at a high level in yeast and readily purified will facilitate detailed analysis of the properties of this and other MTHFRs.

Plant MTHFR proteins resemble those of other eukaryotes in having a catalytic domain homologous to the *E. coli* enzyme, and a long (≈270-residue) COOH-terminal extension. Like their mammalian and yeast counterparts, plant MTHFRs appear to be cytosolic proteins inasmuch as they lack obvious targeting sequences. Despite these overall structural similarities, the plant enzymes have the opposite pyridine nucleotide preference to mammalian MTHFR, and are not inhibited by AdoMet. Because of the far-reaching implications of these conclusions for the regulation of plant one-carbon metabolism, it is important to examine the evidence for them. The conclusion that plant MTHFRs are NADH-dependent rests (i) on the properties of three different recombinant enzymes from *Arabidopsis* and maize (with control experiments in which recombinant human MTHFR expressed in the same system proved to be NADPH-dependent), and (ii) on data for enzymes isolated directly from these and two other plant species. Taken together, this evidence rules out the possibility that the NADH-dependence of the plant enzymes is an artifact of expression in yeast. The same can be concluded for the AdoMet response of the plant enzymes, because neither enzymes from plant sources nor recombinant plant MTHFRs were inhibited by AdoMet, whereas the recombinant human enzyme was inhibited. Moreover, the demonstration that recombinant plant MTHFR has intact subunits excludes the possibility that proteolytic cleavage between the catalytic and COOH-terminal domains causes the AdoMet insensitivity. This interdomain cleavage is the most likely origin of artifactual AdoMet insensitivity (2).

The lack of inhibition of plant MTHFRs by AdoMet seems most likely to be due to absence of an AdoMet binding site. Photoaffinity labeling data (3) locate the binding site in mammalian MTHFR some 50 residues from the junction between the domains (3), so it may be significant that the human and plant sequences diverge substantially in this region (Fig. 1). About 80 residues from the junction, the human enzyme has a seven-residue insertion that is absent from plant and yeast MTHFRs. However, our preliminary data indicate that the yeast Met13 enzyme is NADPH-dependent and inhibited by AdoMet,[2] suggesting that the insert does not relate to AdoMet binding. If plant MTHFRs do not bind AdoMet, the overall sequence conservation between the mammalian, yeast and plant COOH-terminal domains would suggest that these have other functions that remain to be discovered. It is also possible that plant MTHFRs bind AdoMet but are not inhibited by it. The moderate stabilizing or stimulatory effects of AdoMet on the activities of *Arabidopsis* MTHFRs are consistent with such a possibility, and merit further investigation.

That plant MTHFRs use NADH rather than NADPH as reductant suggests that the MTHFR reaction is reversible under physiological conditions. The equilibrium constant (K_{eq}) for the reductive reaction has been determined (8) to be 4.5×10^{10}.

[2] Roje and Raymond, unpublished results.

$$K_{eq} = \frac{[CH_3\text{-THF}][NAD]}{[CH_2\text{-THF}][NADH][H^+]} = 4.5 \times 10^{10}\ \text{M}^{-1} \qquad \text{(Eq. 1)}$$

At a pH of 7.6 ($[H^+] = 2.5 \times 10^{-8}$ M), the cytosolic NADH and NAD concentrations in illuminated spinach leaves have been estimated as 7×10^{-7} and 6×10^{-4} M, respectively (34). Using these values in Equation 1 gives a value of 1.3 for the CH_3-THF/CH_2-THF ratio at equilibrium. A value so close to unity connotes a freely reversible reaction in the cytosol ($\Delta G \approx 0$). A physiologically reversible MTHFR reaction could account for the absence of allosteric inhibition by AdoMet in the plant enzymes, since a reversible reaction could maintain an adequate pool of CH_2-THF for thymidylate and purine synthesis, without need of a feedback signal from methyl metabolism. Similar considerations may apply to *E. coli* MTHFR, which is also NADH-dependent and AdoMet-insensitive, as the NADH/NAD ratio is very low in aerobically grown *E. coli* cells (36). Note that for ready interconversion of CH_3-THF and CH_2-THF to occur, the thermodynamic reversibility of Equation 1 must be accompanied by kinetic reversibility. Thus, the forward and reverse rates of the MTHFR reaction *in vivo* would need to be at least as great as those for other reactions forming and consuming CH_3-THF and CH_2-THF, otherwise the calculated ratio of ≈1 would probably not hold. Because the MTHFR activities measured in plant extracts (5–25 nmol min^{-1} mg^{-1} protein) are similar to or higher than those reported for methionine synthase, cytosolic glycine hydroxymethyl transferase and CH_2-THF dehydrogenase (37–40), this condition may be met. Moreover, indirect evidence indicates that the CH_2-THF level in illuminated leaves may be approximately the same as the CH_3-THF level (37).

The exploratory radiotracer tests that we made for *in vivo* reversibility of the MTHFR reaction establish that leaves readily metabolize the methyl group of CH_3-THF to serine, and thence to carbohydrates. This result is consistent with conversion of $^{14}CH_3$-THF to $^{14}CH_2$-THF through the action of MTHFR, but not proof of it. Plants lack glycine *N*-methyltransferase and sarcosine dehydrogenase (11, 37), whose sequential action in animal tissues provides a route to convert the methyl group of AdoMet, via formaldehyde, to CH_2-THF (9). However, while there are no reports that it occurs in plants, oxidative demethylation of $^{14}CH_3$-THF, or of methylated products derived from it, could potentially generate [^{14}C]formaldehyde and hence $^{14}CH_2$-THF and [^{14}C]serine. Other caveats are that the (necessarily) large dose of $^{14}CH_3$-THF used may have perturbed one-carbon metabolism, and that the monoglutamyl form supplied may not have acted as a faithful tracer for endogenous polyglutamylated forms. The direct conversion of $^{14}CH_3$-THF to $^{14}CH_2$-THF via MTHFR nonetheless remains the simplest explanation of our ^{14}C-tracer results.

Based on the thermodynamic considerations outlined above, together with the $^{14}CH_3$-THF metabolism data, we suggest that the MTHFR reaction is reversible in plants. Support for this comes from early work by Clandinin and Cossins (41), who showed that germinating peas converted supplied $^{14}CH_3$-THF to 5- and 10-[^{14}C]formyl-THF.

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