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Choline Import into Chloroplasts Limits Glycine Betaine Synthesis in Tobacco: Analysis of Plants Engineered with a Chloroplastic or a Cytosolic Pathway

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The biosynthesis of the osmoprotectant glycine betaine (GlyBet) is a target for metabolic engineering to enhance stress resistance in crops. Certain plants synthesize GlyBet in chloroplasts via a two-step oxidation of choline (Cho). In previous work, a chloroplastic GlyBet synthesis pathway was inserted into tobacco (which lacks GlyBet) by expressing spinach choline monooxygenase (CMO). The transformants had low CMO enzyme activity, and produced little GlyBet ($\leqslant 70$ nmol g^{-1} fresh wt). In this study, transformants with up to 100-fold higher CMO activity showed no further increase in GlyBet. In contrast, tobacco expressing a cytosolic GlyBet synthesis pathway accumulated significantly more GlyBet (430 nmol g^{-1} fresh wt), suggesting that subcellular localization influences pathway flux. Modeling of the labeling kinetics of Cho metabolites observed when [^{14}C]Cho was supplied to engineered plants demonstrated that Cho import into chloroplasts indeed limits the flux to GlyBet in the chloroplastic pathway. A high-activity Cho transporter in the chloroplast envelope may therefore be an integral part of the GlyBet synthesis pathway in species that accumulate GlyBet naturally, and hence a target for future engineering. © 2000 Academic Press

Key Words: **glycine betaine; choline; choline monooxygenase; choline oxidase; pathway modeling; *Nicotiana tabacum*.**

INTRODUCTION

Drought and salinity severely reduce agricultural productivity worldwide (Boyer, 1982). One engineering strategy to improve the resistance of crops to these stresses is to introduce or increase the capacity to synthesize osmoprotectants (Holmberg and Bülow, 1998; Nuccio *et al.*, 1999). Osmoprotectants are non-toxic solutes that can raise the osmotic pressure of the cytoplasm without disrupting metabolism; they also stabilize proteins and membranes (Yancey, 1994). Osmoprotectant accumulation is one of the mechanisms whereby plants maintain turgor and the driving gradient for water uptake in the face of osmotic stress (Jain and Selvaraj, 1997).

Glycine betaine (GlyBet) is a potent osmoprotectant produced by certain higher plants and bacteria (Rhodes and Hanson, 1993; Kempf and Bremer, 1998). Plants of the family Chenopodiaceae (e.g., spinach) synthesize GlyBet in chloroplasts by oxidation of choline (Cho) via the intermediate betaine aldehyde (Fig. 1A), the Cho being made outside the chloroplast (Weretilnyk *et al.*, 1995; McNeil *et al.*, 2000a). The first Cho oxidation step is catalyzed by a ferredoxin-dependent Cho monooxygenase (CMO) and the second by an NAD-linked betaine aldehyde dehydrogenase (BADH); both enzymes are present in unstressed plants and are further induced by salinity (Rhodes and Hanson, 1993). Plant BADHs are fairly non-specific enzymes that also occur, at least at low levels, in species that lack GlyBet (Trossat *et al.*, 1997). Bacteria also form GlyBet from Cho but do not use CMO; *Arthrobacter* spp. have instead a soluble Cho oxidase (COX) that generates H_2O_2 (Fig. 1B), and *Escherichia coli* has an electron transfer-linked Cho dehydrogenase (Landfald and Strom, 1986; Rozwadowski *et al.*, 1991; Deshnium *et al.*, 1995).

Transgenic expression of CMO, COX or Cho dehydrogenase is sufficient to introduce GlyBet synthesis into plants that essentially lack it, such as tobacco, Arabidopsis and canola (Huang *et al.*, 2000; Nuccio *et al.*, 1999, and

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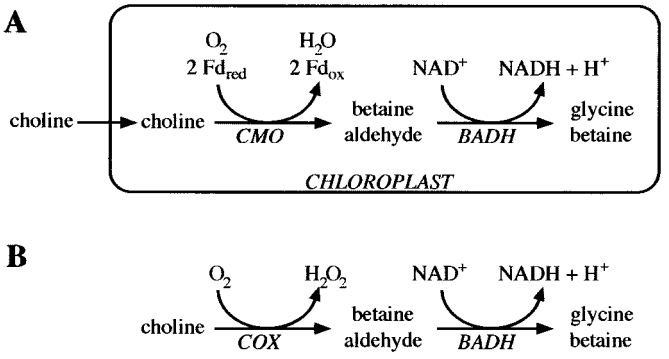

FIG. 1. GlyBet biosynthesis. (A) The chloroplastic pathway that occurs in spinach and other Chenopodiaceae, and that has been engineered in tobacco by introducing spinach CMO. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin. (B) The pathway in *Arthrobacter* spp. Note that COX itself can also mediate betaine aldehyde oxidation although the K_m for betaine aldehyde is much higher than for Cho (Rozwadowski *et al.*, 1991).

references therein). However, GlyBet levels in the transformants ($\approx$0.05–2 μmol g^{-1} fresh wt, Fw) are $\approx$10- to 100-fold below those in plants that accumulate GlyBet naturally. Encouragingly, these transformants typically display small but significant increases in stress tolerance (Nuccio *et al.*, 1999). It is therefore desirable to attain higher GlyBet levels, which requires determination of what limits flux in the engineered biosynthetic pathway. In tobacco, plants expressing CMO in chloroplasts accumulate $\approx$0.05 μmol g^{-1} Fw GlyBet, and plants expressing the *Arthrobacter pascens* Cho oxidase in the cytosol accumulate $\approx$1 μmol g^{-1} Fw GlyBet (Nuccio *et al.*, 1998; Huang *et al.*, 2000). The low GlyBet levels in these transgenics are not due to GlyBet degradation (Nuccio *et al.*, 1998). Nor, at least in plants expressing CMO, do they seem to be due to inadequate endogenous BADH activity as betaine aldehyde does not accumulate (Nuccio *et al.*, 1998).

For transgenic tobacco expressing spinach CMO in chloroplasts at 1–3% of the level found in salinized spinach, we showed that GlyBet accumulation is limited by the level of CMO and by the endogenous Cho supply (Nuccio *et al.*, 1998). For these plants, the flux control coefficient of CMO on GlyBet synthesis was estimated as 0.24 (Nuccio *et al.*, 1998); theory suggests that with a coefficient of this order, the increase in flux to GlyBet achievable by raising CMO activity alone would be quite limited (Fell, 1997). To guide future engineering of the GlyBet pathway, we developed a model to simulate the patterns of [^{14}C]Cho metabolism observed in plants expressing CMO (McNeil *et al.*, 2000b). This modeling work led to the hypothesis that Cho import into chloroplasts could exert significant control over the flux to GlyBet.

