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ORIGINAL ARTICLE

Isolation and characterization of a novel peroxisomal choline monooxygenase in barley

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Abstract Glycine betaine (GB) is a compatible solute accumulated by many plants under various abiotic stresses. GB is synthesized in two steps, choline → betaine aldehyde → GB, where a functional choline-oxidizing enzyme has only been reported in Amaranthaceae (a chloroplastic ferredoxin-dependent choline monooxygenase) thus far. Here, we have cloned a cDNA encoding a choline monooxygenase (CMO) from barley (*Hordeum vulgare*) plants, *HvCMO*. In barley plants under non-stress condition, GB had accumulated in all the determined organs (leaves, internodes, awn and floret proper), mostly in the leaves. The expression of HvCMO protein was abundant in the leaves, whereas the expression of betaine aldehyde dehydrogenase (BADH) protein was abundant in the awn, floret proper and the youngest internode than in the leaves. The accumulation of *HvCMO* mRNA was increased by high osmotic and low-temperature environments. Also, the expression of HvCMO protein was increased by the presence of high NaCl. Immunofluorescent labeling of HvCMO protein and subcellular fractionation analysis showed that HvCMO protein was localized to peroxisomes. [^{14}C]choline was oxidized to betaine aldehyde and GB in spinach (*Spinacia oleracea*) chloroplasts but not in barley, which indicates that the subcellular localization of choline-oxidizing enzyme is different between two plant species. We investigated the choline-oxidizing reaction using recombinant HvCMO protein expressed in yeast (*Saccharomyces cerevisiae*). The crude extract of HvCMO-expressing yeast coupled with recombinant BBD2 protein converted [^{14}C]choline to GB when NADPH was added as a cofactor. These results suggest that choline oxidation in GB synthesis is mediated by a peroxisomal NADPH-dependent choline monooxygenase in barley plants.

Keywords Betaine aldehyde dehydrogenase · Choline monooxygenase · Functional analysis · Glycine betaine · *Hordeum*

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Abbreviations

BADH	Betaine aldehyde dehydrogenase
CMO	Choline monooxygenase
ER	Endoplasmic reticulum
GB	Glycine betaine
GFP	Green fluorescent protein
mRFP	Monomeric red fluorescent protein
PEG	Polyethylene glycol

Introduction

Plants are frequently subjected to environmental stresses such as drought, salinity and low and/or high temperature. They survive these abiotic stresses by responding with a variety of tolerance mechanisms, one of which is the accumulation of compatible solutes. Glycine betaine (GB) is a major compatible solute in bacteria, algae, plants and animals (Blunden et al. 1992; Rhodes and Hanson 1993; Kempf and Bremer 1998). In plants, GB is synthesized by the oxidation of choline via a two-step process: choline → betaine aldehyde → GB (Rathinasabapathi et al.

1997). The second step is catalyzed by betaine aldehyde dehydrogenase (BADH) in GB-accumulating plants (Arakawa et al. 1987; Wood et al. 1996; Hibino et al. 2001; Nakamura et al. 2001). In spinach, both the steps occur in chloroplasts (Weigel et al. 1988) and the first step is catalyzed by a ferredoxin-dependent choline monooxygenase (CMO), which is localized in chloroplast stroma (Brouquisse et al. 1989; Rathinasabapathi et al. 1997). So far, functional CMO has only been found in Amaranthaceae (e.g., amaranth, *Atriplex hortensis*, spinach, sugar beet) (Rathinasabapathi et al. 1997; Russell et al. 1998; Hibino et al. 2002; Shen et al. 2002). Although Arabidopsis is reported to have the *CMO* gene in the genome and its mRNA, the recombinant CMO protein has no activity in *Escherichia coli* (Hibino et al. 2002).

In gramineous plants, including most of the important cereals such as barley, rice, wheat and maize, the enzyme for converting choline to betaine aldehyde has not been found yet. Hanson and Nelsen (1978) reported that [^{14}C]choline is converted to betaine in barley leaves, indicating the existence of a choline-oxidizing enzyme in the GB biosynthetic pathway. We have previously reported that barley plants harbor peroxisomal and cytosolic BADH proteins (BBD1 and BBD2, respectively) and that cytosolic BBD2 shows extremely high affinity for betaine aldehyde compared with BBD1 with an apparent K_m of 18.9 μM and 19.9 mM, respectively (Fujiwara et al. 2008). These results indicate that cytosolic BBD2 has a major role in GB synthesis and that the subcellular location of GB biosynthesis is different between spinach and barley.

Since many crop plants do not accumulate GB, genetic engineering of GB biosynthesis pathway represents a potential way to improve crop plant stress tolerance. Therefore, much research into improving environmental stress tolerance in plants has centered around introducing choline-oxidizing enzymes such as choline dehydrogenase, choline oxidase and spinach CMO into plants (Nomura et al. 1995, 1998; Hibino et al. 2002; Shirasawa et al. 2006; Su et al. 2006). Transgenic plants that have acquired the ability of GB biosynthesis produced smaller amount of GB than found in the plants naturally accumulating GB (Hibino et al. 2002; Park et al. 2007). To make environmental stress tolerance more efficient in plants, it is also necessary to understand how and where GB is produced and distributed in GB-accumulating plants.

In this work, we isolated a *CMO* gene from gramineous barley plants, *HvCMO*. We have shown that *HvCMO* expression is regulated at both mRNA and protein levels under abiotic stress conditions. We have also shown the subcellular localization of HvCMO protein and choline oxidation activity of HvCMO protein in vitro using NADPH as a cofactor, which is the first reported instance of a functional choline-oxidizing protein in Graminaceae.

Materials and methods

Plant materials and growth conditions

The seeds of barley (*Hordeum vulgare* L. cv. Haruna-nijyo; seeds propagated by our university farm) were surface sterilized with 1% (v/v) sodium hypochlorite solution for 3 min and thoroughly washed with distilled water. The germinated seeds were grown in the soil until the panicles appeared (for about 4 months from November to February) in a greenhouse of Nagoya University (Nagoya, Japan, 35°9′N, 136°58′E). The illumination and temperature were naturally controlled.

For abiotic stress treatment, seedlings were grown hydroponically as described previously (Fujiwara et al. 2010). Three-week-old seedlings were treated with the nutrient solution containing 200 mM NaCl, 20% (w/v) PEG 4000, 20 mM H_2O_2 or 50 μM abscisic acid for further 24 h. For treatment at high- or low-temperature, the seedlings were put under 42 and 4°C, respectively, and grown for further 24 h. After treatment, the youngest fully developed leaves were collected and used for further experiments.

The field-grown spinach (*Spinacia oleracea* L.) plants were purchased from a local market and used for the experiments.

