Functional Characterization of Choline Monoxygenase, an Enzyme for Betaine Synthesis in Plants*

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In plants, the first step in betaine synthesis was shown to be catalyzed by a novel Rieske-type iron-sulfur enzyme, choline monooxygenase (CMO). Although CMO so far has been found only in Chenopodiaceae and Amaranthaceae, the recent genome sequence suggests the presence of a CMO-like gene in Arabidopsis, a betaine non-accumulating plant. Here, we examined the functional properties of CMO expressed in Escherichia coli, cyanobacterium, and Arabidopsis thaliana. We found that E. coli cells in which choline dehydrogenase (CDH) was replaced with spinach CMO accumulate betaine and complement the salt-sensitive phenotype of the CDH-deleted E. coli mutant. Changes of Cys-181 in spinach CMO to Ser, Thr, and Ala and His-287 to Gly, Val, and Ala abolished the accumulation of betaine. The Arabidopsis CMO-like gene was transcribed in Arabidopsis, but its protein was not detected. When the Arabidopsis CMO-like gene was expressed in E. coli, the protein was detected but was found not to promote betaine synthesis. Overexpression of spinach CMO in E. coli, Synechococcus sp. PCC7942, and Arabidopsis conferred resistance to abiotic stress. These facts clearly indicate that CMO, but not the CMO-like protein, could oxidize choline and that Cys-181 and His-287 are involved in the binding of Fe-S cluster and Fe, respectively.

Many bacteria, plants, and animals accumulate glycine betaine (betaine) under water or salt stress conditions (1–3). In these organisms, it was shown that betaine is synthesized in two steps, choline → betaine aldehyde → glycine betaine. The enzyme involved in the second step seems to be the same in plants, animals, and bacteria, namely, NAD⁺-dependent betaine aldehyde dehydrogenase (BADH) (4–6). By contrast, different enzymes are involved in the first step. In plants, the first step is catalyzed by a novel Rieske-type iron-sulfur enzyme choline monooxygenase (CMO) (7, 8). CMO is not found in animals or bacteria. In these organisms, the first step is catalyzed by membrane-bound choline dehydrogenase (CDH) or soluble choline oxidase (COX) (9–11). CMO is not well known, having so far been found only in Chenopodiaceae (spinach and sugar beet) and Amaranthaceae (7, 12, 13), and not detected even in some betaine-accumulating plants such as mangrove (14). CMO purified from spinach leaves are shown to be soluble and insensitive to carbon monoxide, contains a Rieske-type iron-sulfur center, and consists of homo-dimer or -trimer of subunit M, 42,000 (7, 8). These properties are completely unrelated to CDH, COX, and cytochrome P-450-type monoxygenases. The activity of purified CMO is extremely low (393 picomol min⁻¹) probably due to the labile property of the enzyme (7), which hampers further characterization.

For functional characterization of CMO, it would be useful to express it in E. coli, which has not yet been reported. In this study, we examined whether CMO could function in E. coli and cyanobacteria. E. coli contains a bet gene cluster consisting of betA, betB, betT, and betI, which encode CDH, BADH, choline transport, and regulatory protein, respectively (9). We constructed the expression vectors in which the CDH gene of E. coli bet gene clusters was replaced with CMO or deleted. The vectors were used for the transformation of E. coli (DH5α) and fresh water cyanobacterium Synechococcus sp. PCC7942, neither of which could synthesize betaine. For operation of CMO in these organisms, the Rieske-type iron-sulfur center must be assembled into the CMO polypeptide, and reduced ferredoxin is required. We tested whether the heterologous expression could be used for the characterization of functional properties of spinach CMO and obtained information on the active site of CMO by a site-directed mutagenesis approach.

A recent genome sequence of Arabidopsis revealed that Arabidopsis contains a CMO-like gene (accession nos. NM_119135 and CAB43664). Because Arabidopsis is a betaine non-accumulating plant, we examined whether the CMO-like gene is expressed in Arabidopsis and its physiological function if any. Here, we show that a CMO-like gene was transcribed in Arabidopsis, but the protein was not detected. Although both spinach CMO and Arabidopsis CMO-like protein could be expressed in E. coli, only spinach CMO was involved in betaine synthesis.