To further investigate limits on GlyBet synthesis in tobacco expressing CMO, in this study we built expression constructs that gave chloroplastic CMO activities from 0.1- to 10-fold that in salinized spinach. Plants expressing both CMO and BADH in chloroplasts were also constructed. We found that above a very low threshold, CMO activity ceases to affect flux to GlyBet, and that co-expressing BADH does not increase flux to GlyBet. These results suggest that the Cho supply in the chloroplast becomes limiting. To test whether the transport of Cho across the chloroplast envelope contributes to this, we compared the metabolism of [^{14}C]Cho in tobacco engineered with a chloroplastic or a cytosolic GlyBet pathway. Modeling of the data established that Cho transport indeed limits the flux to GlyBet in tobacco expressing a chloroplastic GlyBet pathway.

MATERIALS AND METHODS

Tobacco Transgenics with Chloroplastic CMO and BADH Activities

CMO expression cassettes were assembled in the vectors pJD301 (Luehrsen *et al.*, 1992) or pMON10845 (Wong *et al.*, 1992), then excised intact and ligated to the binary vector pBIN19 (Bevan, 1984) for plant transformation. Spinach CMO cDNAs were produced by high-fidelity PCR using *pfu* polymerase (Stratagene) with pRS3 (Rathinasabapathi *et al.*, 1997) as template. Primers 5′-ACGCGTCGACAAAAATGGCAGCAAGCGCAAGC-3′ (*Ω*5) and 5′-GCAGATCTGTTTAAAATTACTGGTAATATTGA-3′ amplified the *Ω*CMO insert, which was ligated to pJD301 as a *Sal*I/*Bgl*II fragment. Primers *Ω*5 and 5′ - ACGCGAGCTCCAATTACTTCAAAGTTTGTTGC - AACC-3′ amplified the *Ω*CMOnos insert, which was ligated to pJD301 as a *Sal*I/*Sac*I fragment. The *ΩΔ*CMO and *Δ*CMOnos inserts were made as above except that 5′-ACGCGTCGACAAAAATGGCCGTCGCGGCACCG-3′ replaced *Ω*5. Each expression cassette was subcloned into pBIN19 as an *Xba*I fragment. The ss*Δ*CMO and ss*Δ*CMOnos cassettes were made in pMON10845, inserting the 3′ part of the sequence first. For ss*Δ*CMO this was a 1004-bp *Bgl*II (blunted by T4 DNA polymerase)/*Nco*I fragment from *Ω*CMO, inserted in *Sma*I/*Nco*I sites, and for ss*Δ*CMOnos it was a 780-bp *Sac*I (blunted)/*Nco*I fragment from *Ω*CMOnos inserted in the *Eco*RI (blunted)/*Nco*I sites. The 5′ sequence, a 384-bp *Nco*I fragment amplified as above with primers 5′-CATGCCATGGCGGCACCGTCCTTTCC-3′ and 5′-ACCCATCCATGGTAAGGGCACACG-3′ was then ligated to the *Nco*I site in each construct. These expression cassettes were excised as *Not*I fragments, ligated to *Not*I–*Xmn*I–*Xba*I adapters (New England Biolabs) and then ligated to the *Xba*I site of pBIN19. All constructs were sequenced and transformed into tobacco (*Nicotiana tabacum* cv. Wisconsin 38) as described (Nuccio *et al.*, 1998). Lines 4 and 6 from Nuccio *et al.* (1998) expressing the native CMO cDNA were used as benchmarks.

Plants expressing both CMO and BADH in the chloroplast were obtained by crossing. Primary transformants harboring ss*Δ*CMOnos were selfed, and progeny homozygous for kanamycin resistance were identified. These were then crossed with *cv*. Wisconsin 38 plants homozygous for a sugar beet BADH transgene (Rathinasabapathi *et al*., 1994). The F_1 progeny from this cross were assayed to confirm expression of both CMO and BADH, and were used for experiments.

Tobacco Transgenics with Cytosolic COX and BADH Activities

The COX^+ transgenic line of tobacco *cv*. Xanthi, obtained with the *Agrobacterium tumefaciens* plasmid pHS993, has been described (Huang *et al*., 2000). Briefly, the open reading frame (ORF) encoding *A. pascens* choline oxidase (Rozwadowski *et al*., 1991) was cloned between a promoter module containing a duplicated CaMV35S-promoter (Datla *et al*., 1993) followed by a synthetic sequence of the alfalfa mosaic virus RNA4 leader to enhance translation, and the CaMV polyadenylation signal sequence from pJIT117 (Guerineau *et al*., 1988). The binary vector pRD400 (Datla *et al*., 1992) was used in deriving pHS993 containing the above construct.

The cassette for cytosolic expression of *E. coli* BADH (*betB*; Boyd *et al*., 1991) was constructed as follows. A *Bam*HI site preceding the initiation codon and an *Eco*RI site following the stop codon in the *betB* ORF were introduced in pLB12-2 (Boyd *et al*., 1991) by the method of Kunkel *et al.* (1987). The resulting 1.5-kb *Bam*HI-*betB*-*Eco*RI segment was cloned into the *Bam*HI/*Eco*RI sites of pJIT117. The pJIT117 derivative was restricted with *Hind*III and *Pst*I and the ends of the plasmid backbone were polished and ligated; this removed the chloroplast transit peptide-encoding sequence of pJIT117 and resulted in the following configuration: *Kpn*I-duplicated CaMV35S promoter-*Bam*HI-*betB* ORF-CaMV polyA signal-*Kpn*I. This 2.9-kb *Kpn*I cassette was cloned into the *Kpn*I site of pBIN19, and the construct was transformed into tobacco (*cv*. Xanthi) according to the method of Horsch *et al.* (1985), using a derivative of *A. tumefaciens* strain GV3101 (Koncz and Schell, 1986). Homozygotes of a $BADH^+$ line and the above COX^+ line, obtained by selfing, were crossed to obtain the F_1 hybrid line 993-208, which was used in this study. Assays confirmed that 993-208 expressed both COX and BADH.

Plant Growth Conditions

Plants were routinely grown in a light soil mixture in a naturally lit greenhouse or a growth chamber (12-h day, 22°C, photosynthetic photon flux density 200–300 μE m^{-2} s^{-1}; 12-h night, 18°C); irrigation was with 0.5 × Hoagland's solution. Half-expanded leaves from plants 4–6 weeks of age were taken for GlyBet determinations, RNA and protein analysis, and [^{14}C]Cho labeling experiments. For experiments in which unlabeled Cho was supplied, plants were cultured for 31 days in Magenta boxes on TSM medium without or with 5 mM Cho at 25°C for 12-h days as described (Nuccio *et al*., 1998). The uppermost 3-5 leaves were harvested for metabolite analysis. Leaf material was frozen in liquid N_2 and stored at −80°C until analyzed.

