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Isolation of a choline monooxygenase cDNA clone from Amaranthus tricolor and its expressions under stress conditions

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ABSTRACT

Plants synthesize the osmoprotectant glycine betaine (GB) via choline→betaine aldehyde→glycine betaine[1]. Two enzymes are involved in the pathway, choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). A full length CMO cDNA (1,643bp) was cloned from Amaranthus tricolor. The open reading frame encoded a 442-amino acid polypeptide, which showed 69% identity with CMOs in Spinacia oleracea L. and Beta vulgaris L. DNA gel blot analysis indicated the presence of one copy of CMO gene in the A. tricolor genome. The expressions of CMO and BADH proteins in A.tricolor leaves significantly increased under salinization, drought and heat stress (42°C), as determined by immunoblot analysis, but did not respond to cold stress (4°C), or exogenous ABA application. The increase of GB content in leaves was parallel to CMO and BADH contents.

Key words: *Amaranthus tricolor, betaine aldehyde dehydrogenase (BADH), choline monooxygenase (CMO), glycine betaine (GB), stress.*

INTRODUCTION

Amaranth is a C_4 dicotyledonous mesophyte crop plant. A. tricolor is a major variety for vegetable and ornamental crops, and is widely cultivated in the world. Osmoprotectant glycine betaine (GB) was detected in Amaranthaceae, A. Hypochondriacus L [2] and A. Caudatus L[3], [4]. GB is widespread and an effective osmoprotectant in many plants[3]. We studied the photosynthetic adaptation mechanism of A. tricolor under salt stress due to accumulation of GB[5].

GB is synthesized by the two-step oxidation of choline. The first step is catalyzed by choline monooxygenase (CMO), which has only been studied in Chenopodiaceae, spinach and sugar beet up to now. Afterward cDNAs encoding CMO proteins were cloned from both species[4],[6], and the deduced amino acid sequences showed 84% identity. Spinach CMO is a stromal, Fd-dependent monooxygenase with a Rieske-type [2Fe-2S] center[7], and is completely different from the bacterial choline dehydrogenase and oxidase enzymes. CMO was assumed to be unique among plant oxygenases[6], [8]. The second step of GB synthesis is mediated by betaine aldehyde dehydrogenase (BADH)[1], which has been well documented in amaranth[2], [9] and other plants[10-14].

CMO and BADH expressions in spinach leaves, and sugar beet leaves and taproots[4], [6], [7] were regulated by salt and drought stresses. A. caudatus L. in addition to Chenopodiaceae was first known to accumulate GB and express CMO protein under salt stress[5].

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Abbreviations: BADH, betaine aldehyde dehydrogenase; CMO, choline monooxygenase; GB, glycine betaine; ABA, abscisic acid; PMSF, phenylmethyl sulfonylfluoride; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; bp, base pair; kb, kilobase; kDa, kilodalton.
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In present work, we investigated CMO proteins in GB-accumulated species of Chenopodiaceae and Amaranthaceae. Furthermore, we examined CMO, BADH proteins expression and GB contents in A. tricolor under conditions of environmental stresses including salt, drought, cold, heat, and exogenous ABA application. We also isolated the CMO cDNA from A. tricolor cDNA library.

MATERIALS AND METHODS

A.tricolor, A. caudatus, A. hypochondriacus, Spinacia oleracea, Suaeda japonica and Atriplex ssp.gmelini seeds were germinated and their seedlings grown under the conditions described by Wang et al[5]. 6-week-old A. tricolor plants were exposed to the following treatments, respectively: 300 mM NaCl for 5 d, drought by withholding water for 10 d, cold (4°C) for 8 d, and heat stress (42°C) for 3 d. 100 mm ABA in water was sprayed on the surface of all leaves until solution runoff, repeated spreading three times, the plants further grew 5 d. Measurements of protein and GB were carried out as previously described[5].

