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AhCMO, regulated by stresses in *Atriplex hortensis*, can improve drought tolerance in transgenic tobacco

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Abstract Choline monooxygenase (CMO) catalyzes the committed step of glycine betaine (GlyBet) biosynthesis in many flowering plants. To investigate its effect on various stress tolerances in plant metabolic engineering, we isolated and characterized the CMO gene from *Atriplex hortensis*, a GlyBet natural accumulator, and introduced it into tobacco to examine the effect of GlyBet on plant drought and salt tolerance, respectively. In *A. hortensis*, the expression of *AhCMO* was induced 3-fold in the root and stem, as well as in the leaf, when plants were treated with 400 mM of NaCl, indicating that the acceleration of GlyBet biosynthesis under salt stress was achieved through the whole plant, including organs without chloroplasts. *AhCMO* transcription was also regulated by drought, ABA and circadian rhythm. Over-expression of *AhCMO* improved drought tolerance in transgenic tobacco when cultured in medium containing PEG-6000. The transgenic plants also have a better performance under salt stress.

Keywords Glycine betaine · Choline monooxygenase · *Atriplex hortensis* · Stress tolerance

Introduction

Most organisms accumulate highly soluble compounds termed osmoprotectants or osmolytes under stresses such as salinity, drought and cold. One of the most-important osmoprotectants is glycine betaine, GlyBet (Rhodes and Hanson 1993), which is widespread and accumulates in many organisms from archaebacteria to higher plants and animals. Previous reports show that betaine in vitro can protect enzyme activity and stabilize the cell membrane (Gorham 1995; Papageorgiou and Murata 1995); betaine in vivo can not only take these effects but also regulates the osmotic pressure in the cell, therefore reducing the damage to the cell caused by abiotic stresses (Yancey 1994; Deshnium et al. 1997). Some flowering plants, e.g. sugar beet, can accumulate high levels of GlyBet (Hanson and Wyse 1982), whereas in many crops, such as rice and wheat, osmoprotectant content is very low. It is thus possible to improve the stress tolerance of crops by introducing genes responsible for GlyBet biosynthesis into these crops. Advances have been made in recent years in plant metabolic engineering of trehalose (Goddijin et al. 1997), polyol (Sheveleva et al. 1997) and GlyBet (Guo et al. 1997; Sakamoto and Murata 2001).

GlyBet is synthesized from choline through different oxidative reactions in different organisms (Hayashi and Murata 1998). In *Arthrobacter globiformis*, choline oxidase (COD) catalyzes choline to GlyBet. In mammalian cells and microorganisms such as *Escherichia coli*, choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH) catalyze the oxidative reaction via two steps: choline → betaine aldehyde → glycine betaine. In higher plants, the pathway is the same, with choline monooxygenase (CMO) catalyzing the first step instead of CDH. CMO activity is very restricted to plant species and is only found in the Chenopodiaceae and the Amaranthaceae (Rathinasabapathi et al. 1997; Russell et al. 1998). The BADH gene has been cloned from many organisms including spinach (McCue and Hanson 1992), *Atriplex hortensis* (Xiao et al. 1995) and wheat (Ishitani et al. 1995). Through enzymatic tests and Western blots, many flowering plants including rice showed the existence of BADH (Weretilnyk et al. 1989). It is then possible to establish the GlyBet biosynthesis pathway in crops by transformation with *CMO*.

In our early reports, a BADH gene has been cloned from *A. hortensis* and introduced into rice, wheat and

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turf grass (Xiao et al. 1995; Guo et al. 1997; Guo et al. 2000). Improvement of salt tolerance in transgenic plants was observed during growth. Even greater improvement in rice introduced with the BADH gene has been achieved when supplied with extraneous betaine aldehyde (Kishitani et al. 1998). This means that production of betaine aldehyde is a limiting step in transgenic plants, the CMO/CDH then become the key catalysts of the biosynthesis route. Plant genetic engineering with CDH/COD has proved effective on stress resistance in various plants (Sakamoto and Murata 2001); recently one report showed that tobacco introduced with the spinach CMO gene could synthesize GlyBet in vivo (Nuccio et al. 1998). Whether CMO$^+$ plants show resistance to stresses such as cold or water deficiency is not yet clear. We thus cloned and characterized the CMO gene from *A. hortensis* and introduced it into *Nicotiana tobacum* var. Xanthi to test its effect on various stress tolerances. *A. hortensis* belongs to a family that naturally accumulates GlyBet to 320 mM in the cytoplasm, much higher than in other species (Matoh et al. 1987). Our evaluation of AhCMO$^+$ transgenic plants shows a considerable improvement in drought tolerance.