RNA Gel Blot Analyses

Total RNA was isolated using RNAeasy plant mini kits (Qiagen). Ten-microgram samples of RNA were separated in formaldehyde/1.5% agarose gels and transferred to supported nitrocellulose membrane (NitroPure, MSI), hybridized, and washed according to the manufacturer's protocols. The CMO probe was the 1.2-kb *Sal*I/*Sac*I fragment from the *ΩΔ*CMOnos construct described above and the 18S rRNA probe was from *Zamia pumila* (Nairn and Ferl, 1988); both were labeled with ^{32}P by the random primer method. Radioactive bands were detected by autoradiography.

Enzyme and Protein Analyses

For CMO analyses, proteins were extracted and fractionated with polyethylene glycol 8000 as described by Russell *et al.* (1998). CMO activity was assayed radiometrically (Burnet *et al*., 1995). Immunoblot procedures were as described previously (Nuccio and Thomas, 1999); blots were probed with rabbit antibodies to spinach CMO (Rathinasabapathi *et al*., 1997) or BADH (Weretilnyk and Hanson, 1989). For COX assays, proteins were extracted from leaf tissue (0.5 g Fw) by grinding with sand in 2 mL of extraction buffer (100 mM Tris–HCl, pH 8.0, 2 mM EDTA, 50 mM β-mercaptoethanol, 20 μM FAD, 4% w/v polyvinylpolypyrollidone) in an ice-cold mortar. After clearing by centrifugation (12,000*g*, 10 min) extracts were desalted on Sephadex G-25 columns equilibrated with assay buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 2 mM DTT, 10% v/v glycerol). COX activity was assayed as described for CMO (Burnet *et al*., 1995) except that thylakoids, ferredoxin, and $MgCl_2$ were omitted, 1 μL of 2 mM FAD was included, and the [*methyl*-^{14}C]Cho concentration in the assay was 2.4 mM (0.22 μCi $\mu$$mol^{-1}$). Incubation was for 30 min at 30°C in darkness. The recovery of COX activity (Sigma C4405) added before extraction was 30%; COX and CMO activity data were corrected accordingly. BADH activity was assayed as described previously (Weretilnyk and Hanson, 1989).

Determination of Choline, Phosphocholine, and Glycine Betaine

Cho, phosphocholine (P-Cho), and GlyBet were isolated from 0.5-g Fw leaf samples as described previously (Nuccio *et al*., 1998). GlyBet was converted to its *n*-butyl ester and

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quantified by fast atom bombardment MS (Rhodes *et al.*, 1987). Cho and P-Cho (after acid hydrolysis) were determined enzymatically using Cho oxidase (Sigma C4405) as described (Nuccio *et al.*, 1998).

Radiolabeling Experiments

[*methyl*-^{14}C]Cho (54 mCi mmol^{-1}; New England Nuclear) was purified as described by Weigel *et al.* (1988). Leaf disks (10 mm diameter) received eight shallow cuts on the abaxial surface. Batches of three disks ($\approx$50 mg Fw) were supplied via the cuts with 178 nCi (3.3 nmol) of [*methyl*-^{14}C]Cho per disk, in a 2 μL volume, and incubated (cut surface up) on moist filter paper in petri dishes for up to 10 h in fluorescent light (photosynthetic photon flux density 150 μE m^{-2} s^{-1}) at 25 $\pm$3°C. The disks were frozen in liquid N_2 and kept at $-$80°C until extraction. GlyBet, Cho, P-Cho, and phosphatidyl-Cho (Ptd-Cho) were extracted and separated as described (McNeil *et al.*, 2000a), and their ^{14}C contents were quantified by scintillation counting.

Computer Modeling

The models used were basically the same as that developed by McNeil *et al.* (2000b) to simulate [^{14}C]Cho labeling experiments with tobacco expressing low levels of CMO. Key parameters are initial pool sizes and specific activities, and flux rates between the pools. McNeil *et al.* (2000b) have discussed the model's assumptions, which are briefly restated in the text (below). During 0.1-min intervals, material of defined specific activity is drawn from one pool to another at rates determined by the kinetic characteristics assigned to the processes involved, and by existing substrate pool sizes. During each iteration new specific activities and pool sizes are computed, and total radioactivity in each pool is plotted as a function of time. Further details on development of models of this type are available at http://www.hort.purdue.edu/cfpesp/models/models.htm. Specific model assumptions for the various experiments are given in the text.

RESULTS

Engineering Tobacco for High CMO Expression

The CMO cDNA used in our earlier work (Nuccio *et al.*, 1998) (Fig. 2A) had features that may have limited its expression in tobacco. These were eliminated in the cassettes built for this study (Fig. 2B). The native CMO 5′-untranslated region (UTR) is GC-rich, which can reduce mRNA stability and ribosome recruitment (Gallie, 1996); in constructs designated Ω it was replaced with the tobacco

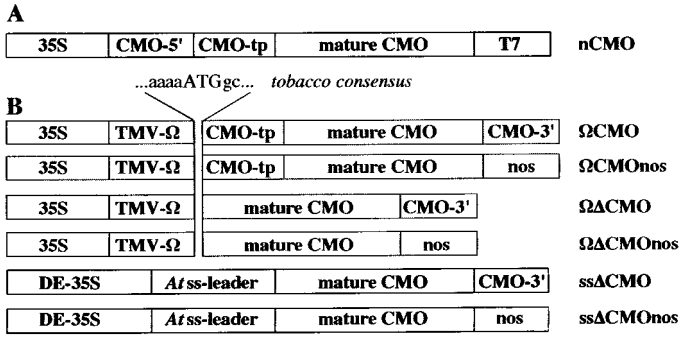

FIG. 2. Comparison between the spinach CMO expression cassette used by Nuccio *et al.* (1998) (A) and those built for this study (B). All constructs include the CaMV 35S promoter; those designated ss have in addition a dual-enhancer (DE) upstream of the promoter. In constructs designated Ω the CMO 5′-UTR was replaced by the Ω sequence from tobacco mosaic virus (TMV-Ω), and the sequence context of the start codon was altered to the tobacco consensus as described in the text. In constructs with a nos terminus designation the nopaline synthase terminus replaced the CMO 3′-UTR. Constructs with a Δ designation lack the CMO transit peptide (CMO-tp). In constructs designated ss the CMO 5′-UTR and transit peptide were replaced with corresponding sequences (*At*ss-leader) derived from the Arabidopsis RuBisCo small subunit.

mosaic virus Ω sequence, a translational enhancer which can increase chimeric gene expression by four- to six-fold (Dowson-Day *et al.*, 1993). The CMO open reading frame begins with two methionine codons (5′-...GGTTAATGATGGC...-3′), which could lower translational efficiency. This was altered to 5′-...AAAAATGGC...-3′, a tobacco consensus that conforms to that for dicotyledons (Koziel *et al.*, 1996). The CMO transit peptide, while typical in structure, may be inefficient in tobacco (Cline and Henry, 1996). In the ssΔCMO constructs, the CMO transit peptide and 5′-UTR were replaced by those derived from an Arabidopsis RuBisCo small subunit cDNA (Wong *et al.*, 1992). The CMO 3′-UTR contains the mRNA instability motif ATTA (Green, 1993) and may have a suboptimal polyadenylation signal. Constructs were therefore produced in pairs: one with the native CMO 3′-UTR and the other with the nopaline synthase terminus (NOS). All the above constructs were designed to target CMO to the chloroplast. To target CMO to the cytosol, in $\Omega\Delta$CMO constructs the transit peptide was deleted.