cDNA cloning

Total RNA from salinized A. tricolor leaves was extracted as described by Yamamoto et al[15]. A 947-bp DNA fragment was generated using reverse transcription-PCR with degenerate primers. The reverse and forward primers were: 5'-TCYAARGGNTGGCARGTNGCNGG-3'; and 5'-CCARCARTGRAARTGRTGDATNCC-3', respectively. Poly(A) RNA was isolated using PolyATtract Series 9600^{TM} mRNA isolation system (Promega, USA), and used to construct a cDNA library (1×10^6 plaque-forming units) in UniZap XR (Stratagene, USA). The specific primers were synthesized for screening the cDNA library. The forward primer and reverse primer were: 5-GATGGCAAGTCGCAGGATATAG-3 and 5'-AGCAATGGAAATGATGGATTCCT-3', respectively. The library was screened using a PCR-96-well plate method as previously described[16]. DNA sequences were determined with a BigDye Terminator Cycle Sequencing ready Reaction Kit (PE-ABI, Warrighton, UK). Sequence was analyzed using PC-GENE (TM IntelliGentics Inc. Switzerland).

DNA gel blot analysis

DNA was isolated from the leaves of A. tricolor according to the method described by Chen et al[17]. DNA was cleaved and denatured in the gel and transferred to Hybond-N^+ membrane (Amersham, UK). A 1308-bp fragment of CMO cDNA digested by BamH I and Xba I, was used as a probe. A standard protocol was then used for DNA hybridization[18].

SDS-PAGE, native PAGE and immunoblot analysis

Rabbit antisera were raised against BADH and CMO as previously described[5]. To determine both proteins expression under various stresses, total proteins were extracted from A. tricolor leaves, according to an established method[5], and were separated by 12.5% SDS-PAGE. To compare molecular weight of CMO and its subunits between A. tricolor and spinach, the proteins were extracted from salinized leaves in previously described buffer[5] supplemented with 10-25 mM PMSF and 1-10 mM NEM, then separated by 12.5% SDS-PAGE or 10% native PAGE, and transferred to nitrocellulose membrane (Amersham, UK).

RESULTS

Isolation of choline monooxygenase cDNA clone

A cDNA library was prepared using mRNA from salinized A. tricolor leaves, and screened with PCR methods[16]. A full-length cDNA of 1,643 bp encoding a 442-amino acid polypeptide was obtained. This cDNA has 5' and 3' untranslated region of 54 bp and 260 bp. The DNA and deduced amino acid sequence are shown in Fig 1. When the nucleotide sequence and deduced amino acid sequence were aligned with those of spinach and sugar beet CMOs, their identities attained 73.6% and 75.7% at nucleotide level; 69.4% and 69.5% at amino acid level, respectively. Comparison of the N-terminal region of A. tricolor CMO with those of sugar beet and spinach suggests that they are very similar and share typical amino acid composition (Fig 2). The amino acid sequence of A. tricolor CMO possesses characteristics of transit peptide which target polypeptide to chloroplast[19]. A. tricolor CMO clone shares two consensus sequences with spinach[6] and sugar beet CMO[4]: one is Cys-X-His-$X_{15\text{-}17}$-Cys-X-X-His which coordinates with a Rieske-type [2Fe-2S] cluster. The other is a sequence containing coordination sites for mononuclear nonheme Fe: Glu/A_{sp}-$X_{3\text{-}4}$-A_{sp}-X_2-His-$X_{4\text{-}5}$-His[20, 21].

Genomic DNA Gel Blot analysis

A.tricolor genomic DNA was digested with BamH I, EcoR I, Hind III and Xba I. Blot analysis revealed single bands which represent the BamH I, EcoR I and Xba I digestion fragments, respectively, and two bands representing the Hind III digestion fragment (Fig 3a). A restriction endonuclease cleavage sites of CMO cDNA indicates that there are two Hind III sites, one at 743 and the other at 1430 bp (Fig 3b). This revealed that the CMO gene contained an intron(s) between the two Hind III sites, and one copy of CMO gene was present within A. tricolor genome.