Materials and methods

Plant materials, growth condition and stress treatments

Seeds of *Atriplex hortensis* L. were germinated hydroponically at 25 °C for 2 days, and then grown in the greenhouse at 22 °C in natural daylight during October and November. Mature plants were irrigated with solutions containing 400 mM of NaCl, 20 μM of ABA (abscisic acid) or grown without irrigation in drought-stress treatments. Young plants were irrigated daily with a solution containing 200 mM of NaCl for 4 days as salt treatment. The tissues harvested were quickly frozen in liquid nitrogen and stored at –70 °C for DNA and RNA extraction.

Construction and screening of a cDNA library

Total RNA was isolated from leaves of *A. hortensis* after being stressed with 400 mM of NaCl for 4 days, and 2 micrograms of poly(A$^+$) RNA were used for cDNA library construction as described previously (Zhang et al. 2001). Approximately 250,000 plaques were screened with spinach CMO cDNA which was generated by PCR. Positive plaques were obtained from the third-round screening and then excised in vivo into pExCell plasmids (Amersham) following the instructions, and the plasmid with the longest insert was subjected to sequencing analysis.

RNA gel-blot analysis

Total RNA was extracted using the guanidinium isothiocyanate/acidic phenol method (Zhang et al. 1995). Thirty micrograms of total RNA were transferred to a nylon membrane (Hybond N$^+$, Amersham) as described previously (Li and Chen 2000). Northern Hybridization was carried out overnight at 65 °C by using the α-^{32}P-dCTP-labeled AhCMO cDNA as a probe. Filters were washed with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 15 min at 42 °C, respectively, and then washed with 1 × SSC, 0.1% SDS for 15 min at 55 °C. After stripping the probe, the same blots were re-probed with the 18S rDNA gene. The mRNA levels were quantified using the Imaging DensitoMeter (Model GS-670 Bio-Rad). The resulting values were normalized with those obtained from 18S rDNA hybridization.

Genomic Southern-blot analysis

DNA extraction and Southern-blot analysis were carried out as described previously (Zhang et al. 1999). High-stringency hybridizations were carried out for 16 h at 65 °C with α-^{32}P-dCTP-labeled AhCMO cDNA as a probe. The membrane was washed with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS for 15 min at 65 °C, respectively.

DNA sequencing and data analysis

DNA sequences were determined using the *Taq* Dye Primer Cycle Sequencing Kit (Amersham) and the ABI 373A automatic sequencer. The nucleotide and amino-acid sequences were compared with those released in GenBank databases by using the GAPPED BLAST analysis program. The full-length sequence of *AhCMO* has been deposited in GenBank databases under the accession number AF270651. The alignment report was produced by software DNASTAR.

Tobacco transformation and analysis of stress tolerance

A *SmaI*/*SacI* fragment encoding AhCMO was ligated into the *SmaI*/*SacI* site of pBI121 (Clontech) (see Fig. 6A) under the control of the 35S promotor, and introduced into tobacco (*Nicotiana tobacum* L. var. Xanthi) by *Agrobacterium*-mediated transformation. The transgenic plants were selected on MS media containing 50 mg/l of kanamycin and confirmed by Southern and Northern analysis.

To test salt tolerance, MS medium containing 0.9%, 1.2% and 1.5% NaCl were used as salt-grads; to test drought tolerance, 5% and 10% PEG-6000 was added into MS medium (Pilon-Smits et al. 1995). Primary transgenic tobaccos (T_0) and control plants were propagated vegetatively in various stress mediums by cutting into several seedlings. All surviving plants were transplanted into soil tanks to give seeds. T1 seeds from the transgenic lines were plated on MS medium containing 0.9% and 1.2% NaCl, or 5% and 10% PEG-6000, to test the germination under salt or drought stresses, respectively.