Cloning of *HvCMO* gene

Total RNA was isolated from the green leaves of barley plants using the guanidine thiocyanate procedure (McGookin 1984). Reverse transcription of RNA to cDNA was performed with an oligo(dT) primer using a Superscript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). A fragment of *HvCMO* cDNA was obtained from the total RNA preparation by RT-PCR, using degenerate primers designed for the highly conserved region of *CMO* genes in higher plants (Supplemental Table S1). The cDNA sequence containing the entire coding region was obtained by 5′- and 3′-RACE. The 5′- and 3′-RACE-PCRs were performed with the isolated total RNAs by a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Sequence data from this article has been deposited at DDBJ/EMBL/GenBank under accession number AB434467.

Sequence alignment and construction of a phylogenetic tree

The deduced amino acid sequences of plant CMOs were aligned by the N-J method using ClustalX software (Larkin et al. 2007). An N-J tree was produced from the results of 100 bootstrap replicates using ClustalX, and the

phylogenetic tree was displayed with the MEGA4 program (Tamura et al. 2007).

Southern-blot analysis

Genomic DNA was extracted from the leaves of barley and rice (*Oryza sativa* L. cv. Nipponbare). As much as 30 μg of genomic DNA was digested with *Eco*RI, *Hind*III, *Eco*RV or *Bam*HI, and then separated on a 0.8% (w/v) agarose gel. Blotting and hybridization were carried out as described previously (Ueda et al. 2001). A PCR-amplified fragment using the primer set for cloning of the fragment of *HvCMO* was used as a probe (Supplemental Table S1).

Real-time PCR

RNA isolation and real-time PCR were done as described previously (Mitsuya et al. 2009). The primers used in this study are shown in Supplemental Table S1. *HvEF1* (accession No. Z23130) was used as the internal control (Fujiwara et al. 2010). The transcript level of target genes was normalized to that of *HvEF1* (value = 1).

Protein gel-blot analyses of CMO and BADH proteins

Total soluble protein was extracted from the indicated organs of barley plants and spinach leaves as reported previously (Burnet et al. 1995) and precipitated by PEG 6000 or ammonium sulfate. A polyclonal antibody for HvCMO was raised against the mixture of two peptides representing intermediate sequences of HvCMO (n-CESA PAEQEDIDRLGTKA-c and n-ATRISGIKNFNKNDFGL-c) (Medical and Biological Laboratories, Nagoya, Japan). For protein gel-blot analysis, proteins were electrophoresed on SDS-polyacrylamide gels, transferred to a polyvinylidene fluoride membrane and developed using an ECL Advance Western Blotting Detection Kit (GE healthcare biosciences, Piscataway, NJ, USA). Primary (rabbit anti-HvCMO peptides, anti-spinach CMO (Hibino et al. 2002), anti-pumpkin catalase (Yamaguchi et al. 1986), anti-*Eleusine coracana* aspartate aminotransferase (Taniguchi and Sugiyama 1990) and anti-spinach BADH IgGs) and secondary (peroxidase-conjugated donkey anti-rabbit IgG, GE Healthcare) antibodies were diluted in Can Get Signal solution (Toyobo, Osaka, Japan) at 1:1,000, 1:1,000, 1:10,000, 1:1,000, 1:5,000 and 1:5,000, respectively. Protein concentration was determined with the Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as a standard.

Determination of glycine betaine (GB) content

The content of GB was determined as reported previously (Jagendorf and Takabe 2001).

Immunofluorescent labeling of HvCMO protein

Immunodetection of HvCMO protein was performed mainly as described by Kobayashi et al. (2009) but with slight modification. Small leaf segments (5 mm^2) were excised from leaf blades and vacuum infiltrated for 10 min with fixation buffer [50% (v/v) ethanol, 5% (v/v) acetic acid, 3.5% (v/v) formaldehyde]. After incubation at 4°C overnight, the fixed segments were embedded in 3% (w/v) agar and sectioned at 70–80 μm with a micro-slicer (DTK-3000W, Dosaka EM, Kyoto, Japan). The sections were soaked in phosphate-buffered saline (PBS: 20 mM Na-Pi, pH 7.0, 150 mM NaCl) containing 0.25% (v/v) Triton X-100 for 20 min, washed with PBS three times, and then incubated in blocking buffer [5% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween-20 in PBS] for 30 min. After washing with PBS three times, the sections were incubated overnight at 4°C with anti-HvCMO peptides antibody diluted 1:200 in incubation buffer [1% (w/v) BSA, 0.05% (v/v) Tween-20 in PBS]. The sections were washed with PBS for 2 h and then incubated at room temperature for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Wako Pure Chemical Industries, Osaka, Japan) diluted 1:200 in the incubation buffer. After rinsing again with PBS, the sections were mounted on microscope slides in PBS containing 0.1% (w/v) *p*-phenylenediamine (Wako Pure Chemical Industries). The sections were observed using a confocal microscope (Carl Zeiss LSM 5 PASCAL).

Sucrose density gradient centrifugation

Subcellular fractionation by sucrose density gradient centrifugation was performed as described by Nishimura et al. (1976). Leaf blades (1 g) were chopped into small segments on ice for 10 min. The grinding medium was 0.5 M sucrose and 1 mM EDTA in 150 mM Tris–HCl (pH 7.5). The homogenate was hand squeezed through three layers of Miracloth and centrifuged at 1,600*g* for 5 min at 4°C. The supernatant was layered on top of 14 ml of a linear sucrose gradient (30–60%, w/w) dissolved in 1 mM EDTA (pH 8) and spun at 100,000*g* for 2.5 h at 4°C using a Hitachi SRP28SA rotor. At the end of the run, 0.5 ml fractions were collected and the aliquots were used for measuring the enzymic activities. The activity of catalase and cytochrome *c* oxidase was measured as described by Nishimura et al. (1976). An equal volume of each of the peroxisomal (No. 7 in Fig. 7b) and the mitochondrion (No. 14 in Fig. 7b) fractions was electrophoresed on SDS-polyacrylamide gels and used for protein gel-blot analysis as described above.

[^{14}C]choline oxidation assay using barley and spinach chloroplasts

Chloroplasts were isolated from spinach and barley leaves as described by Weigel et al. (1988). Chloroplast integrity measured by ferricyanide reduction (Lilley et al. 1975) was >94%. [Methyl-^{14}C]choline chloride was obtained from Amersham Biosciences (Pittsburg, PA, USA). Standard reaction mixtures (76 µl) contained 50 µl of resuspension buffer, chloroplasts equivalent to 20 µg Chl, 1 µl of 0.5 M $NaHCO_3$ and 5 µl of [^{14}C]choline solution (3,700 Bq, final concentration 0.23 mM) as described by Weigel et al. (1988). Incubation was carried out at 25°C under light (about 500 µmol photons m^{-2} s^{-1}) or darkness (covered with aluminum foil). Reactions were stopped in liquid N_2 immediately. After freeze–thaw for three times, the reaction solution was centrifuged, and the supernatant (10 µl) and standards (4 µl of the mixture of 0.5 M choline chloride, betaine aldehyde and GB) were electrophoresed on filter papers (Whatman 1 chr) in 70 mM sodium tetraborate (400 V, 40 min). Radioactive compounds were located by autoradiography, and standards with the Dragendorff's reagent spray solution (Merck, Darmstadt, Germany). Then the betaine aldehyde and GB were eluted and ^{14}C was counted in a scintillation counter at an efficiency of 49%.