CMOs in different species

We examined CMO protein in several species of Amaranthaceae and Chenopodiaceae by immunoblot analysis. CMO proteins could be detected and showed subunits in different molecular weights (Fig 4A and 4B). The subunit of A. tricolor CMO had a lower molecular weight than that of the other two Amaranthaceae plants, A. caudatus L. and A. hypochondriacus L. (Fig 4A). Spinach CMO subunits showed higher molecular weight in three species of Chenopodiaceae, Spinacia oleracea L. Suaeda japonica and Atriplex ssp. Gmelini (Fig 4B). Mean-

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CGGCACCAGAAAAAAACATAGAAGAGTTTTGAGTGGTATTATTAGTATTGTGCGATGGCA    60
                                                          M  A     2
TCATCAGCTTCAATGTTGATAAATTATCCAACTACTTTTTGTGGAGTTAGAAATTCATCA   120
S  S  A  S  M  L  I  N  Y  P  T  T  F  C  G  V  R  N  S  S      22
AATCCAAATAATGATCAATTTTCTGATCAAATTAACATTCCTTCTTCATTAAATAATAAT   180
N  P  N  N  D  Q  F  S  D  Q  I  N  I  P  S  S  L  N  N  N      42
ATTAATATTAGTAAAATTACAAGTAAAACCAATAAAATAATCCCAAAAGCAGTAGCATCC   240
I  N  I  S  K  I  T  S  K  T  N  K  I  I  P  K  A*  V  A  S     62
CCTGTGATTCCATCTTCTATAAATAGTAATAATATAACAACAACAACACCAAATATCAAA   300
P  V  I  P  S  S  I  N  S  N  N  I  T  T  T  T  P  N  I  K      82
AGAATAATTCATGAATTTGATCCAAAAGTTCCAGCTGAAGATGGTTTCACTCCTCCTTCT   360
R  I  I  H  E  F  D  P  K  V  P  A  E  D  G  F  T  P  P  S     102
ACTTGGTACACTGACCCTTCCCTTTATTCTCATGAACTTGACCGTATCTTTTCCAAAGGA   420
T  W  Y  T  D  P  S  L  Y  S  H  E  L  D  R  I  F  S  K  G     122
TGGCAAGTCGCAGGATATAGTGATCAAATAAAGGAGCCTAATCAATATTTCACCGGAAGT   480
W  Q  V  A  G  Y  S  D  Q  I  K  E  P  N  Q  Y  F  T  G  S     142
CTAGGAAATGTTGAATATTTGGTATGCCGAGATGGTCAGGGGAAAGTTCATGCATTCCAC   540
L  G  N  V  E  Y  L  V  C  R  D  G  Q  G  K  V  H  A  F  H     162
AATGTTTGTACTCATCGTGCATCAATTCTTGCATGTGGAACTGGCAAGAAGTCTTGTTTT   600
N  V  C  T  H  R  A  S  I  L  A  C  G  T  G  K  K  S  C  F     182
GTCTGCCCTTATCATGGATGGGTATTTGGCTTAGATGGATCACTCATGAAAGCCACTAAA   660
V  C  P  Y  H  G  W  V  F  G  L  D  G  S  L  M  K  A  T  K     202
ACTGAAAATCAAGTATTTGACCCTAAAGAACTTGGGCTAGTAACTCTAAAGGTAGCAATA   720
T  E  N  Q  V  F  D  P  K  E  L  G  L  V  T  L  K  V  A  I
TGGGGGCCATTTGTTCTGATAAGCTTAGATAGATCAGGCTCTGAAGGAACTGAAGATGTT   780