These constructs were transformed into tobacco (*cv.* Wisconsin 38) and plants that accumulated CMO protein were vegetatively propagated. Eight to thirteen independent transformants per construct were obtained, all of which appeared normal. Plants harboring the new constructs (Fig. 2B; ΩCMO, ΩCMOnos, $\Omega\Delta$CMO, $\Omega\Delta$CMOnos, ssΔCMO and ssΔCMOnos) were compared to plants expressing the native CMO cDNA (Fig. 2A; nCMO). Western analysis (Fig. 3A) showed that the new constructs all gave much higher levels of CMO protein than the

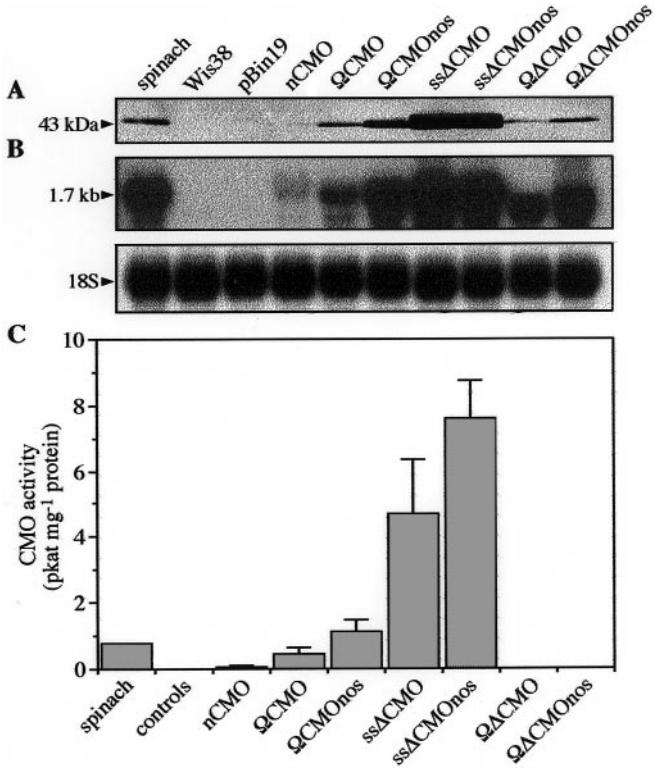

FIG. 3. CMO expression in leaves of tobacco plants carrying various CMO constructs. Data for wild type *cv.* Wisconsin 38 plants (Wis 38), empty-vector controls (pBIN19) and salinized spinach are included for comparison. (A) Immunoblot showing CMO protein levels; lanes contained 50 μg of protein. (B) RNA gel blots showing CMO mRNA levels (above) and loading controls with 18S rRNA (below); lanes contained 10 μg of total RNA. A and B show data for one representative transformant per construct. (C) CMO activities. Except for nCMO, values are means and SE for 8-13 independent transformants per construct; nCMO values are for line 4 from Nuccio *et al.* (1998). The wild type and empty-vector controls had no detectable CMO activity, and so are shown together. The CMO activity data for salinized spinach are from Rathinasabapathi *et al.* (1997).

benchmark nCMO construct. The ssΔCMO lines accumulated the most protein followed by ΩCMO and $\Omega\Delta$CMO lines; in both the latter replacing the CMO 3′-UTR with the NOS terminus roughly doubled the level of CMO protein. RNA gel blot analysis (Fig. 3B) demonstrated that CMO transcript levels were positively correlated with the levels of protein accumulated. As expected, no CMO protein or mRNA was detected in wild type or empty-vector controls.

CMO activities paralleled CMO protein levels in plants harboring the constructs designed to direct CMO to the chloroplast (Fig. 3C). Activity was highest in ssΔCMOnos plants, the mean value (8 pkat mg^{-1} protein) being $>$100-fold that in nCMO plants and 10-fold that in salinized spinach (Rathinasabapathi *et al.*, 1997). The native CMO transit peptide (present in the ΩCMO and ΩCMOnos constructs) was shown previously to direct CMO to tobacco chloroplasts (Nuccio *et al.*, 1998). The ssΔCMOnos and ssΔCMO constructs include the Arabidopsis RuBisCo small subunit-transit peptide for chloroplast expression; immunoblot analysis of protoplast-derived chloroplastic and extrachloroplastic fractions from a representative ssΔCMOnos transformant verified that CMO was correctly targeted (not shown).

The $\Delta\Omega$CMO and $\Delta\Omega$CMOnos constructs, designed for cytosolic CMO expression, gave moderate levels of CMO protein (Fig. 3A), but this protein had no enzymatic activity (Fig. 3C). Failure to assemble the [2Fe-2S] prosthetic group required by CMO for catalytic activity may have caused this, since this process probably occurs in chloroplasts (Nuccio *et al.*, 1998). In any case, this result precludes relocating CMO to the cytosol as an engineering strategy.

High CMO Expression in Chloroplasts Has Little Effect on Flux to GlyBet

Of the four new constructs designed to express CMO in the chloroplast, none yielded transformant populations with significantly ($P = 0.05$) higher mean GlyBet levels than the benchmark nCMO transgenics (69 nmol g^{-1} Fw). Because there was much variation for CMO enzyme level (Fig. 3C) as well as for GlyBet level within the populations of transformants harboring each construct, the data were analyzed as a scatter plot (using logarithmic scales) of GlyBet level *vs.* CMO activity (Fig. 4). This plot reveals that, above a very low threshold, CMO activity has no control over the flux of endogenous Cho to GlyBet. Previous work showed that tobacco expressing CMO does not accumulate detectable betaine aldehyde (Nuccio *et al.*, 1998), there being some endogenous BADH activity in tobacco (Rathinasabapathi *et al.*, 1994; Trossat *et al.*, 1997). To confirm that BADH did not limit GlyBet synthesis in the present experiments, plants with high chloroplastic activities of both CMO (from the ssΔCMOnos construct) and sugar beet BADH were obtained by crossing homozygotes. Their BADH activities (21 nmol min^{-1} g^{-1} Fw) were about 10-fold those in plants expressing CMO alone, but their GlyBet levels were no higher.