MATERIALS AND METHODS

Construction of Expression Plasmids—The spinach CMO gene was isolated by PCR technique from the spinach cDNA library, which was a kind gift from Prof. K. Saito, Faculty of Pharmacy of Chiba University. Using the forward primer SpPCMO-F and reverse primer SpPCMO-R (Table I), a 1320-bp fragment was amplified and ligated into the EcoRV site of pBlueScript II SK+. The resulting plasmid pSPCMO encodes the precursor CMO (8). For the construction of mature CMO expression vector, the forward primer SpMCMO-F, containing the 121–133 bases of spinach CMO cDNA (8) and NdeI site, and the reverse primer SpPCMO-R, containing the stop codon, were used. The amplified fragment, 1145 bp, was ligated into the EcoRV site of pBlueScript II SK+ and then digested with the NdeI and BamHI. The resulting fragment

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§ The abbreviations used are: BADH, betaine aldehyde dehydrogenase; CDH, choline dehydrogenase; CMO, choline monooxygenase; COX, choline oxidase; RT, reverse transcription.
was ligated into the corresponding site of pET3a plasmid vector, which generated the plasmid, pSMC20, encoding the mature CMO.

The transcription of Arabidopsis CMO-like gene was examined by the reverse transcription (RT) reaction technique. For this, total RNA was extracted from Arabidopsis leaves by using cetyltrimethylammonium bromide as described previously (14). The single strand DNA was constructed by RT-PCR reaction using the forward primer ATPCMO-F and reverse primer ATPCMO-R, which matched to the 5′- and 3′-terminal sequences of genomic DNA of Arabidopsis CMO-like gene, respectively. The amplified fragment was ligated into the EcoRV site of pBluescript II SK+.

The E. coli bet gene cluster was ligated into the BamHI site of an E. coli/Synechococcus shuttle vector pUC303-Bm (15), which was designated as pBET+CDH. For the subtraction of betA gene with spinach CMO or Arabidopsis CMO-like gene, first, the NdeI site was introduced at the 5′-terminal region of mature spinach CMO or Arabidopsis CMO-like gene. For the mature spinach CMO, the forward primer SpMCMO-Nae-F, containing NaeI site and 187–199 bases of spinach CMO cdna (8) and reverse primer SpPCMO-R, were used. For the Arabidopsis CMO-like gene, the forward primer AtCMO-Nae-F, containing NaeI site and 121–133 bases of Arabidopsis CMO-like gene, and the reverse primer AtCMO-R, containing BamHI site and stop codon, were used. The amplified fragments were ligated into the EcoRV site of pBluescript II SK+. The resulting plasmids were digested with NaeI and also with KpnI derived from the pBluescript II SK+. On the other hand, the bet gene cluster was ligated with BamHI and ligated into the BamHI site of pUC18. The resulting plasmid pHCBET was digested with NaeI and KpnI, which removed most of the betA gene (nucleotides 31–1430 of betA). Then, the NaeI/KpnI-digested mature spinach CMO or Arabidopsis CMO-like gene was ligated into the corresponding site of pHCBET. The resulting plasmids were digested with HindIII (derived from the plBluescript II SK+) and XbaI (derived from the pUC18) and ligated into the centre of SpCMO-Sol3-Bm. The generated plasmid, pHET+SpCMO or pHET+AtCMO, encoded a protein containing the extra 9 N-terminal residues of CDH fused to mature protein, but the second residue Val in the mature protein was changed to Gly. For deletion of betA from the bet gene cluster, pHCBET was digested with NaeI and KpnI, filled in with Klenow DNA polymerase, and then digested with BamHI and XbaI (derived from the pUC18). The resulting fragment was ligated into the corresponding site of pUC983-Bm, and the plasmid pHET-CDH was generated. The plasmids pHET+CDH, pHET+SpCMO, pHET+AtCMO, and pHET-CDH were expressed in E. coli DH5α cells in which the bet gene cluster was deleted from the host genome.