W  G  P  F  V  L  I  S  L  D  R  S  G  S  E  G  T  E  D  V     242
GGAAAAGAGTGGATTGGTTCATGTGCTGAAGAAGTTAAAAAACATGCTTTTGATCCTTCT   840
G  K  E  W  I  G  S  C  A  E  E  V  K  K  H  A  F  D  P  S     262
CTTCAATTCATTAATAGGAGTGAATTCCCCATGGAATCCAATTGGAAGGTATTTTGCGAC   900
L  Q  F  I  N  R  S  E  F  P  M  E  S  N  W  K  V  F  C  D     282
AATTATTTGGACAGTGCATATCATGTTCCTTATGCTCACAAATACTATGCTGCTGAACTC   960
N  Y  L  D  S  A  Y  H  V  P  Y  A  H  K  Y  Y  A  A  E  L     302
GACTTTGACACCTATAAAACTGATTTGTTGGAGAAAGTTGTGATTCAAAGAGTAGCAAGC  1020
D  F  D  T  Y  K  T  D  L  L  E  K  V  V  I  Q  R  V  A  S     322
AGTTCAAACAAGCCAAATGGGTTTGATAGACTTGGATCAGAAGCATTCTATGCTTTTATT  1080
S  S  N  K  P  N  G  F  D  R  L  G  S  E  A  F  Y  A  F  I     342
TATCCAAACTTTGCTGTGGAAAGGTATGGCCCTTGGATGACCACAATGCACATTGGTCCA  1140
Y  P  N  F  A  V  E  R  Y  G  P  W  M  T  T  M  H  I  G  P     362
TTAGGACCCAGGAAGTGTAAACTTGTGGTGGACTATTATCTTGAAAATGCCATGATGAAC  1200
L  G  P  R  K  C  K  L  V  V  D  Y  Y  L  E  N  A  M  M  N     382
GACAAACCTTACATTGAAAAAAGCATAATGATCAACGACAACGTCCAGAAAGAAGATGTA  1260
D  K  P  Y  I  E  K  S  I  M  I  N  D  N  V  Q  K  E  D  V     402
GTGTTATGTGAAAGTGTGCAGAGGGGTCTAGAAACACCAGCATATAGAAGTGGAAGATAT  1320
V  L  C  E  S  V  Q  R  G  L  E  T  P  A  Y  R  S  G  R  Y     422
GTGATGCCAATTGAGAAAGGAATCCATCATTTCCATTGCTGGTTGCACCAAACTTTGAAC  1380
V  M  P  I  E  K  G  I  H  H  F  H  C  W  L  H  Q  T  L  N     442
TGATCCTTCTTCATCTTCCTCTTCCTCTAGTTGGGTCCAATATTAATTAAGCTTATGCTT  1440
ATGAGTTTCATGTTAAAAATATGTATGAAGGGCAAGTATGTCTACTTGAAGATTATGGTA  1500
TGGTGCTAAATGCTAATGCATGTTATTGTAATATTTTTAATAAACACCATCAACCTTCCT  1560
TGGCCCTTGCATTAAACTTAGAACTTCTAATAATTAATCTTATTAATGTTTATGATTTTC  1620
ATCCAAAAAAAAAAAAAAAAAAA                                       1643
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Fig 1. Nucleotide and deduced amino acid sequences of CMO cDNA from A. tricolor. Single-Rieske-type iron-sulfur [2Fe-2S] cluster-binding region is underlined, boxes mark the conserved Cys-His pairs residues. The conserved residues of the mononuclear Fe-binding motif are double underlined, and the gray boxes indicate the conserved residues. The asterisk shows processing site of N-terminal residue corresponding to the mature CMO polypeptide of spinach[6]. The accession number for A. tricolor CMO nucleotide sequence is AF290974.