Results

Isolation and characterization of CMO cDNA from *A. hortensis*

Spinach CMO cDNA was generated by RT-PCR and identified by sequencing. Using this 1.3-kb cDNA fragment as a probe, we screened a cDNA library constructed from salt-treated *A.hortensis* plants; 37 clones with different insertions from 1.4 kb to 1.8 kb were isolated and the longest one of them was sequenced. Analysis of this cDNA sequence revealed an ORF of 1,314 bp, with a 136 bp 5′-UTR and a 314 bp 3′-UTR. The deduced amino-acid sequence of the ORF shows a similarity of 81% and 72% with the CMO of spinach and sugar beet, respectively. Thus, the gene corresponding to this cDNA was designated *AhCMO*. The alignment of this AhCMO with other CMO proteins identified four conserved domains (Fig. 1): (1) one conserved motif in chloroplast-targeting peptides; (2) three conserved residues “AVA” at

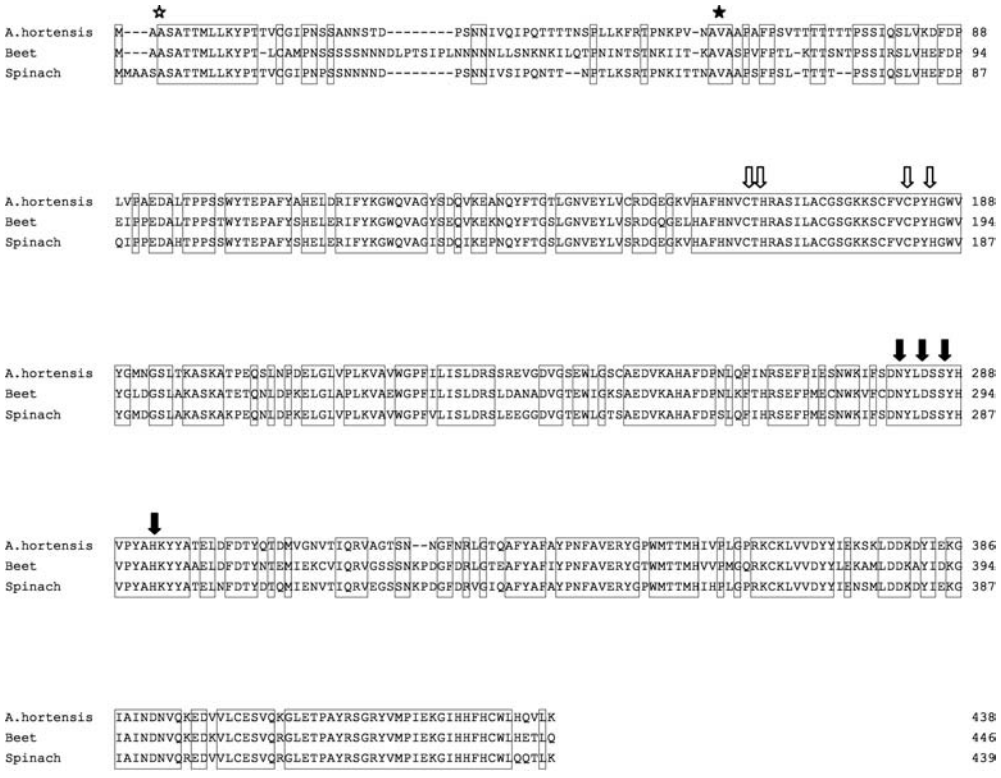

Fig. 1 Comparison of the deduced amino-acid sequence of AhCMO with the CMO sequences of spinach and sugar beet. Gaps are introduced to maximize the alignment, *Shaded residues* indicate the identity. Four conserved domains are marked. The *solid asterisk* marks the conserved residues "AVA" at the N terminal of mature peptides. The *open asterisk* marks the conserved chloroplast-targeting peptide sequence. The *solid arrows* show the two Cys-His pairs of the Rieske-type [2Fe-2S] domain. The *open arrows* indicate the conserved residues in the mononuclear Fe-binding motif. The accession number of *AhCMO* in GeneBank is AF270651

the N terminal of mature peptides; (3) two Cys-His pairs of the Rieske-type [2Fe-2S] domain; and (4) the conserved motif for the mononuclear Fe-binding domain. Except for these domains, the sequence of AhCMO shows more similarity with spinach CMO.