Expression and extraction of HvCMO protein in yeast

The *HvCMO* cDNA was amplified by PCR and inserted into a pYES2 vector (Invitrogen) via the *Kpn*I and *Bam*HI sites. For protein expression, the resulting *HvCMO*-pYES and empty pYES2 plasmids were transferred into *Saccharomyces cerevisiae* strain BJ5458 (*Mat a*, *ura3-52*, *trp1*, *lys2-801*, *leu2Δ1*, *his3Δ200*, *pep4::HIS3*, *prbΔ 1.6R, can1* and *GAL*), which is deficient in the major vacuolar proteases (Jones 1991) and which was provided by Dr. M. Maeshima (Nagoya University, Japan). To express the *HvCMO* gene, the transformants were grown in complete medium, YP, supplemented with 2% (w/v) raffinose at 30°C. When OD_{600} reached at around 1, galactose was added to 2% and grown for a further 12 h at 30°C. The cells were harvested by centrifugation at 5,000*g* for 5 min at 4°C and resuspended in the extraction buffer containing 100 mM Tris–HCl (pH 8), 1 mM EDTA, 2 mM DTT and 0.1 mM PMSF. The cells were broken with glass beads by vortexing and centrifuged at 18,800*g* for 15 min at 4°C. The supernatants were mixed with equal volume of 50% (w/v) PEG 6000 solution in 50 mM Tris–HCl (pH 8) and incubated at 4°C to precipitate protein. Then the PEG-precipitated protein was collected by centrifugation at 18,800*g* for 15 min at 4°C and resuspended in 1 ml of buffer A containing 50 mM Tris–HCl (pH 8), 10% (v/v) glycerol and 0.1% (v/v) β-mercaptoethanol.

In vitro [^{14}C]choline oxidation assay using recombinant HvCMO protein

The standard reaction mixture (65 µl) contained 1 µl of 0.6 M $MgCl_2$, 1 µl of 0.35 M DTT, 0.4 µl of 0.2 M NAD^+, 0.4 µl of 0.2 M NADPH, 3 µl of 2.5 nmol $µl^{-1}$ min^{-1} recombinant BBD2 protein (Fujiwara et al. 2008), 1 µl of 25 mg ml^{-1} catalase (Sigma-Aldrich, Irvine, UK), 5 µl of [^{14}C]choline solution (3,700 Bq, final concentration 0.23 mM), 20 µl of protein (about 800 µg), and 33.2 µl of buffer A. The reaction was incubated at 25°C for 1 h under dark and stopped in liquid N_2 immediately. The reaction solution was then centrifuged, and the supernatant (10 µl) was subjected to paper electrophoresis as described above. The dried paper was exposed and analyzed on a bioimaging analyzer (BAS 2500, Fujix, Tokyo). The GB was eluted and ^{14}C was counted in a scintillation counter.

Results

Cloning of *HvCMO* gene in barley

The *HvCMO* cDNA contained an open reading frame of 1,224 bp encoding a deduced 407 amino acid polypeptide (Fig. 1) with a predicted molecular mass of 44.8 kDa and an isoelectric point of 6.2. A phylogenetic analysis showed that the deduced amino acid sequence of HvCMO protein was closely related to gramineous CMOs (corn, rice, sorghum) and does not cluster with amaranthaceous CMOs (amaranth, *Atriplex hortensis*, spinach, sugar beet) (Fig. 2). However, HvCMO has conserved putative amino acid residues essential for binding the Rieske-type [2Fe-2S] center and mononuclear Fe (Fig. 1), which is necessary for CMO activity (Rathinasabapathi et al. 1997; Hibino et al. 2002). The primary structure of HvCMO was quite different from that of other choline-oxidizing proteins such as choline dehydrogenase and choline oxidase (Fig. 2).

To determine the copy number of the *HvCMO* gene in the barley genome, Southern-blot analysis was performed with the HvCMO fragment, which is a highly conserved region of *CMO* genes in higher plants, as a probe (Supplemental Table S1). Only a single band in the *Eco*RI and *Hind*III digests of barley genomic DNA was detected (Fig. 3). The result showed that *HvCMO* was encoded by a single gene in the barley genome (Fig. 3). The copy number of the *CMO* gene in rice genome using the same probe was also determined. Only a single band in the *Eco*RI, *Eco*RV and *Hind*III digests and two bands in the *Bam*HI digest of rice genomic DNA were detected because the rice genome had an internal *Bam*HI restriction site at almost the center of the hybridized region using the present probe [2,261 bp downstream from the translation initiation site in the fifth intron of rice *CMO*

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   1 ACGCGGGGACTCCAATTCCTCCCAACCCGCACGCAGCCAGCCCGGGCCGGCGATGGCGACCGCGCAGTTCCGACCCCTGTCCTCCTCCTCC   91
   1                                                     M  A  T  A  Q  F  R  P  L  S  S  S  S   13

  92 TCCGCCTCCGCAGCCGCCCGGCCCAGGGCCTTCCGCGCCGCGCCGTCCCGCGTGGCCGCGGCGGCGTCGGCGTCGGGCGAGCCCGCGCGG  181
  14 S  A  S  A  A  A  R  P  R  A  F  R  A  A  P  S  R  V  A  A  A  A  S  A  S  G  E  P  A  R   43

 182 CGGCTCGCGGCGGAGTTCGACCCGGCGGTCCCGCTGGCCTCCGCGGTGACGCCGCCCAGCGGGTGGTACACCGACCCGGGGTTCCTCCGT  271
  44 R  L  A  A  E  F  D  P  A  V  P  L  A  S  A  V  T  P  P  S  G  W  Y  T  D  P  G  F  L  R   73

 272 CTCGAGCTAGACCGCGTCTTCCTCCGCGGGTGGCAGGCCGTTGGTCACATAGGACAAGTCAAGAACCCAAATGATTTCTTCACAGGAAGC  361
  74 L  E  L  D  R  V  F  L  R  G  W  Q  A  V  G  H  I  G  Q  V  K  N  P  N  D  F  F  T  G  S  103

 362 CTAGGAAATGTAGAATTTGTGATATGCCGGGATGCAAATGGGAAACTTCAAGCTTTTCACAATGTGTGTCGTCATCATGCCTCACTCCTC  451
 104 L  G  N  V  E  F  V  I  C  R  D  A  N  G  K  L  Q  A  F  H  N  V  C  R  H  H  A  S  L  L  133
            [2Fe-2S] cluster
 452 GCATGTGGAAGTGGTCAGAAGACTTGCTTCCAATGCCCTTATCACGGTTGGACGTATGGTTTAGATGGTACCCTCCTGAAAGCTACAAGA  541
 134 A  C  G  S  G  Q  K  T  C  F  Q  C  P  Y  H  G  W  T  Y  G  L  D  G  T  L  L  K  A  T  R  163