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A. tricolor    MASSAS---MLINYPTTFCGVRN---SSNPNNDQFSDQINIPSSLNNNINI    45
Spinach        MMAASASATTMLLKYPTTVCGIPNP--SSNNNND--------PS---NNIV-   38
Sugar beet     MA--ASATTMLLKYPTL-CAMPNSSSSSNNN-DLPTS---IP--LNNNNNL    42

A. tricolor    -SKI---TS-----K--T-NKIIPKA*VASPVIPSSINSNNITTTTPN-IKRI   85
Spinach        -S-IPQNTTNPTLKSRTPNKITTNA*VAAPSFPSL------TTTTPSSIQSL   82
Sugar beet     LSNKNKILQTPNINTST-NKIITKA*VASPVFPTL----KTTSNTPSSIRSL   89
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Fig 2. Comparison of the deduced of 5'-transit peptide amino acid sequences of CMOs from A. tricolor, Spinacia oleracea L. [6] and Beta vulgaris L.[4]. The gray boxes indicate identical amino acids. The asterisk indicates processing site of the N-terminal residue corresponding to the mature CMO polypeptide of spinach[6]. Number on the right refer to amino acid residues.

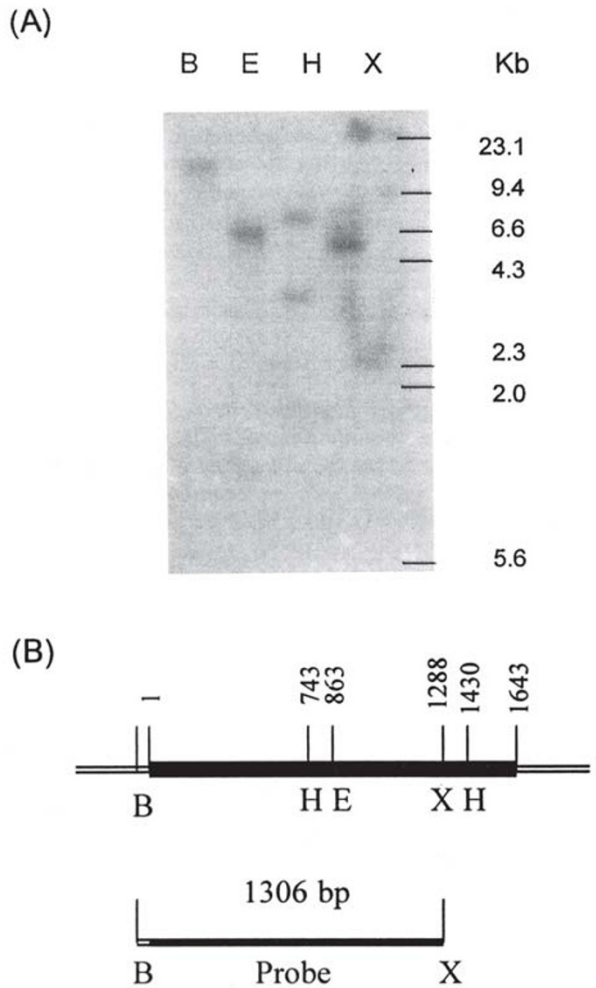

Fig 3. (**A**)DNA gel blot analysis of A. tricolor genomic DNA. 10mg genomic DNA was digested with Bam H I, Eco R I, Hind III and Xba I, respectively, separated on 1% agarose gel, transferred to a Hybond-N^+ membrane, and hybridized with a ^{32}P-labeled CMO cDNA probe. The probe was a 1308 bp fragment digested by Bam H I- Xba I. Lanes are as follows: B, Bam H I; E, Eco R I; H, Hind III and X, Xba I. Numbers on the right are the molecular weight markers of λ-DNA digested with Hind III in kb. (**B**) The full length of A. tricolor cDNA and its partial restriction map. The bold lines indicate the cDNA, double lines are the vector DNA. The numbers indicate the sites of restriction endonuclease cleavage in the cDNA. Restriction cleavage site: B, Bam H I; E, Eco}R I; H, Hind III and X, Xba I.

while we compared the subunits of A. tricolor and spinach CMOs, and found the molecular weight of A. tricolor CMO subunit was lower than that of spinach CMOs. On the contrary, the results of native PAGE showed that the molecular weight of A. tricolor CMO was higher than that of spinach (Fig 5).