Construction of Spinach CMO Mutants—The mutant spinach CMO genes were constructed by the PCR technique using the plasmid pSMC20 as the template. The Cys in the putative consensus sequence (CXXH15-X15CXH) for coordination of Rieske-type [2Fe-2S] cluster was changed to Ser, Ala, and Thr. The forward primer was SpMC20-Nae-F. For the reverse primer, the Ncol site at position 549 in the CMO gene was used. The reverse primer C181STA-R, corresponding to the 526–558 bases of spinach CMO gene, contains the Ncol site and mixed nucleotides (T/A/G/C/C, which encode Ser, Thr, and Ala in place of TGC encoding Cys-181. The amplified fragment was ligated into the EcoRV site of pBluescript II SK+, double digested with Nael/Ncol, and ligated into the Nael/Ncol sites in pSMC20. The mutant CMO genes were transferred to the pHET+SpCMO as described above. The resulting plasmids, designated as pBET+SpCMO/S, pHET+SpCMO/T, and pHET+SpCMO/A, respectively.

Next, the first His in the putative consensus sequence for coordination of mononuclear non-heme Fe, GIX12_DIX12H12, was changed to Gly, Ala, and Val. For this, a Ndel site was introduced at position 854 in the spinach CMO gene. The 5′-terminal part in the CMO gene was amplified by PCR using the plasmid pSMC20 as the template. The forward primer SpMC20-Nae-F and the reverse primer H287Nde-R, containing the introduced Ndel site were used. The 3′-terminal part of the CMO gene was amplified by using the forward primer H287Nde-R and the reverse primer pSPCMO-R. The forward primer primers the introduced NdeI site and mixed nucleotides G/G/T/C, which encode Gly, Val, and Ala in place of His encoding CAT encoding Cys-181. Both amplified fragments were mixed, annealed, and amplified by using the primer sets SpMC20-Nae-F and pSPCMO-R. The amplified fragment was ligated into the EcoRV site of pBluescript II SK+ and then transferred to the pBET+SpCMO as described above. The generated plasmids that encode the CMO mutants, H287G, H287V, and H287A, were designated as pBET+SpCMO/HG, pBET+SpCMO/V, and pBET+SpCMO/A, respectively.

Expression of bet and CMO Genes in E. coli and Synechococcus sp. PCC7942—For the expression of betaine synthesis genes in E. coli and Synechococcus sp., E. coli DH5α and Synechococcus sp. PCC7942 cells were transformed with pBET+CDH, pBET+SpCMO, pHET+AtCMO and pBET+CDH. E. coli cells were grown at 30 °C in L-broth supplemented with 100 μg/ml chloramphenicol and 50 μg/ml streptomycin. Synechococcus cells were cultured at 30 °C under continuous fluorescent white light at 80 μE m−2 s−1 in BG 11 liquid medium supplemented with 100 μM choline and 10 μg/ml streptomycin and bubbled with air. Cells at the logarithmic phase were transferred into fresh medium containing various concentrations of NaCl. The cultures were incubated for various times as indicated.

For the expression of mature spinach CMO, E. coli BL21(DE3) cells transformed with pSMCMO were grown at 30 °C in Terrific-broth with 50 μg/ml ampicillin until the optical density at 600 nm reached 0.6, and then 0.5 mM isopropyl 1-thio-β-galactopyranoside was added (16). After induction, 25 μM ferric ammonium citrate was added four times to a final concentration of 30 μM. Cells were allowed to grow overnight at 15,000 rpm. The pellets were dissolved in 20 ml of 10 mM Tris-HCl (pH 8.0), 0.02% β-mercaptoethanol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (buffer A) per liter of culture medium and sonicated for 5 min. After centrifugation at 15,000 rpm for 30 min, the supernatant was used for the measurement of CMO activity.