 542 ATATCAGGAATCAAGAACTTTAACAAGAATGATTTCGGTCTTTTACCAATTAAGGTGGCTACCTGGGGACCTTTTGTGCTTGCCAGATTT  631
 164 I  S  G  I  K  N  F  N  K  N  D  F  G  L  L  P  I  K  V  A  T  W  G  P  F  V  L  A  R  F  193

 632 GATGACTCCTCTCAAGATACTGTTCATGATGTGGTTGGAGATGAATGGCTGGGTAGTGCTTCAGATCTGTTGAGTAGAAGTGGCATTAAC  721
 194 D  D  S  S  Q  D  T  V  H  D  V  V  G  D  E  W  L  G  S  A  S  D  L  L  S  R  S  G  I  N  223
                                                                                  Fe-binding
 722 ACTTCACTACCCCATATTTGCAGGCGAGAATATATTATCGAATGTAACTGGAAGGTATTTTGTGACAACTATCTAGATGGTGGATATCAT  811
 224 T  S  L  P  H  I  C  R  R  E  Y  I  I  E  C  N  W  K  V  F  C  D  N  Y  L  D  G  G  Y  H  253

 812 GTTCCATATGCACATGGGGCCCTTGCATCCGGTCTTCAGCTTCAATCCTATGAAACACTTACATACGAAAAAGTTAGTGTTCAAAGATGT  901
 254 V  P  Y  A  H  G  A  L  A  S  G  L  Q  L  Q  S  Y  E  T  L  T  Y  E  K  V  S  V  Q  R  C  283

 842 GAAAGTGCCCCAGCAGAACAAGAAGATATCGATCGCTTAGGGACAAAAGCTACCTATGCCTTTGTTTATCCAAACTTTATGATCAATAGG  991
 284 E  S  A  P  A  E  Q  E  D  I  D  R  L  G  T  K  A  T  Y  A  F  V  Y  P  N  F  M  I  N  R  313

 902 TATGGTCCATGGATGGACACTAATCTAGCT GTCCCATTGGATGCAACCAGGTGCAAAGTGGTTTTTGATTATTTTCTGGACAAATCTCTT 1081
 314 Y  G  P  W  M  D  T  N  L  A  V  P  L  D  A  T  R  C  K  V  V  F  D  Y  F  L  D  K  S  L  343

1082 CTGGATGACCAGGATTTCATCAACAGAAGCTTAAAAGACAGCGAACAAGTACAGATTGAAGACATTGCACTGTGCGAGGGAGTCCAGCGG 1171
 344 L  D  D  Q  D  F  I  N  R  S  L  K  D  S  E  Q  V  Q  I  E  D  I  A  L  C  E  G  V  Q  R  373

1172 GGCTTGGCGTCGCCGGCCTACGGCGTGGGCAGGTACGCGCCGTCCGTGGAGATGGCCATGCACCACTTCCACTGCCTCCTGCACGCCAAT 1261
 374 G  L  A  S  P  A  Y  G  V  G  R  Y  A  P  S  V  E  M  A  M  H  H  F  H  C  L  L  H  A  N  403

1262 CTCAGTGGCCAGTGATGATTATGTGCGGCAC                                                            1292
 404 L  S  G  Q                                                                                 407
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Fig. 1 The nucleotide sequence and the deduced amino acid sequence of *HvCMO*. The putative sites for binding the Rieske-type [2Fe-2S] cluster and mononuclear non-heme Fe are shown by *bars*

homolog (accession number NM_001188048)] (Fig. 3). This result indicates that the *CMO* gene was encoded by a single gene in the rice genome, which is consistent with the single *CMO* homolog in the rice genome database.

Distribution of GB and protein gel-blot analysis of HvCMO in barley

We performed protein gel-blot analysis of the CMO protein in the youngest leaves of 4-month-old barley plants grown under normal condition with an antibody raised against a mixture of two peptides representing the intermediate sequence of HvCMO protein as described in "Materials and methods". A signal was detected at between 37 and 50 kDa in ammonium sulfate 30–40% (w/v) and polyethylene glycol (PEG) 6000 0–16% (w/v) fractions of barley protein (Fig. 4a). When using the antibody against spinach CMO protein (Hibino et al. 2002), the CMO signal was detected in 16–24% (w/v) PEG fractions of spinach protein but not in any fractions of barley protein (Fig. 4b).

Next, we determined the distribution of GB content and CMO and BADH proteins in the various organs of barley plants at the reproductive stage (2 days after the emergence of the spikelet) grown under non-stress condition. In Fig. 5,

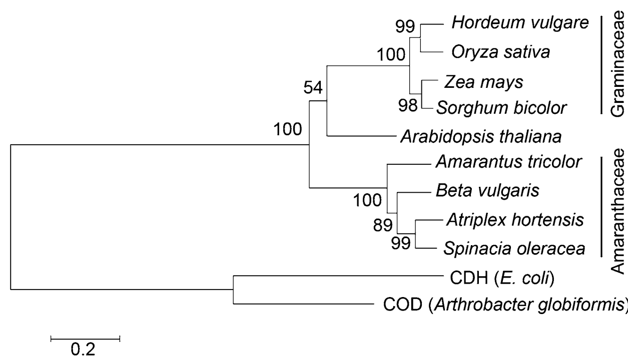

Fig. 2 Phylogenetic tree of plant CMOs. Analyzed amino acid sequences of CMO are from *Amaranthus tricolor* (GenBank accession BAF93188), *Atriplex hortensis* (AAF76895), *Beta vulgaris* (AF023132), *Sorghum bicolor* (XP_002437525), *Spinacia oleracea* (U85780) and *Zea mays* (NP_001105926). The amino acid sequences of *Hordeum vulgare*, *Oryza sativa* and *Arabidopsis thaliana* CMO were deduced from the cDNAs (GenBank accession AB434467, AJ578494, and NM_119135), respectively. The amino acid sequence of choline dehydrogenase (CDH) (*E. coli*; GenBank accession YP_001729254) and choline oxidase (COD) (*Arthrobacter globiformis*; AAP68832) was also analyzed for comparison with HvCMO. Multiple sequence alignment and the generation of the phylogenetic tree were performed with the program of Clustal W. Bootstrap values for 100 resamplings are shown on each branch. The *scale bar* represents 0.2 substitutions per site