To study the genomic organization of the AhCMO gene, the genomic DNA from *A. hortensis* was digested with different restriction enzymes and hybridized with the AhCMO cDNA probe. In Fig. 2, the result showed that each digestion gave 1–4 hybridization bands. Since there is one *Eco*RI and one *Hin*dIII site in AhCMO cDNA, the hybridization pattern probably indicates the presence of one copy of the AhCMO gene in the genome of *A. hortensis*, although other possibilities exist.

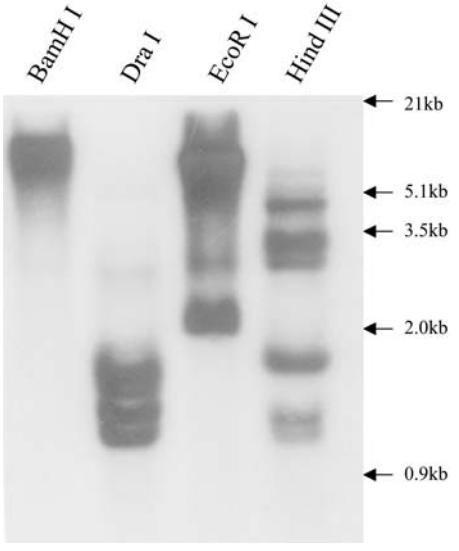

Fig. 2 The Southern-blot analysis of *AhCMO*. The genomic DNA was completely digested with *Bam*HI, *Dra*I, *Eco*RI and *Hin*dIII, and subjected to hybridization using the full-length sequence of *AhCMO* as a probe

AhCMO transcription in response to various stresses

Young plants were used to extract RNA from roots, stems and leaves, and all samples of tissues were collected at 9 AM to avoid the possible influence of circadian rhythm on expression. The Northern blot shows that, in all three organs, there is no expression of the *AhCMO* gene in untreated controls. However, when plants were treated with 200 mM of NaCl (Fig. 3A, B, C), the *AhCMO* transcript increased significantly and the induction was raised about 3-fold in root, stem as well as in leaf during the following days.

To study the effect of drought stress on the transcription of *AhCMO*, we withheld water from the plants for 1 week. It is shown in Fig. 4A that expression of *AhCMO* was raised about 3-fold. The transcription of *AhCMO* was only induced slightly by a 24-h ABA treatment.

Total RNA was extracted from the mature leaves of *A. hortensis* every 6 h to monitor the expression level of *AhCMO* during the whole day. It can be seen from Fig. 4B that the transcription peaked at about 12 AM and almost disappeared at about 12 PM. This expression pat-

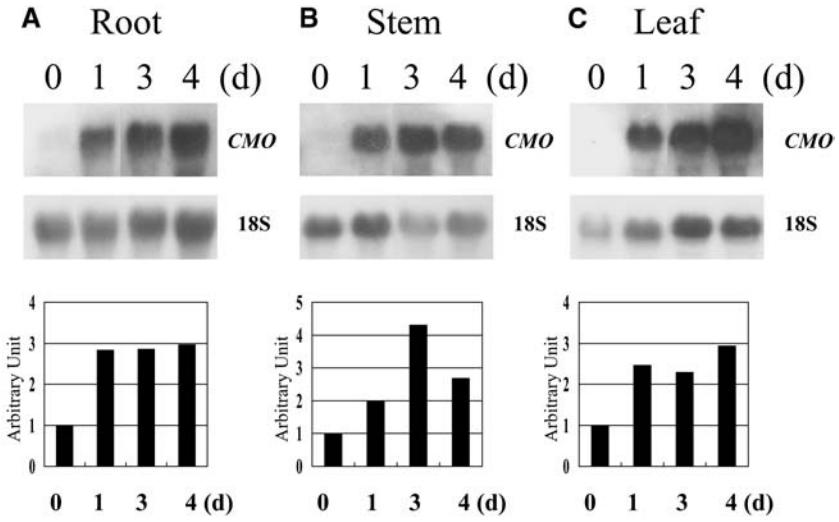

Fig. 3 Time-course RNA gel-blot analysis of the *AhCMO* transcript in response to salinity in roots (**A**), stems (**B**) and leaves(**C**) of *A. hortensis*. Total RNA was extracted at 9 AM from young plants stressed with 200 mM of NaCl for 4 days. Northern blots were carried out as described in Materials and methods. The same blots were stripped of probes and re-hybridized with the 18S rDNA gene. The mRNA levels were quantified and the resulting values were normalized with those obtained from 18S rDNA hybridization