Construction of Transgenic Arabidopsis—For the construction of binary vector of spinach CMO, the pSPCMO plasmid was double digested with NdeI and BamHI. The resulting fragment was blunt-ended and ligated into the Smal/Sacl-digested and blunt-ended pBi121 vector. The resulting vector was designated as pHSPCMO. For the construction of binary vector coexpressing spinach CMO and BADH, the full-length spinach BADH gene was amplified by PCR as previously described (16) and ligated into the HindIII site of pUC119. The resulting plasmid was digested with XbaI and BamHI. The digested fragment was ligated into the corresponding site of pHSPCMO. Then, a Smal/Sacl fragment containing the glucuronidase gene was removed and blunt-ended, and ligated into the partially digested EcoRI site of pHSPCMO with pBADH. Arabidopsis plants (ecotype Columbia) were transformed by the Agrobacterium-mediated method as described previously (17). Two transformants homozygous for the introduced genes and showing high level expression of the CMO gene were selected. The analysis of stress tolerance. Plants were treated with Murashige & Skoog medium containing 1 molar choline at a growth chamber (San Joaquin MLR-3500HT, Japan) at 22 °C with a 16-h light (150 μE m−2 s−1) /8-h dark cycle and 60% relative humidity. For salt stress, 3-week-old Arabidopsis plants were treated with 200 mM NaCl.
For the experiments of exogenous betaine, 1 mM betaine was added to carried out with the incubation at 5°C. The ages of surviving plants were counted. Low temperature treatment was stressed, water was withheld for 24 h and then resupplied, and percent-quantum yield in photosystem II (PSII) was measured. For drought, D-effeltrich, Germany) (17). Before the measurements, cells were dark-adapted for 10 min, and then the quantum yield in photosystem II measured after esterification with time of flight mass spectroscopy.

The sequences were aligned by the program ClustalW. The accession numbers of spinach CMO (accession no. U85780) and sugar beet CMO (accession no. AF023132), respectively. The different amino acids between At cDNA and At genome are shown by asterisks, and conservative substitutions are shown by dots. The putative consensus sequence (CXXX2H) for coordination of Rieske-type [2Fe-2S] cluster and that for coordination of mononuclear non-heme Fe (G/DFX2H) are shown by a closed downward arrow. The full-length CMO-like gene was transcribed in Arabidopsis.

We also amplified the spinach CMO gene and sequenced it. Its nucleotide sequence completely coincided with the published one (8). Alignment of amino acids between Arabidopsis CMO-like protein, spinach CMO, and sugar beet CMO (12) revealed that all these proteins contain the putative consensus sequences for coordination of the Rieske-type [2Fe-2S] cluster and that for coordination of mononuclear non-heme Fe (G/DXX4, DXH2H) are shown by a bar. The processing site of spinach CMO is shown by a closed downward arrow.

Functional Characterization of Choline Monooxygenase

Expression of the CMO-like Gene in Arabidopsis—To examine whether the CMO-like gene is expressed in Arabidopsis, we carried out RT-PCR. Using the primer sets, which completely matched the 5′- and 3′-terminal regions of putative CMO-like gene, a 1.3-kb fragment was amplified. The nucleotide sequence revealed that the amplified fragment was the putative CMO-like protein deduced from the genome sequence. Another CMO-like protein, spinach CMO, and sugar beet CMO (12) revealed that all these proteins contain the putative consensus sequences for coordination of the Rieske-type [2Fe-2S] cluster and that for coordination of mononuclear non-heme Fe (G/DXX4, DXH2H) are shown by a bar. The processing site of spinach CMO is shown by a closed downward arrow.

RESULTS

Expression of the CMO-like Gene in Arabidopsis—To examine whether the CMO-like gene is expressed in Arabidopsis, we carried out RT-PCR. Using the primer sets, which completely matched the 5′- and 3′-terminal regions of putative CMO-like gene, a 1.3-kb fragment was amplified. The nucleotide sequence revealed that the amplified fragment was the putative CMO-like gene. However, the CMO-like protein deduced from the amplified cDNA was different from that of the genome sequence in two points. As shown in Fig. 1, ten amino acids (115RLGDVDFVVC124) deduced from the cDNA were missing in the putative CMO-like protein deduced from the genome. By contrast, 14 amino acids (279TLVASCDL-)

LYGLR292) deduced from the genome sequence were missing in our cDNA. These differences were probably due to the inaccurate prediction of exon in the genome sequence. Another difference was the change of Ile-371 (ATC) to Gly-Leu-Met in Arabidopsis CMO-like protein. The tripeptide TyrTyrAla (8) are U85780 (8) and AF023132 (19), respectively. The different amino acids between At cDNA and At genome are shown by asterisks, and conservative substitutions are shown by dots. The putative consensus sequence (CXXX2H) for coordination of Rieske-type [2Fe-2S] cluster and that for coordination of mononuclear non-heme Fe (G/DXX4, DXH2H) are shown by a bar. The processing site of spinach CMO is shown by a closed downward arrow.