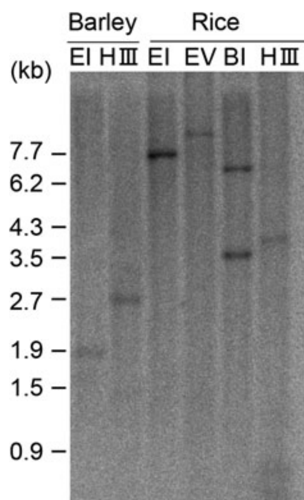

Fig. 3 Southern-blot analysis of the *CMO* gene in barley and rice plants. A 30 μg aliquot of genomic DNA was digested with *Eco*RI (EI), *Hind*III (HIII), *Eco*RV (EV) or *Bam*HI (BI) and separated on a 0.8% (w/v) agarose gel. The blot was hybridized at 65°C with the cDNA of the conserved region as a probe and washed in 0.5% SSC and 0.5% SDS at 65°C

organs are numbered to indicate the order counted from the youngest part with the first organ meaning the youngest part of the organs. GB had accumulated in all the determined organs (Fig. 5a). In the internodes, GB content was the highest in the youngest ones and was lower toward the older ones. In the leaf blades, the secnd youngest leaf blade

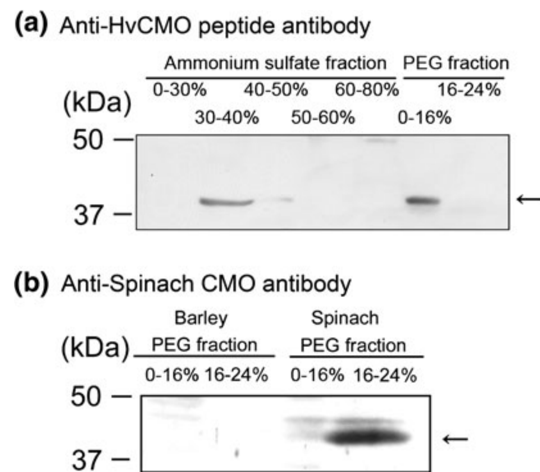

Fig. 4 Protein gel-blot analysis of CMO protein in the youngest leaves of 4-month-old barley plants. A 50 μg aliquot of the redissolved ammonium sulfate and PEG precipitates was loaded in each *lane*. *Arrow* shows a CMO signal. **a** Protein gel-blot analysis of barley proteins with an antibody raised against partial polypeptides of HvCMO. **b** Protein gel-blot analysis of barley and spinach proteins with an antibody raised against spinach CMO (Hibino et al. 2002)

accumulated the most GB, but overall the leaf blades accumulated more GB than the internodes and florets. In the florets, the awn had accumulated more GB than the floret proper. The CMO protein was detected in each of the leaf blades and more in the older leaf blades, but little in the florets and internodes (Fig. 5b). The amount of BADH protein was also determined by protein gel-blot analysis using anti-spinach BADH antibody which binds to both BBD1 and BBD2 proteins. The amount of BADH proteins was abundant in the florets and younger internodes (Fig. 5b). BADH proteins were also detected in leaf blades but less than in the other organs.

Expression pattern of *HvCMO* gene and its encoding protein under abiotic treatments

The effect of abiotic stress treatment on the expression pattern of *HvCMO* and *BBD2* gene was also determined. As abiotic stresses, 3-week-old barley seedlings were treated with the indicated amount of NaCl, PEG, abscisic acid, H_2O_2, and heat- or low-temperature for 24 h and the fully developed leaves were used for further analyses. The mRNA of *HvCMO* gene was significantly increased by PEG and low-temperature treatments, whereas *BBD2* mRNA was increased by the treatment of NaCl, PEG, ABA and low temperature, and decreased by heat (Fig. 6a). The mRNA of *HvCMO* gene was comparable among control, NaCl-, H_2O_2-, ABA- and heat-treated plants (Fig. 6a). The treatment of H_2O_2 did not increase the expression amount of *BBD2* gene. In addition, we determined the GB content and the protein level of HvCMO in the leaves of NaCl-treated

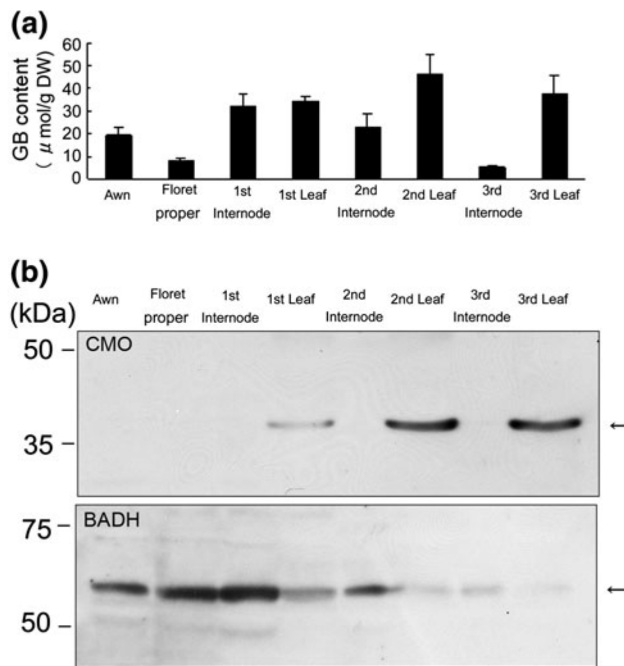

Fig. 5 Relationship between the distribution of GB and expression of CMO and BADH proteins in various organs of barley plants at the reproductive stage. **a** The content of GB in various organs of barley plants. The numbers attached to each organ indicate the order counted from the youngest part. Data are means ± SE ($n = 3$). Vertical bars represent SE. The experiment shown is representative of triplicate measurements. **b** Protein gel-blot analyses of CMO and BADH in various organs of barley plants. The *lanes* were loaded with protein extracted from various organs equivalent to 12 mg FW. The 0–40% (for CMO) or 55–80% (for BADH) ammonium sulfate-fractionated protein was loaded in each *lane*

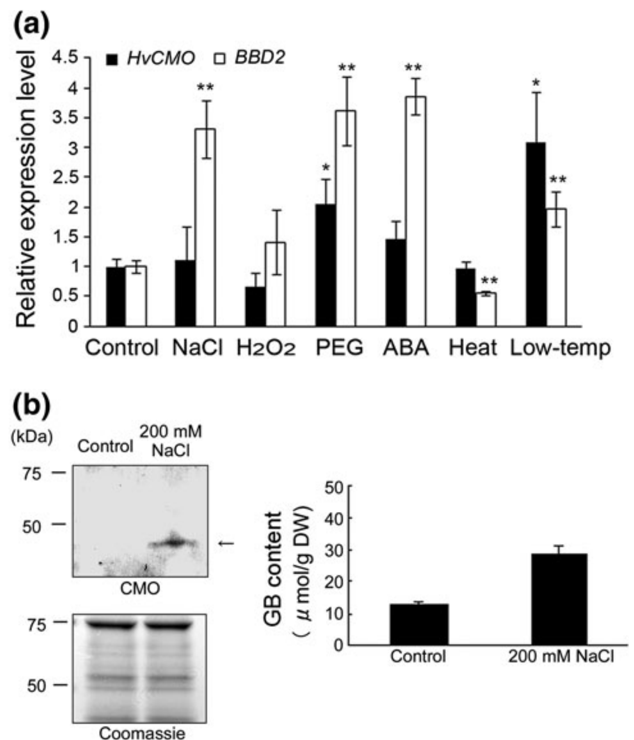

Fig. 6 Effect of abiotic stress treatment on the expression of *HvCMO* and *BBD2* genes. **a** Real-time PCR analysis of the expression of *HvCMO* and *BBD2* under some abiotic stress treatment in the youngest fully developed leaves of barley plants. *HvEF1* was used as an internal control to normalize for variation in the amount of cDNA template. Data show the mean of relative values with one unit being control ± SD of three biological replicates. **b** The content of GB and protein gel-blot analysis of CMO in the youngest fully developed leaves of barley plants treated with 0 and 200 mM NaCl for 24 h. Data are mean ± SE ($n = 3$). The experiment shown is representative of triplicate measurements. The 0–16% PEG-fractionated protein was loaded in each *lane*. *Arrow* shows a CMO signal. A Coomassie brilliant blue-stained gel is shown to the bottom as a loading control

barley plants. The GB content and CMO protein were concomitantly increased by salt treatment (Fig. 6b).