CMO expression under stress conditions

In order to obtain more information about the regulation of CMO expression, the effects of several types of abiotic stresses on the CMO and BADH expressions were assessed by immunoblot analysis. The results showed that the expression of CMO protein increased significantly by salt, drought and heat stress (Fig 6A). A maximum response was observed in the heat stressed leaves, where 3 d after treatment, the content of CMO protein was the highest among that under other

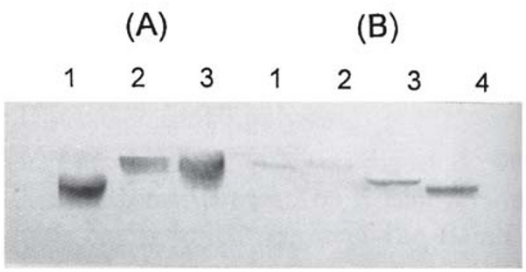

Fig 4. Immunoblot analysis of CMO. Total proteins (100 μg) from leaves were separated by SDS-PAGE and immunoblotted using anti-CMO antibodies. The plants were treated with 300mM NaCl for 5 d. Lanes are as follows: (**A**) A. tricolor (lane 1), A. caudatus L. (lane 2), A. hypochondriacus L. (lane 3). (**B**) Spinach (Spinacia oleracea L.) (lane 1), Suaeda japonica (lane 2), Atriplex ssp. Gmelini (lane 3), A. tricolor (lane 4).

treatments. Compared with the changes of CMO contents, the changes of BAD contents were not so significant. The basal amount of BADH protein was higher in non-stressed plants. The highest levels of GB were also detected in heat stress (Fig 6C), followed by salt and drought stresses, accompanied by an altered expression pattern of CMO and BADH proteins (Fig 6A, 6B). Such changes were not observed in cold and ABA treatment.

DISCUSSION

CMO protein was detected in A. tricolor, and its cDNA was isolated from A. tricolor in addition to Chenopodiaceae family. Since Amaranthaceae are phylogenetically close to Chenopodiaceae, the nucleotide and deduced amino acid sequences of their CMO reveal a high identity with spinach and sugar beet CMOs. Spinach and sugar beet CMOs have 60 and 65-residues which target the stroma[4]. Through judging from the processing sites of spinach and sugar beet, A. tricolor might have a transit peptide of 58 residues.

The results of SDS-PAGE and native PAGE implied that A. tricolor CMO consisted of subunits(Fig 5). The quaternary structure of CMO is not clear. Spinach CMO might be a homodimer consisting of subunits around 43 kDa, but the possibility of additional subunits cannot be ruled out[7],[8]. Although the deduced amino acid sequence of A. tricolor CMO cDNA is similar to that of spinach in molecular weight, its subunit weighed less than spinach CMO subunit from this study (Fig 5). When PMSF and NEM were added into the extracting buffer in order to prevent protein denaturation and degradation, the same results reappeared. Molecular weight difference of CMO subunits in several species of Amaranthaceae and Chenopodiaceae implied a possibility of different processing sites among CMO

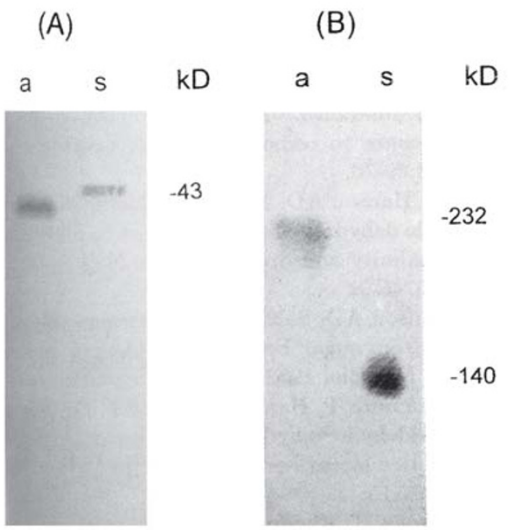

Fig 5. Immunoblot analysis of CMO and its subunit. Total proteins (100 μg) from leaves were separated by SDS-PAGE (A) and Native gel (B) followed by immunoblotting with anti-CMO antibodies. The six-week old seedlings of A. tricolor (a) and Spinacia oleracea L. (s) were treated with 300 m M NaCl for 5 d. The reported molecular weights were averages of 3-5 replicate measurements.