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treated for 12 h with 0.2 M NaCl; Arabidopsis like protein could not be detected in either the root or leaf of CMO showed that unlike spinach CMO, the blotting analysis using an antibody raised against spinach betaine proteins were analyzed by Western blotting. DH5 cells transformed with pBET-CDH; lane 2, E. coli (DH5α) transformed with pBET+SpCMO; lane 3, E. coli (DH5α) transformed with pBET+AtCMO.

 soluble proteins were extracted from Arabidopsis. Western blotting analysis using an antibody raised against spinach CMO showed that unlike spinach CMO, the Arabidopsis CMO-like protein could not be detected in either the root or leaf of Arabidopsis (Fig. 2A). These results suggest that the CMO-like protein does not accumulate in Arabidopsis or cannot be detected by the antibody raised against spinach CMO.

Expression of Spinach CMO and Arabidopsis CMO-like Proteins in E. coli—We examined whether spinach CMO and Arabidopsis CMO-like protein could be expressed in E. coli. The expression vectors pBET+CDH, pBET+SpCMO, pBET+AtCMO, and pBET-CDH were constructed as shown in Fig. 3A and used for transforming E. coli DH5α cells. The expressed proteins were analyzed by Western blotting. DH5α cells transformed with pBET+SpCMO or pBET+AtCMO accumulated a protein that cross-reacted with the antibody raised against spinach CMO (Fig. 2B, lanes 2 and 3), whereas E. coli cells expressing pBET+CDH (Fig. 2B, lane 1) or pBET-CDH (data not shown) did not show the cross-reaction band. When the mature spinach CMO (8) and Arabidopsis CMO-like protein were expressed in E. coli, the mature-size proteins accumulated in the soluble fraction, whereas when the precursor spinach CMO was expressed in E. coli, most of the precursor protein accumulated in the insoluble fraction without processing (data not shown). These results indicate that both spinach CMO and Arabidopsis CMO-like protein could be expressed in E. coli and that the failure of detection of CMO-like protein in Arabidopsis (Fig. 2A) was not due to the weak reactivity of the spinach CMO antibody with Arabidopsis CMO-like protein.

Accumulation of Betaine in Transformed E. coli Cells—Next, we examined the accumulation of betaine in the transformants. As shown in Fig. 4, the E. coli DH5α cells transformed with pBET+CDH accumulated betaine and its contents increased as the concentration of NaCl increased. However, the betaine levels in DH5α cells or DH5α cells expressing pBET-CDH were negligible even if the cells were grown at high salinity (0.5 M NaCl). They were about 2% of those produced by the bet gene cluster. By contrast, the E. coli cells expressing pBET+SpCMO accumulated betaine at about 37% of that expressing pBET+CDH at 0.5 M NaCl. The E. coli cells expressing pBET+AtCMO did not accumulate betaine. These results indicate that spinach CMO catalyzes the oxidation of choline to betaine aldehyde in E. coli, whereas Arabidopsis CMO-like protein is not involved in betaine synthesis. Because reduced ferredoxin is required for the function of CMO (7, 8), the present results suggest that E. coli ferredoxin (21) could replace spinach ferredoxin.

Salt Tolerance of E. coli Cells Expressing Spinach CMO—Due to the absence of the bet gene cluster, the E. coli DH5α
cells are relatively salt-sensitive. We examined the effects of CMO expression on the salt tolerance of DH5α cells. All the DH5α cells transformed with pBET+SpCMO, pBET+CDH, and pBET-CDH and plated on agar plates which contain 100 mM NaCl (A) or 500 mM NaCl (B). C, growth curves. The control and transformant cells at logarithmic phase in minimum medium were inoculated into fresh medium containing indicated concentrations of NaCl for salt stress. Open circle, E. coli (DH5α) control cells; open square, pBET+CDH-transformed cells; open triangle, pBET-CDH-transformed cells; closed triangle, pBET+SpCMO-transformed cells. Each value shows the average of three independent measurements.