Subcellular localization of HvCMO protein

Leaf blades of barley plants were used to determine the subcellular localization of HvCMO protein. Immunodetection using an anti-HvCMO peptide antibody and a fluorescein-conjugated secondary antibody showed strong fluorescent signals for HvCMO in the dot-shaped organelles, which did not overlap with the autofluorescence of chloroplasts (Fig. 7a). To identify the dot-shaped organelles, we further performed subcellular fractionation by sucrose density gradient centrifugation. Marker enzyme activities of peroxisomes (catalase) and mitochondria (cytochrome *c* oxidase) were clearly present in peroxisomal ($d = 1.25$) (No. 7) and mitochondrial fractions ($d = 1.2$) (No. 14), respectively (arrows in Fig. 7b), although the activity of these enzymes was also detected in the supernatant fractions (from No. 22 to the top fraction). Next, protein gel-blot analysis of the HvCMO and marker proteins using peroxisomal and mitochondrial fractions was performed (Nos. 7 and 14, respectively) (Fig. 7c). The signal of peroxisomal catalase and mitochondrial aspartate aminotransferase was detected in peroxisomal (No. 7) and mitochondrial (No. 14) fractions, respectively. The signal of HvCMO was detected in the same fraction as the one of catalase, which indicated that HvCMO was localized to peroxisomes (Fig. 7c). The subcellular localization of HvCMO- and BBD2-green fluorescent protein (GFP) fusion proteins was determined by transiently expressing in onion epidermal cells. The ER-marker protein, sp-monomeric red fluorescent protein (mRFP)-HDEL (Tamura et al. 2005), was simultaneously expressed with the HvCMO-GFP fusion protein. Cells expressing sp-mRFP-HDEL showed RFP fluorescence in ER (Supplemental Fig. S1a). GFP fluorescence of HvCMO-GFP corresponded to RFP one of sp-mRFP-HDEL, which indicated that HvCMO-GFP was localized to ER (Supplemental Fig. S1 b, c).

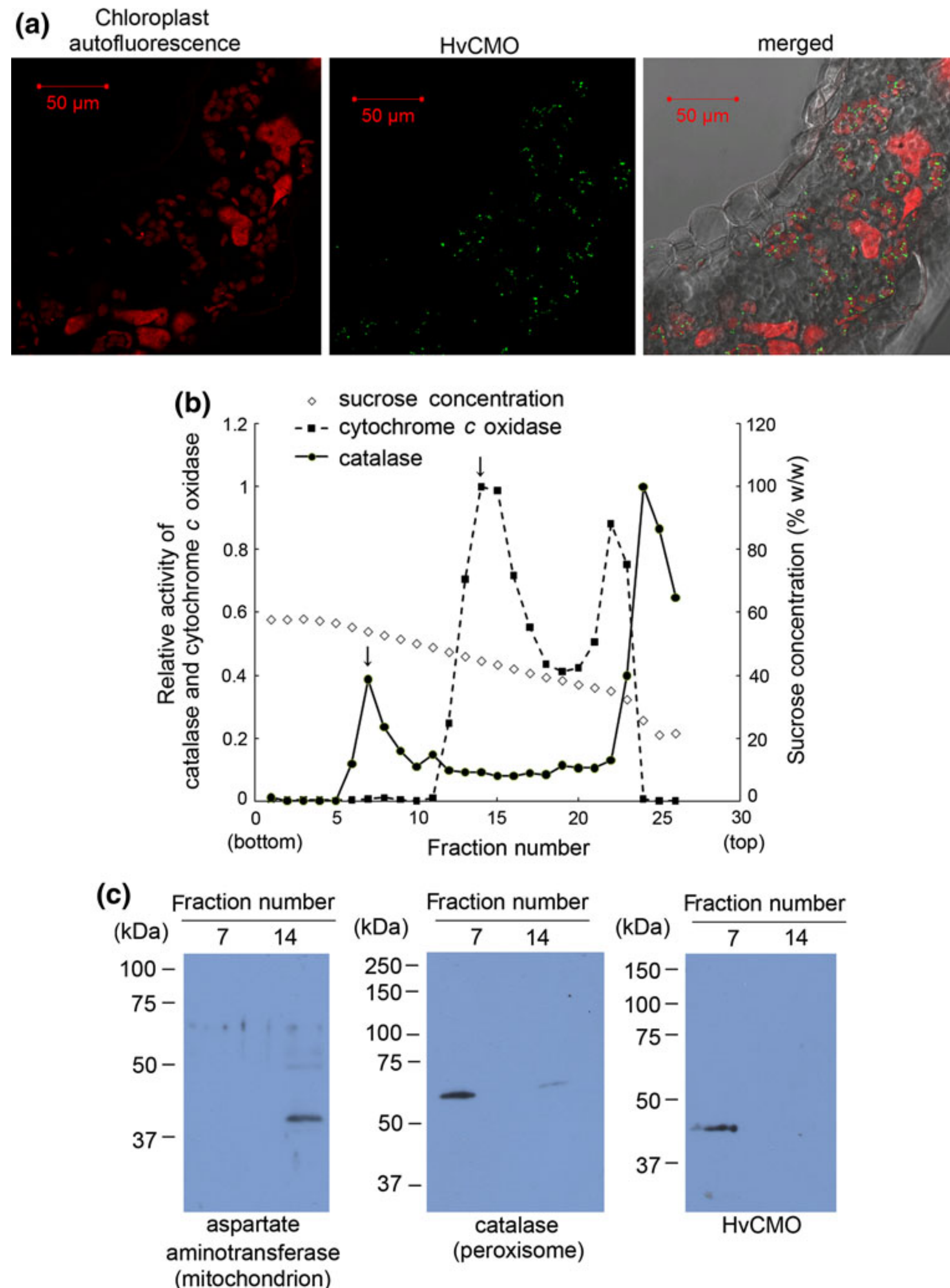

Fig. 7 Subcellular localization of HvCMO protein in barley plants. **a** Subcellular immunodetection of HvCMO protein in the leaf blades. The *red* autofluorescence emitted by chloroplasts, the *green* fluorescence of HvCMO detected with fluorescein-conjugated goat anti-rabbit antibody and merged image are shown. *Scale bars* represent 50 μm. **b** Localization of enzyme activities in the separated fractions after sucrose density gradient centrifugation of the extract from the leaf blades. The leaf blade extract was subjected to sucrose density gradient centrifugation and the 0.5 ml fractions separated were used for the following assays. The activity of catalase and cytochrome *c* oxidase is expressed as relative values with one unit being maximum activity of the enzymes among the fractions. **c** Protein gel-blot analyses of HvCMO, aspartate aminotransferase and catalase proteins in the peroxisomal (No. 7) and mitochondrial (No. 14) fractions. Equal volume of these fractions was loaded in each *lane*