CMO also had no control over the flux of supplemental Cho to GlyBet (Fig. 5). When transformants were cultured on medium with 5 mM Cho, the GlyBet level increased $>$40-fold, as reported for the nCMO construct (Nuccio *et al.*, 1998). However, none of the new constructs resulted in significantly ($P = 0.05$) more GlyBet accumulation than did nCMO. To verify that this lack of control in Cho-supplemented plants was not due simply to consumption of all

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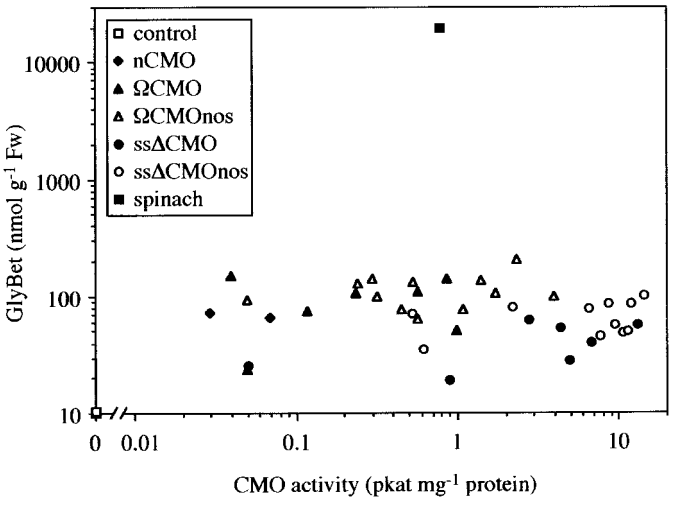

FIG. 4. GlyBet accumulation in leaves of tobacco plants as a function of CMO enzyme activity. Data are included for wild type (Wis 38) and empty-vector (pBIN19) controls, and for salinized spinach. Data points are values for individual transformants carrying the constructs indicated; that for salinized spinach leaves is based on literature values (Weigel *et al.*, 1986; Rathinasabapathi *et al.*, 1997). The nCMO values are for lines 4 and 6 from Nuccio *et al.* (1998). Note that GlyBet level and CMO activity are plotted on logarithmic scales, and that the CMO activity axis is broken because the controls lack detectable CMO activity.

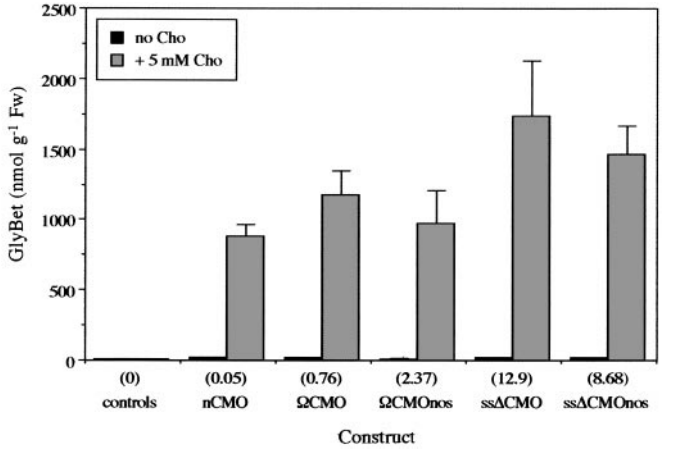

FIG. 5. Effect of Cho supplementation on GlyBet levels in leaves of tobacco expressing CMO. Triplicate plants derived by vegetative propagation from one representative transformant per construct were cultured axenically for 31 days minus or plus 5 mM Cho. The nCMO values are for line 4 from Nuccio *et al.* (1998). Wild type (Wis 38) and empty-vector (pBIN19) controls were included; the data for these did not differ significantly and were pooled. Cho did not significantly ($P = 0.05$) affect Fw of controls or CMO^+ plants. GlyBet values are means and SE; the data for nCMO plants are from Table 1 of Nuccio *et al.* (1998). CMO activities in leaves of cultured plants are given in parentheses (pkat mg^{-1} protein, means of duplicates).

the Cho available, Cho and P-Cho pools were measured for a representative transformant with high CMO activity (ss*Δ*CMOnos). The Cho and P-Cho pools (4090 ± 130 and 1050 ± 55 nmol g^{-1} Fw, respectively) were large compared to the GlyBet pool (1470 ± 200 nmol g^{-1} Fw, Fig. 5). Taken with the results in Fig. 4, this implies that a process downstream of Cho synthesis and upstream of Cho oxidation in the chloroplast controls flux in the GlyBet pathway. This process could be transport of Cho into the chloroplast, as hypothesized by McNeil *et al.* (2000b).

Engineering a Cytosolic GlyBet Pathway

To evaluate the role of Cho transport, we compared tobacco expressing the ss*Δ*CMOnos construct (CMO^+) and the ss*Δ*CMOnos construct plus sugarbeet BADH ($CMO^+/BADH^+$) in the chloroplast with tobacco engineered to have a cytosolic GlyBet synthesis pathway. When CMO was targeted to the cytosol, it was found to be inactive (Fig. 3C). Consequently, we instead used the *A. pascens* COX gene in conjunction with *E. coli* BADH to install a cytosolic pathway. Tobacco primary transformants (*cv.* Xanthi) expressing COX alone have been shown to accumulate up to ≈ 1 μmol g^{-1} Fw GlyBet (Huang *et al.*, 2000). To assess the extent to which BADH influences flux through the cytosolic pathway, tobacco expressing both COX and *E. coli* BADH were constructed by crossing *cv.* Xanthi lines homozygous for one copy of each transgene. The F_1 progeny coexpressing COX and BADH will henceforth be termed COX^+ for convenience; the BADH activity in these plants was 340 nmol min^{-1} g^{-1} Fw. GlyBet content in these plants is similar to those in plants expressing COX alone (compare Table 1 with Fig. 3 of Huang *et al.*, 2000).

The GlyBet, Cho, and P-Cho levels in COX^+ and CMO^+ plants are shown in Table 1, along with values for the corresponding wild types (*cvs.* Xanthi and Wisconsin 38). Consistent with the data of Fig. 4 and those of Huang *et al.* (2000), the COX^+ plants had ten-fold more GlyBet than the CMO^+ transgenic. Table 1 also shows that the Cho oxidizing activity in COX^+ plants is at most one-fourth that of CMO, when assayed at saturating or near-saturating Cho concentrations. Since COX has a higher K_m for Cho than does CMO (1200 *vs.* 100 μM; Brouquisse *et al.*, 1989; Rozwadowski *et al.*, 1991), the catalytic potential of COX may be much less than one fourth that of CMO at physiological (i.e., low) Cho concentrations. In any case, that CMO^+ plants have more Cho oxidizing activity but less GlyBet than COX^+ plants is qualitatively consistent with a limitation on GlyBet synthesis imposed by Cho import into the chloroplast.