A) putative consensus sequence for Rieske-type [2Fe-2S] cluster

spinach CMO 162 QTHRASILACGGKKSFCVPHGW 186

1: pETSMCMO
2: pBET-CDH
3: pBET+CDH
4: pBET+SpCMO
5: pBET+SpCMO/C
6: pBET+SpCMO/T
7: pBET+SpCMO/A

B) putative consensus sequence for mononuclear non-heme Fe

spinach CMO 279 DNYLDSSYHVPAHKKYYA 296

G,V,A

1: pETSMCMO
2: pBET-CDH
3: pBET+CDH
4: pBET+SpCMO
5: pBET+SpCMO/HG
6: pBET+SpCMO/HV
7: pBET+SpCMO/H/A

Fig. 5. Effects of salt on the growth of the control and three kinds of transformant cells. A and B, complementation test. E. coli (DH5α) cells were transformed with pBET+SpCMO, pBET+CDH, and pBET-CDH and plated on the agar plates, which contain 100 mM NaCl (A) or 500 mM NaCl (B). C, growth curves. The control and transformant cells at logarithmic phase in minimum medium were inoculated into fresh medium containing indicated concentrations of NaCl for salt stress. Open circle, E. coli (DH5α) control cells; open square, pBET+CDH-transformed cells; open triangle, pBET-CDH-transformed cells; closed triangle, pBET+SpCMO-transformed cells. Each value shows the average of three independent measurements.

Fig. 6. Effects of mutagenesis on the expression and accumulation of betaine. A, Cys-181 in the putative consensus sequence for coordination of the Rieske-type [2Fe-2S] cluster was changed to Ser, Ala, and Thr. B, His-287 in the putative consensus sequence for coordination of mononuclear non-heme Fe was changed to Gly, Ala, and Val. The control and mutants were expressed in DH5α cells. The level of betaine accumulation was measured as in Fig. 4.

Cys-181 and His-287 Are Essential for the Activity of CMO—The nucleotide sequence showed that the CMO gene contains a
Rieske-type [2Fe-2S] cluster motif as well as mononuclear non-heme Fe binding motif (8, 12). However, no experimental evidence on the specific amino acids has been reported. Therefore, we modified the second Cys in the putative consensus sequence for coordination of Rieske-type [2Fe-2S] cluster to Ser, Ala, and Thr. The C181S, C181A, and C181T mutants were expressed in E. coli. The accumulation levels of protein in mutants were almost the same as that of the wild-type CMO (Fig. 6A). However, these mutants could not accumulate betaine in DH5α cells as shown in Fig. 6A. The mutants also did not complement the salt-sensitive phenotype of DH5α (data not shown). These results indicate that Cys-181 is essential for the function of CMO.

We also modified the first His in the putative consensus sequence for coordination of mononuclear non-heme Fe to Gly, Ala, and Val. The mutants H287G, H287A, and H287V were also expressed at similar levels to that of wild-type CMO in DH5α cells (Fig. 6B). Again, these mutants could not accumulate betaine in DH5α cells (Fig. 6B), indicating that His-287 is essential for the activity of CMO. Taken together, the above results indicate that Cys-181 and His-287 are involved for the Rieske-type [2Fe-2S] cluster and mononuclear non-heme Fe, respectively.

Expression of CMO in a Fresh Water Cyanobacterium Synechococcus sp. PCC7942—We examined whether CMO could be expressed in Synechococcus sp. PCC7942. When Synechococcus sp. PCC7942 cells transformed with pBET/CDH, pBET+SpCMO, and pBET-CDH were grown in liquid medium BG11, which contains a low level of NaCl (<100 mM NaCl), the control and transformant cells could grow at almost the same rate (data not shown). However, at a high salinity (300 mM NaCl), Synechococcus cells transformed with pBET/CDH and pBET+SpCMO had a higher growth rate than pBET-CDH (Fig. 7A). Western blotting analysis indicated that Synechococcus sp. PCC7942 cells transformed with pBET+SpCMO accumulated CMO, whereas those of pBET/CDH and pBET-CDH did not (Fig. 7B). These results clearly indicate that spinach CMO could be expressed in Synechococcus sp. PCC7942 cells and confer the tolerance to salt stress.