However, protein gel-blot analysis showed that HvCMO was not detected in the ER-containing microsome fraction of barley leaves (Supplemental Fig. S1 g). Cells expressing BBD2-GFP showed GFP fluorescence in the cytosol (Supplemental Fig. S1 d–f).

[^{14}C]choline oxidation assay using barley and spinach chloroplasts

To determine the activity of choline oxidation of barley chloroplasts, the chloroplasts of barley leaves were isolated and used in an in vitro [^{14}C]choline oxidation assay with spinach chloroplasts used as a positive control. A representative result is shown in Fig. 8a. After a 30-min incubation of chloroplasts with [^{14}C]choline, the metabolites in the reaction solution were separated by electrophoresis using filter papers as described in "Materials and methods". When a reaction solution of spinach chloroplasts incubated under light was electrophoresed, major and minor products co-migrated with betaine aldehyde and GB, respectively (Fig. 8a). This indicated that spinach chloroplasts oxidized [^{14}C]choline to betaine aldehyde and GB under light but less under dark, which corresponded to Weigel et al. (1988), and that barley chloroplasts showed

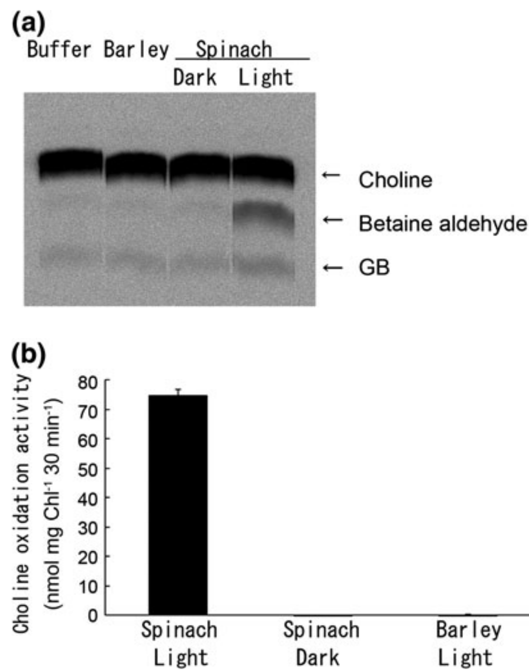

Fig. 8 [^{14}C]choline oxidation assay using barley and spinach chloroplasts. **a** An autoradiograph of reaction mixes separated by paper electrophoresis. Isolated chloroplasts (20 μg) of barley and spinach leaves were used in the assay. The reaction was incubated under light or darkness at 25°C for 30 min. The chloroplast isolation buffer was also used as a negative control instead of chloroplasts. **b** [^{14}C]choline oxidation activity of barley and spinach chloroplasts. Data are mean ± SE ($n = 3$). The experiment shown is representative of triplicate measurements

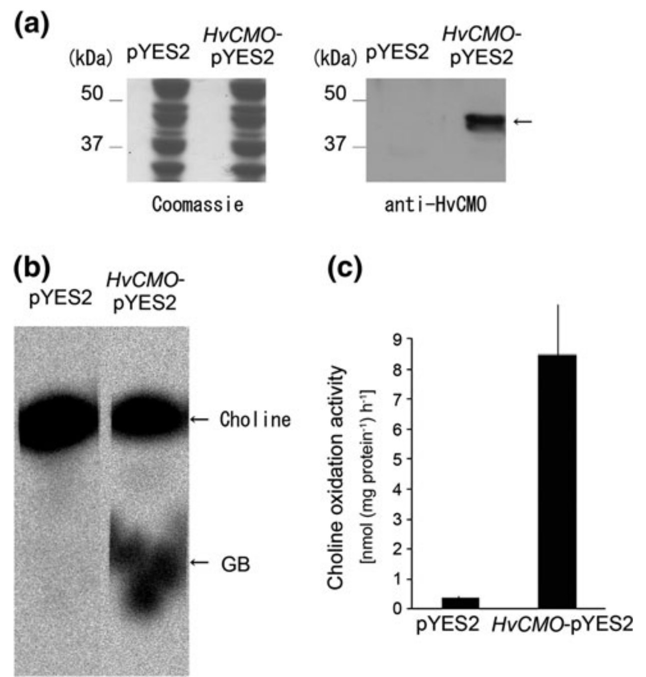

Fig. 9 In vitro [^{14}C]choline oxidation assay using recombinant HvCMO protein. **a** Protein gel-blot analysis of recombinant HvCMO protein. The protein was extracted from yeast harboring pYES2 or *HvCMO*-pYES2 vector, precipitated with 25% (w/v) PEG 6000, and a 20 μg aliquot was loaded. A Coomassie brilliant blue-stained gel is shown as a loading control. **b** An autoradiograph of reaction mixes separated by paper electrophoresis. The protein extracts of yeast harboring pYES2 or *HvCMO*-pYES2 (about 800 μg) were used in the assay. NADPH was added as a coenzyme of CMO. Purified recombinant BBD2 protein plus NAD^+ was also added to the assay to oxidize [^{14}C]betaine aldehyde to [^{14}C]GB. The reaction mix was incubated for 1 h at 25°C under darkness. **c** [^{14}C]choline oxidation activity of the protein extract of yeast harboring pYES2 or *HvCMO*-pYES2. Data are mean ± SE ($n = 3$). The experiment shown is representative of triplicate measurements

no oxidation activity of [^{14}C]choline to betaine aldehyde or GB under light and dark (Fig. 8a, b).