When COX^+ plants were cultured with 5 mM Cho, the GlyBet level increased to 4760 ± 1810 nmol g^{-1} Fw, which is four times that in Cho-supplemented plants expressing

TABLE 1

Cho Metabolite Levels and CMO or COX Activity in Wild-Type and Transgenic Tobacco

Plant	Cho metabolites (nmol g^{-1} Fw)[a]			Activity[a] (nmol min^{-1} g^{-1} Fw)
	Cho	P-cho	GlyBet	
Wis 38[b]	112 (3)	96 (6)	14 (2)	—
Wis 38/CMO	117 (24)	158 (3)	43 (7)	4.3 (0.3)
Xanthi	247 (46)	113 (7)	11 (1)	—
Xanthi/COX	173 (25)	91 (13)	434 (40)	$\leqslant 1$[c]

[a] Means and SE (in parentheses) of three replicates.
[b] Wis 38, Wisconsin 38.
[c] Activity was too close to the detection limit to be quantified accurately.

the chloroplastic GlyBet pathway (Fig. 5). The Cho and P-Cho levels in COX^+ plants were respectively 3810 ± 600 and 401 ± 90 nmol g^{-1} Fw, so that their combined total was less than the GlyBet level. This contrasts with the situation in CMO^+ plants (see above), where the (Cho + P-Cho) total was 3.5-fold greater than the GlyBet level. It is therefore probable that, compared to CMO in the chloroplast, COX had freer access to exogenously supplied Cho as well as to endogenous Cho.

[^{14}C]*Cho Metabolism and Modeling of Experimental Data*

To test the hypothesis that Cho transport into the chloroplast limits GlyBet synthesis in CMO^+ transgenics, radiotracer experiments were combined with computer modeling. A [^{14}C]Cho dose of 200 nmol g^{-1} Fw, which is comparable to the endogenous Cho content (Table 1), was supplied to leaf disks of wild type plants, COX^+, CMO^+, and $CMO^+/BADH^+$ plants. The $CMO^+/BADH^+$ plants were included to provide a more direct comparison with COX^+ plants. The labeling kinetics of Cho, P-Cho, Ptd-Cho and GlyBet were monitored, and the resultant data were used as inputs for modeling. Other inputs were the pool sizes of Cho metabolites (Table 1 and McNeil *et al.*, 2000a), and the measured V_{max} and K_m values for CMO, COX and BADH. Since extractable COX activity was too low to quantify accurately ($\leqslant 1$ nmol min^{-1} g^{-1} Fw, Table 1), a V_{max} value (0.2–0.5 nmol min^{-1} g^{-1} Fw) was estimated from the GlyBet content of COX^+ transgenics cultured on Cho-containing medium (see above) by assuming a growth rate of 10% d^{-1} (McNeil *et al.*, 2000a).

The models used are closely related to those presented by McNeil *et al.* (2000b) and are shown schematically in Fig. 6A. Briefly stated, their main features are: (a) single pools of P-Cho, Ptd-Cho, betaine aldehyde and GlyBet, in the compartments indicated in Fig. 6A; (b) a large vacuolar storage pool of Cho (Cho_{vac}) and small metabolically active Cho pools in the cytoplasm (Cho_{cy}) and chloroplast (Cho_{chl}); (c) treatment of Ptd-Cho synthesis from P-Cho as a single step; (d) catabolism of Ptd-Cho both to free Cho and to P-Cho; (e) endogenous (unlabeled) biosynthetic fluxes to P-Cho and Ptd-Cho that remain constant at 0.12 and 0.02 nmol min^{-1} g^{-1} Fw, respectively; (f) [^{14}C]Cho uptake at a rate proportional to its apoplastic concentration; (g) a flux of Cho from cytosol to chloroplast that is proportional to Cho_{cy} and significant only in CMO^+ plants (in which Cho entering the chloroplast is rapidly oxidized); (h) a flux of Cho from vacuole to cytosol that is proportional to Cho_{vac}; (i) other fluxes that respond to substrate pool size according to Michaelian kinetics and that are each represented by a V_{max} (nmol min^{-1} g^{-1} Fw) and a K_m (nmol g^{-1} Fw). K_m values measured *in vitro* (μM) were converted to K_m values for the model by assuming cytoplasmic, stromal and vacuolar volumes of 24, 66, and 546 μL mg^{-1} chlorophyll, respectively (Winter *et al.*, 1994) and a chlorophyll content of 1 mg g^{-1} Fw.

In the modeling process, the pool sizes, V_{max} and K_m values were progressively adjusted (within limits imposed by measured values) until the simulated time courses of radiolabeling of the various Cho metabolites closely matched the experimental values. The results are summarized in Fig. 6B–F, in which the points are experimental data, and the lines are model-generated, best-fit curves. The parameters used to generate these curves are given in Tables 2 and 3.

Inspection of the labeling kinetics shows that the main fate of [^{14}C]Cho in wild-type tobacco (Wis 38 and Xanthi) is phosphorylation to P-Cho, and subsequent incorporation into Ptd-Cho, and that a small but significant amount of [^{14}C]Cho remains unmetabolized at the end of the experiment (Fig. 6B and 6D). These data and those in Table 1 establish that the wild type plants are comparable with respect to Cho metabolism. In CMO^+, $CMO^+/BADH^+$, or COX^+ plants, there is in addition a flux to GlyBet, larger for COX than for CMO (Fig. 6C, 6D and 6F), which is consistent with the results from the Cho supplementation experiments described above. As expected from their similar GlyBet levels (above), there was little difference in the labeling patterns for CMO^+ or $CMO^+/BADH^+$ tissue.