Expression of Spinach CMO in Arabidopsis—Recently, CDH (22, 23), COX (24–26), and CMO (26, 27) have been expressed in several plants. However, the expression of CMO in Arabidopsis has not been reported. We constructed the binary vec-
tors for the expression of spinach CMO and for the coexpression of spinach CMO and BADH (Fig. 3B) and introduced into Arabidopsis. Twelve independent primary transformants (T_0) for CMO and eight independent transformants for CMO and BADH (CMO/BADH) were allowed to flower and set seeds. In the T_2 generation, two of them, CMO3 and CMO/BADH1 and CMO/BADH2 for the latter, showing high levels of expression were used.

As shown in Fig. 8A, Western blotting analysis using an antibody raised against spinach CMO revealed a single band in both the root and leaf of CMO3 and CMO/BADH1. No band was detected in wild-type Arabidopsis. These results indicate that the cross-reaction band was due to the spinach CMO expressed in Arabidopsis. The betaine accumulation level was slightly higher in transformants than in wild-type Arabidopsis. BADH enzyme was detected in some betaine non-accumulating plants (14, 28). Therefore, the cross-reaction band in the transformant was due to the Arabidopsis BADH as well as spinach BADH. Accumulation levels of betaine in the transformants were low, although their levels increased upon the addition of choline, which is consistent with the previous reports (25–27, 29). Interestingly, the betaine accumulation levels were higher in roots than in leaves.

When the control and transgenic plants were treated with 0.2 M NaCl the quantum yield of photosystem II started to decrease, although they remained at high level without salt treatment (Fig. 9A). However, the rate of decrease was faster in the control plant than those in transgenic plants. Similar re-

![Figure 9: Stress tolerance of the transformed Arabidopsis. A, quantum yield of photosystem II with or without salt stress (0.2 M NaCl). B, survival after drought stress by withdrawing water for 24 h and resupply. C, quantum yield of photosystem II with or without low temperature stress. D, effects of exogenous addition of betaine on the quantum yield of photosystem II with or without salt stress (0.2 M NaCl). Each value shows the average of three independent measurements.](image)

![Figure 10: Putative active site of spinach CMO. A, alignment of the amino acids of spinach CMO (spinach), sugar beet CMO (beet), Arabidopsis CMO-like protein (AtCMO), and naphthale 1,2-dioxygenase (NDO). The amino acid residues conserved in all sequences are shown in bold. The putative ligands for the Rieske-type [2Fe-2S] center and mononuclear Fe are boxed. The Asp that might be important for electron transfer (ET) from the Rieske-type [2Fe-2S] center to Fe is also boxed. B, schematic model of putative active site of spinach CMO.](image)
results were observed for drought and cold stresses as shown in Fig. 9, B and C, respectively. Betaine applied exogenously completely blocked the decrease of the quantum yield in photosystem II in both control and transformant plants (Fig. 9D). These results indicate that spinach CMO could be expressed in Arabidopsis and could produce betaine, but its effect was relatively small due to the low level of betaine.

**DISCUSSION**

The data presented here clearly indicate that CMO could be expressed in E. coli as a soluble protein (Fig. 2B) and could produce betaine in combination with BADH (Fig. 4). The betaine accumulation level in CMO-expressing cells was high, accounting for about 37% of CDH-expressing cells. As a result, CMO could complement the salt-sensitive phenotype of E. coli DH5α cells (Fig. 5). Considering the requirement of reduced ferredoxin and assembly of the Rieske-type [2Fe-2S] center for the activity of CMO (7, 8), high accumulation of betaine in CMO-expressing cells is remarkable. Conversely, the above facts indicate that E. coli contains all the components necessary for the assembly of soluble the Rieske-type [2Fe-2S] center. Indeed, the genes essential for cluster assembly were recently discovered in Azotobacter vinelandii and E. coli (30, 31). The E. coli iron-sulfur cluster assembly operon (30, 31) and ferredoxin (21) are probably involved in the assembly and function of CMO. Compared with the betaine-accumulating transgenic plants, in which the betaine accumulation level was less than 10% of that in betaine-accumulating plants (22–27), the accumulation level of betaine in CMO-expressing E. coli cells was very high. The CMO-expressing E. coli system will be used for detailed functional analysis of CMO.