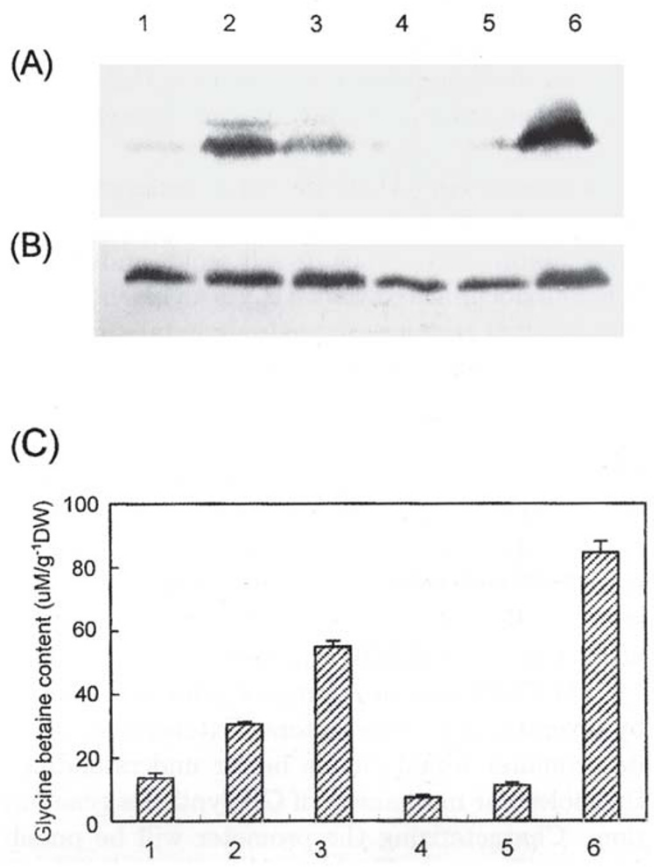

Fig 6. CMO (A), BADH (B) and glycine betaine (C) accumulation in A. tricolor under several abiotic stresses. Equal amounts (100 μg for CMO, 50 μg for BADH) of total protein were run on SDS-PAGE and immunoblotted with anti-CMO and anti-BADH antibodies. Control plants (lane 1), and plants under salt stress induced by 300 m M NaCl for 5 d (lane 2), drought stress for 10 d (lane 3), 100m M ABA treatment for 5 d (lane 4), cold treatment (4°C) for 8 d (lane 5), and 42°C heat stress for 3 d (lane 6) were studied. GB contents were average of 5 replicate measurements.

precursors (Fig 4). Because of the low amount of CMO in A. tricolor, determination of the processing site and subunit composition of its CMO was hindered.

In this study, the expressions of CMO and BADH in A.tricolor under heat stress, salt and drought stresses (Fig 6), showed that both expressions of CMO and BADH were inducible. These results indicated that the enzymes involved in GB synthesis increase by de novo protein synthesis under stress conditions. Compared with the changes of CMO contents, the changes of BADH contents were not so significant. BADHs were not a substrate-specific enzyme that had side effects of polyamine and 3-dimethylsulfonylpropionate metabolism than it did on altering the GB pathway itself[22], [23]. The present results and those of an additional study (to be published elsewhere) suggest that CMO plays a key role and is directly involved in GB synthesis in A. tricolor.

Abscisic acid (ABA) mediates desiccation tolerance in plants, and is involved in their response to other abiotic stress such as salt, cold, and heat[24]. It is well documented that ABA is an essential mediator in plant response to environmental stresses [25]. ABA and drought treatments can trigger an increase in BADH mRNA levels and the content of BADH protein in barley leaves at low temperature, but the results showed a low level accumulation of GB[12], [26]. Naidu et al[27] reported that concentration of accumulated betaine in cold-stressed wheat seedlings increased more than doubled in the controls. In this study, cold stress (4°C) and ABA treatment did not affect CMO and BADH expression in A. tricolor.

The CMO gene in A. tricolor genome is inducible by several types stress factors. Extensive studies on its promoter would enable better understanding of the molecular mechanism of GB synthesis gene function. Characterizing the promoter will be possible for the efficient genetic engineering of stress tolerance in plants.

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