Modeling of the data produced the following findings pertaining specifically to Cho transport into the chloroplast. In CMO^+ or $CMO^+/BADH^+$ plants, the flux from Cho via betaine aldehyde to GlyBet could only be reconciled with the measured V_{max} and K_m of CMO by interposing a low-flux step between the cytosolic pool of Cho (Cho_{cy}) and CMO in the chloroplast. This step could be modeled satisfactorily by assuming that flux through it is linearly proportional to Cho_{cy}, i.e., that it is not saturable, or at least not by Cho_{cy} values in the physiological range. Lack of saturability

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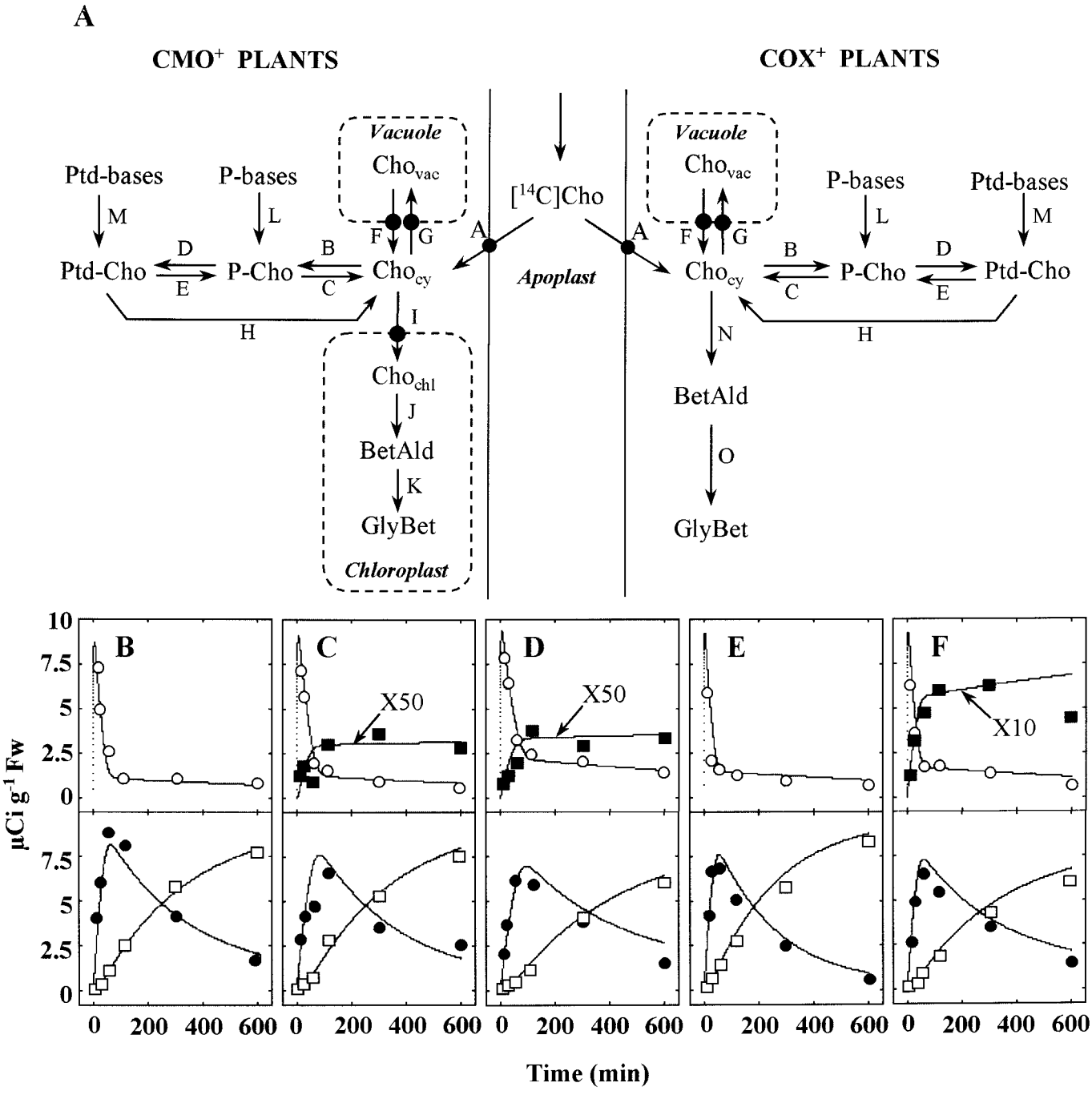

FIG. 6. Computer modeling of [^{14}C]Cho metabolism by leaf disks of tobacco expressing chloroplastic or cytosolic GlyBet biosynthetic pathways, and the corresponding wild type controls. (A) Scheme showing the reaction networks for transgenics expressing CMO or COX plus BADH. Pool sizes are given in Table 2. Letters next to arrows denote fluxes. L and M represent the endogenous phosphobase (P-base) and phosphatidylbase (Ptd-base) *N*-methylation pathway fluxes, which are assumed to remain constant at 0.12 and 0.02 nmol min^{-1} g^{-1} Fw, respectively. Fluxes A, F and I were modeled as processes with first order dependence on the Cho substrate pool size; other fluxes were assumed to show Michaelian responses to pool size. The first order rate constants, V_{max} and K_m values for these fluxes are given in Table 3. BetAld, betaine aldehyde. (B-F) Comparisons between model output (curves) and actual data (points) for tobacco *cv.* Wisconsin 38 (B), *cv.* Wisconsin 38 expressing ss*Δ*CMOnos (C) or ss*Δ*CMOnos plus beet BADH (D), tobacco *cv.* Xanthi (E), and *cv.* Xanthi expressing COX plus *E. coli* BADH (F). ○, Cho; ●, P-Cho; □, Ptd-Cho; ■, GlyBet. Note that the data for GlyBet are multiplied ×10 or ×50.

TABLE 2

Pool Sizes of Cho Metabolites Used to Model [^{14}C]Cho Labeling Experiments

	Concentration (nmol g^{-1} Fw)			
Metabolite	Wis 38	Wis 38/CMO[a]	Xanthi	Xanthi/COX
Cho_{cy}[b]	2	2	2	2
Cho_{chl}[b]	—	0.2	—	0.2
Cho_{vac}[c]	110	120	250	170
P-Cho[c]	100	160	110	90
Ptd-Cho[d]	1800	1800	1800	1800
BetAld[b]	—	0.2	—	0.2
GlyBet[c]	—	40	—	420

[a] These values were also used to model data from plants coexpressing CMO and BADH.
[b] Model-derived values from McNeil *et al.* (2000b).
[c] Values obtained from Table 1.
[d] Experimental value from McNeil *et al.* (2000a).

connotes a simple diffusion process rather than a carrier-mediated mechanism. This result agrees with that obtained by McNeil *et al.* (2000b), which was based on radiolabeling data for transformants with $\approx$100-fold less CMO activity than those in Fig. 6C and 6D. The situation for COX$^+$ plants was completely different: the Cho $\rightarrow$ GlyBet flux could be satisfactorily simulated just by using the measured V_{max} and K_m values for COX. This result strongly reinforces the view that the low-flux step upstream of CMO in the model for CMO$^+$ plants corresponds to a biological process, not a modeling artifact, that this process is Cho transport into the chloroplast, and that it exerts substantial control over the Cho $\rightarrow$ GlyBet flux in CMO$^+$ plants.

DISCUSSION

By replacing up to four suspect elements of the spinach CMO cDNA with sequences known to enhance transgene expression in tobacco, we obtained transformants with chloroplastic CMO levels that were between 0.1- and $>$10-times that in spinach (a species rich in GlyBet). Calculations based on the specific activity of purified CMO (393 pkat mg^{-1}; Burnet *et al.*, 1995) indicate that CMO in the highest-expressing plants constituted as much as 2% of the soluble leaf protein, which is a very high level of transgenic expression for a metabolic enzyme. This emphasizes the engineering utility of the pMON10845 vector used to achieve this (Wong *et al.*, 1992).