Fig. 7 shows that spinach CMO could be expressed in Synechococcus sp. PCC7942 cells, could catalyze the oxidation of choline to betaine aldehyde, and could confer the tolerance for salt stress. These facts also indicate that Synechococcus can assemble foreign soluble Rieske-type proteins, and the cyanobacterium ferredoxin can be replaced by chloroplastic ferredoxin. The presence of genes homologous to E. coli iron-sulfur cluster assembly operon has been suggested very recently in Synechocystis sp. PCC6803 (32), which might be involved in the assembly of CMO. However, the betaine accumulation level was low (data not shown), and the effects of CMO expression on the growth rate were similar to those obtained by CDH (15, 33) and COX (34) expression (Fig. 7). This might be due to the inefficient recognition of E. coli bet gene promoters in Synechococcus. The data obtained in E. coli and cyanobacterium clearly indicate that CMO consisted of a single polypeptide that could exhibit the full activity for choline oxidation, although the majorities of oxygenases have dissimilar subunits (35).

Although CMO has been used to transform tobacco plants (26, 27, 29), its molecular characterization largely remains to be clarified. The site-directed mutagenesis of spinach CMO clearly indicated that Cys-181 and His-287 are essential for the activity of CMO (Fig. 6). CMO shows some similarity to bacterial oxygenases (8, 35), which contain a Rieske-type [2Fe-2S] cluster and mononuclear non-heme Fe. In one of the most homologous oxygenases, naphthalene 1,2-dioxygenase, it is believed that Cys-81, His-83, Cys-101, and His-104 coordinate the Rieske [2Fe-2S] center. Fe at the active site is coordinated by His-208, His-213, and Asp-362, and Asp-205 is necessary for electron transfer from the Rieske [2Fe-2S] center to Fe (20, 36). All of these amino acids are conserved in plant CMO and Arabidopsis CMO-like proteins as shown in Fig. 10A. The site-directed mutagenesis experiments strongly suggest that Cys-181 and His-287 in spinach CMO are involved in the coordination of the Rieske-type [2Fe-2S] center and Fe, respectively (Figs. 6 and 10). Based on these similarities and from the structural information of active site of naphthalene 1,2-dioxygenase, the active site of spinach CMO could be described as shown in Fig. 10B. However, many questions remain to be clarified. For instance, it is unclear why only spinach CMO was involved in betaine production (Fig. 4), although both spinach CMO and Arabidopsis CMO-like proteins could be expressed in E. coli as soluble proteins (Fig. 2D). Our attempt to purify the active CMO was unsuccessful. Further characterization of specific amino acids, in vivo and in vitro, is an interesting future subject.

Recent genome sequence of Arabidopsis revealed that Arabidopsis contains a CMO-like gene (accession nos. NM_119135 and CAB43664). The present results clearly showed that the Arabidopsis CMO-like gene is transcribed, but its protein could not be detected by the antibody raised against spinach CMO (Fig. 2A). This was not due to the heterologous hybridization of antibody because the Arabidopsis CMO-like protein expressed in E. coli could be detected by the same antibody (Fig. 2B). After completing this study, we knew that the cDNA of Arabidopsis CMO-like gene was registered in DDBJ/EMBL/GenBank (accession no. AY090377), although its relation to the CMO-like gene was not described. The cDNA (AY090377) completely matched our cDNA except one nucleotide (Fig. 1). Therefore, the most probable explanation for the results in Fig. 2 would be that the Arabidopsis CMO-like protein accumulates at a very low level in Arabidopsis and plays some physiological function different from choline oxidation.

Fig. 8 shows that CMO protein could be detected in the transformed Arabidopsis, but not in the wild-type Arabidopsis, which is consistent with the results shown in Fig. 2. Accumulation levels of betaine were very low as previously reported (23–27). Consequently, the effects of CMO expression on the tolerance for salt, drought, and low temperature stresses were small (Fig. 9). By contrast, exogenous supply of betaine significantly improved the tolerance to abiotic stresses. These results suggest that betaine synthesis, but not betaine transport, is the most important step for increasing the betaine level in betaine non-accumulating plants. Therefore, targeting of the choline transporter in the chloroplast and increased expression of CMO might be important steps for increasing the betaine level in betaine non-accumulating plants.

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