In vitro [^{14}C]choline oxidation assay using recombinant HvCMO protein

For an in vitro [^{14}C]choline oxidation assay using recombinant HvCMO protein, HvCMO protein was expressed in yeast strain BJ5458, precipitated with 25% (w/v) PEG 6000, and the expression was confirmed by protein gel-blot analysis (Fig. 9a). The CMO assay was similar to that described by Burnet et al. (1995), apart from the addition of the cofactor for CMO and the alteration of incubation conditions. In the present CMO assay, affinity-purified recombinant BBD2 (Fujiwara et al. 2008), supplemented with NAD^+ as a cofactor, was added to the assay to oxidize betaine aldehyde to GB because betaine aldehyde is unstable (Burnet et al. 1995). When the extract of HvCMO-expressing yeasts and NADPH were added to the assay with the reaction incubated in the dark for an hour and the reaction solution was separated by electrophoresis using filter papers, a main metabolite co-migrated with GB (Fig. 9b), which indicated that [^{14}C]GB was produced as a result of the oxidation of [^{14}C]choline. However, when the extract of the yeasts containing empty pYES2 vector was used, small amounts of [^{14}C]GB was detected. The choline oxidation activity of the protein extracts of HvCMO-expressing yeasts was 8.6 nmol mg protein^{-1} h^{-1}, but less in the protein extracts of empty vector-introduced yeasts (Fig. 9c). In addition, when ferredoxin reduced by spinach thylakoid under light was used as a cofactor of CMO instead of NADPH, the protein extract of HvCMO-expressing yeast showed less activity of choline oxidation (data not shown).

Discussion

In this study, *HvCMO* has been cloned and identified as a gene encoding a functional CMO protein in GB biosynthesis of barley. As mentioned previously, a functional CMO protein has been reported only in Amaranthaceae, such as spinach (Weigel et al. 1988; Hibino et al. 2002) so

far. Therefore, this is the first report of a functional CMO protein in gramineous plants.

When [^{14}C]choline was applied to spinach and barley chloroplasts in vitro, [^{14}C]choline-derived betaine aldehyde and GB were produced by spinach chloroplasts but not by barley chloroplasts (Fig. 8). Also, here we have shown that HvCMO is localized to peroxisomes (Fig. 7) and previously we have shown that the BBD2 protein is localized to cytosol and is involved in GB production in barley (Fujiwara et al. 2008). These results indicate that the choline-oxidizing enzyme does not localize to chloroplasts, and GB is produced in a cooperative way in the cytosol and peroxisomes of barley plants. This indicates that GB is produced in peroxisomes in a reaction catalyzed by HvCMO and BBD1, another peroxisomal betaine aldehyde dehydrogenase in barley, although BBD1 shows extremely low affinity for betaine aldehyde compared with BBD2 (Fujiwara et al. 2008), and the overflowed betaine aldehyde is converted to GB in the cytosol. Although the protein for translocation of betaine aldehyde is not known so far, it may be assumed that betaine aldehyde overflowed from peroxisomes to the cytosol via porin-like channel, which is highly permeable to low molecular mass compounds including many substrates and products of peroxisomal metabolism (Reumann et al. 1995; Visser et al. 2007). Kishitani et al. (2000) also reported that when peroxisomal BBD1 was introduced into rice, exogenously applied betaine aldehyde entered into the peroxisomes and was converted to GB in a reaction mediated by BBD1 in transgenic rice plants. We cannot exclude the possibility that the oxidation of choline might occur mediated by the other enzymes in the cytosol (Chen and Murata 2011), although, to our knowledge, there is no report so far which shows the activity of choline oxidation in the cytosol of plants.

Moreover, our results suggest that HvCMO protein requires NADPH directly or indirectly as a cofactor and absence of light for choline oxidation (Fig. 9), while spinach CMO protein is known to require reduced ferredoxin and light (to reduce ferredoxin via photosystem I activity) for its activity. Therefore, it is indicated that the intracellular site and mechanism of GB biosynthesis using HvCMO in barley seem unique compared with those in spinach. The reaction mechanism of HvCMO has to be clarified in more detail such as other factors, since we used partially purified crude extract of HvCMO-expressing yeasts for enzyme assay and failed to detect enzyme activity using purified HvCMO. The HvCMO protein appeared to be fragile.

The expression pattern of *HvCMO* and *BBD2* genes was analyzed under a range of abiotic stress conditions. In this study, the treated concentration of PEG is osmotically equivalent to that of 200 mM NaCl (Ueda et al. 2004). However, the expression of HvCMO gene was increased by high osmotic treatment, but not altered by the application of NaCl (Fig. 6a). Salinity stress causes both osmotic and ionic stress. Under salt stress, osmotic stress is triggered by an excess salt in the soil, and ionic stress is caused by the over-accumulation of salt in the cells. These indicate that the changes of the expression level of HvCMO gene expected from the osmotic component of salt stress may have been suppressed by other salt-mediated signals. Therefore, the regulatory mechanism of the expression level of HvCMO gene would be a good target to dissect further signaling controls for the differentiation of osmotic from ionic stress. Nevertheless, the protein level of HvCMO was increased by salinity stress (Fig. 6). These results indicate that the expression of HvCMO is regulated mainly at a translocation level compared with a transcriptional level under salinity. In addition, the expression of *BBD2* gene was up-regulated by the treatment of NaCl, PEG, ABA and low temperature. In a previous study, we have shown that GB is synthesized in vascular tissues of leaves in barley plants grown under saline conditions (Hattori et al. 2009). Therefore, it is suggested that barley plants increase GB synthesis in the cytosol of vascular tissues of the leaves under saline conditions. On the other hand, amaranthaceous plants synthesize GB in the chloroplasts using stromal CMO and BADH proteins and protect photosynthetic proteins such as RuBisCO and D1 protein under saline conditions (Nomura et al. 1998; Ohnishi and Murata 2006). These results indicate that the physiological role of GB is different between amaranthaceous and gramineous plants under saline conditions. Further study is needed to elucidate the physiological function of GB in the cytosol of gramineous plants.

We have also determined the distribution of GB and its biosynthetic proteins at an organ level of barley plants grown under normal condition. Although GB was most abundant in the leaves, it was also detected in all the determined organs including spikelet and internodes. However, CMO protein was expressed mainly in the leaves and less in spikelet and internodes (Fig. 5). These results suggest that GB is mainly produced in the leaves and transported to the sink organs such as spikelet and internodes. In fact, it was also reported that exogenously applied GB to mature leaves during water-deficit conditions was translocated to the other organs such as expanding leaves, shoot apical meristem, tillers and roots after rewatering (Ladyman et al. 1980). We suggest that, when the ability of GB biosynthesis is introduced into plants, it is necessary not only to introduce GB biosynthetic enzymes, but also to regulate the transport of GB to the growing part of plants. Dissection of the molecular mechanism of the regulation of GB transport in plants is now under investigation in our group. Moreover, two

BADH isozyme (BBD1 and BBD2) proteins were expressed in all the determined organs, and were abundant in the spikelet and younger internodes compared with that in the leaves. In our previous study, BBD1 and BBD2 proteins functioned not only in the oxidation of betaine aldehyde, but also in the oxidation of other aminoaldehydes such as 4-aminobutyraldehyde, 3-aminopropionaldehyde, 4-*N*-trimethylaminobutyraldehyde and 3-*N*-trimethylaminopropionaldehyde (Fujiwara et al. 2008). Together, it is indicated that BADH proteins may have physiological roles in the biosynthesis of GB in the leaves together with HvCMO, but also in the oxidation of the aminoaldehydes in the leaves, spikelet and younger internodes of barley plants.

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