The $>$100-fold range of CMO expression levels that we obtained enabled us to show that, above a very low threshold, CMO exerts no control on the Cho $\rightarrow$ GlyBet flux in tobacco. As we have previously reported (Nuccio *et al.*, 1998; Huang et al., 2000), an inadequate endogenous supply of Cho to support GlyBet synthesis is undoubtedly

TABLE 3

Flux Parameters Used to Model [^{14}C]Cho Labeling Experiments[a]

	Wis 38		Wis38/CMO		Wis 38/CMO–BADH		Xanthi		Xanthi/COX	
Flux	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
B	5.2[b]	40	3.8[b]	40	3.1[b]	40	5.6[b]	40	5.0[b]	40
C	0.15	200	0.15	200	0.15	200	0.15	200	0.15	200
D	1.1[b]	80	1.3[b]	80	1.0[b]	80	1.5[b]	80	1.0[b]	80
E	0.7	2000	0.7	2000	0.7	2000	0.7	2000	0.7	2000
G	0.6[b]	40	0.5[b]	40	0.8[b]	40	0.9[b]	40	1.0[b]	40
H	0.5	2000	0.5	2000	0.5	2000	0.5	2000	0.5	2000
J			4[c]	6	4[c]	6				
K			1	3	21[c]	3				
N									0.3[c]	29[d]
O									340[c]	80[d]
A[e]	0.4		0.4		0.4		0.6		0.6	
F[e]	0.0011		0.0011		0.0011		0.0011		0.0005	
I[e]	—		0.00017		0.00017		—		—	

[a] Units are V_{max} values, nmol min^{-1} g^{-1} Fw; K_m values, nmol g^{-1} Fw; first-order rate constants (fluxes A, F, and I only), min^{-1}. Parameters not followed by a superscript were taken from McNeil *et al.* (2000b) and literature cited therein.
[b] Values derived in this study from modeling.
[c] Values taken from Table 1 or the text.
[d] K_m values for COX and *E. Coli* BADH calculated from Rozwadowski *et al.* (1991) and Landfald and Strom (1986), respectively.
[e] Tabulated values are first-order time constants.

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in part responsible for this, as evidenced by the large increase in GlyBet accumulation brought about by providing Cho in the medium. Our present results show that in tobacco expressing CMO in the chloroplast, the inadequacy of the Cho supply has two components: a limited capacity to synthesize Cho, and a limited capacity to transport it across the chloroplast envelope. Plants expressing the cytosolic GlyBet pathway have only the former limitation and, when reliant upon endogenously-produced Cho, accumulate 10-fold more GlyBet than CMO^+ plants despite having a lower Cho oxidizing capacity.

Modeling of Cho metabolism in CMO^+ plants suggests that Cho may enter tobacco chloroplasts by simple diffusion, because simulation of the experimental data does not require the transport process to be saturable. However, the modeling results do not rule out a carrier-mediated process with a K_m well above the physiological range of Cho_{cy}, for such a process would behave *in vivo* as if it were nonsaturable. Regardless of its exact nature, the transport step in tobacco is clearly inefficient inasmuch as it competes poorly for Cho against Cho kinase and Cho transport into the vacuole, and captures only a small amount of Cho unless the system is flooded with exogenously supplied Cho. In contrast, in GlyBet-accumulating species such as spinach or beet, Cho import and oxidation by chloroplasts clearly compete effectively against Cho kinase and Cho transport into the vacuole (Hanson and Rhodes, 1983; Summers and Weretilnyk, 1993). There is no evidence that spinach Cho kinase has a markedly higher K_m value than that in tobacco or other plants that lack GlyBet (Tanaka *et al*., 1966; Bligny *et al*., 1989; Gawer *et al*., 1991). From this it follows that the chloroplasts of species such as spinach and beet may have a high-affinity, high-activity Cho transporter that tobacco chloroplasts lack. Indirect support for this comes from considering the origin of free Cho in spinach and beet; it appears to be derived directly from P-Cho (Rhodes and Hanson, 1993), yet there is no evidence for a P-Cho phosphatase specific to these plants (Tanaka *et al*., 1966). Cho uptake via a high-affinity transporter in the chloroplast envelope would resolve this paradox, for it could permit the cytosolic Cho concentration to be maintained low enough to run the Cho kinase reaction in reverse. The equilibrium constant for this reaction ($Cho + ATP = P\text{-}Cho + Pi$) is $\approx 10^4$ (Guynn, 1976), and P-Cho formation is normally strongly favored in the cytosol (Bligny *et al*., 1989).

Because little is known about chloroplast transporters other than those for triose phosphate/phosphate, dicarboxylates, and glycolate/glycerate, if a high-affinity Cho transporter can be demonstrated in spinach or beet, it would become an interesting target for isolation and characterization. If cloned, it could be valuable in future engineering of the GlyBet pathway. An alternative, short-term engineering solution to the problem of getting Cho across the chloroplast envelope might be to express the high-affinity BetT Cho transporter of *E. coli* (Lamark *et al*., 1991) in the chloroplast inner envelope membrane. BetT mediates secondary transport of Cho via H^+ symport, and there is evidence that an electrochemical proton gradient across the chloroplast inner envelope membrane can drive metabolite transport via H^+ symport (Howitz and McCarty, 1991). Besides their implications for engineering Cho transport into the chloroplast, our present data as well as previous results (Nuccio *et al*., 1998; Huang *et al*., 2000; McNeil *et al*., 2000b) indicate that it will be necessary to engineer a higher capacity for Cho synthesis if GlyBet levels in transgenic tobacco or other GlyBet-deficient species are to approach those in spinach or beet.

Another engineering solution is to avoid the problem of Cho transport altogether by using the cytosolic GlyBet synthesis pathway, and for the short term we advocate this strategy. However, for the longer term there are two good reasons to seek to improve Cho uptake by chloroplasts so that CMO can be used as the Cho-oxidizing enzyme. First, the ferredoxin-dependence of CMO links GlyBet synthesis to the reduction state of the chloroplast electron transport chain, which may help match the supply of GlyBet with the demand for osmoprotection in stressed plants (Rathinasabapathi *et al*., 1997); COX cannot do this. Second, COX is an H_2O_2-generating enzyme, and there is evidence that *Arabidopsis* expressing COX in the chloroplast has elevated levels of the H_2O_2-scavenging enzymes ascorbate peroxidase and catalase (Alia *et al*., 1999). This implies that chloroplast expression of COX entails a degree of oxidative stress that it might be best to avoid.

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