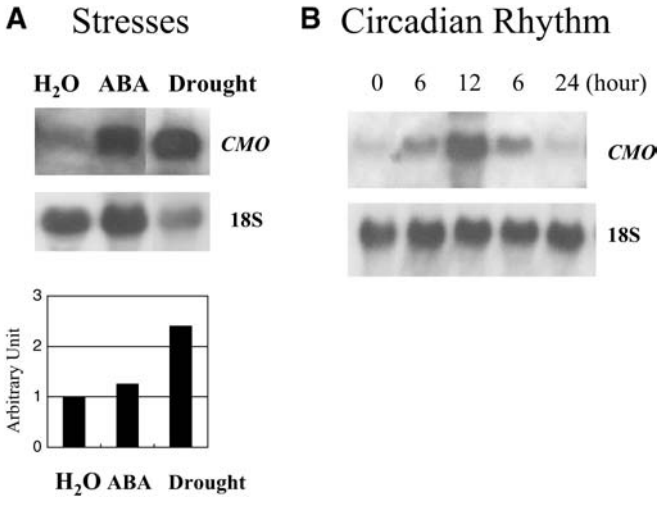

Fig. 4A, B Differential expression of *AhCMO* in mature leaves. **A** Expression of *AhCMO* in response to ABA and drought stresses. Total RNA was extracted from plants stressed with 20 μM of ABA for 24 h or un-irrigated for 1 week, respectively. **B** Expression of *AhCMO* under a normal greenhouse environment during a 24-h period. Total RNA was extracted from mature leaves harvested with a 6-h interval. Northern-blot analysis and normalization of the hybridization signal were the same as described in Fig. 3

tern probably indicated the existence of circadian rhythm in *AhCMO* expression.

Performance of transgenic tobacco upon salt and drought stresses

The AhCMO gene in a binary vector pBI121 was transferred into the tobacco leaf discs using *Agrobacterium*-mediated transformation. Seven primary transformants were obtained after selection on Kanamycin medium and confirmed by Southern blots (data not shown). The expression of the AhCMO gene in one representative line was also detected by Northern blots using *AhCMO* as a probe (Fig. 5B). To test the performance of the transgenic plants under salt- and drought-stress conditions, each of the seven primary transformants was cut into segments that contained one potential bud between petiole and stem to propagate them vegetatively. These segments were cultured in media containing different concentrations of NaCl for 6 weeks, and then the states of development and biomass of the corresponding shoots were recorded. It can be seen from Fig. 5C and D that, in 0.9% NaCl, the representative transgenic line developed large and dark green leaves plus a relatively stronger rooting system, whereas the regenerated non-transgenic control line had smaller and light green leaves plus a weaker rooting system. In 1.2% NaCl, the growth of the regenerated control was retarded whereas the transgenic lines rooted during culture with slowly growing leaves. In 1.5% NaCl, the regenerated controls died finally, but the transgenic lines still had yellow leaves although no roots developed. These results indicated that the *AhCMO* transgenic plants showed more tolerance to salt-stress than the controls.

To test the performance of the transgenic lines under drought stress, a drought environment was simulated by adding 10% PEG-6000 into the MS medium. Segments from the seven primary transgenic lines and controls were transferred into the medium and cultured for 2 months. It is shown that the controls died very soon after transfer, suggesting that the simulated drought stress caused water deficiency and thus seriously damaged the metabolism and development. In contrast, all the transgenic lines that developed roots, shoot and leaves during culture kept increasing the biomass (Fig. 5E). This result demonstrated that the AhCMO gene conferred transgenic tobacco-plant drought tolerance under drought-stress conditions.

We also tested the germination rate of the transgenic T1 seeds under stress conditions (Alia et al. 1998). The control seeds and the T1 seeds from the representative transgenic line were spread on 0.9% and 1.2% NaCl me-

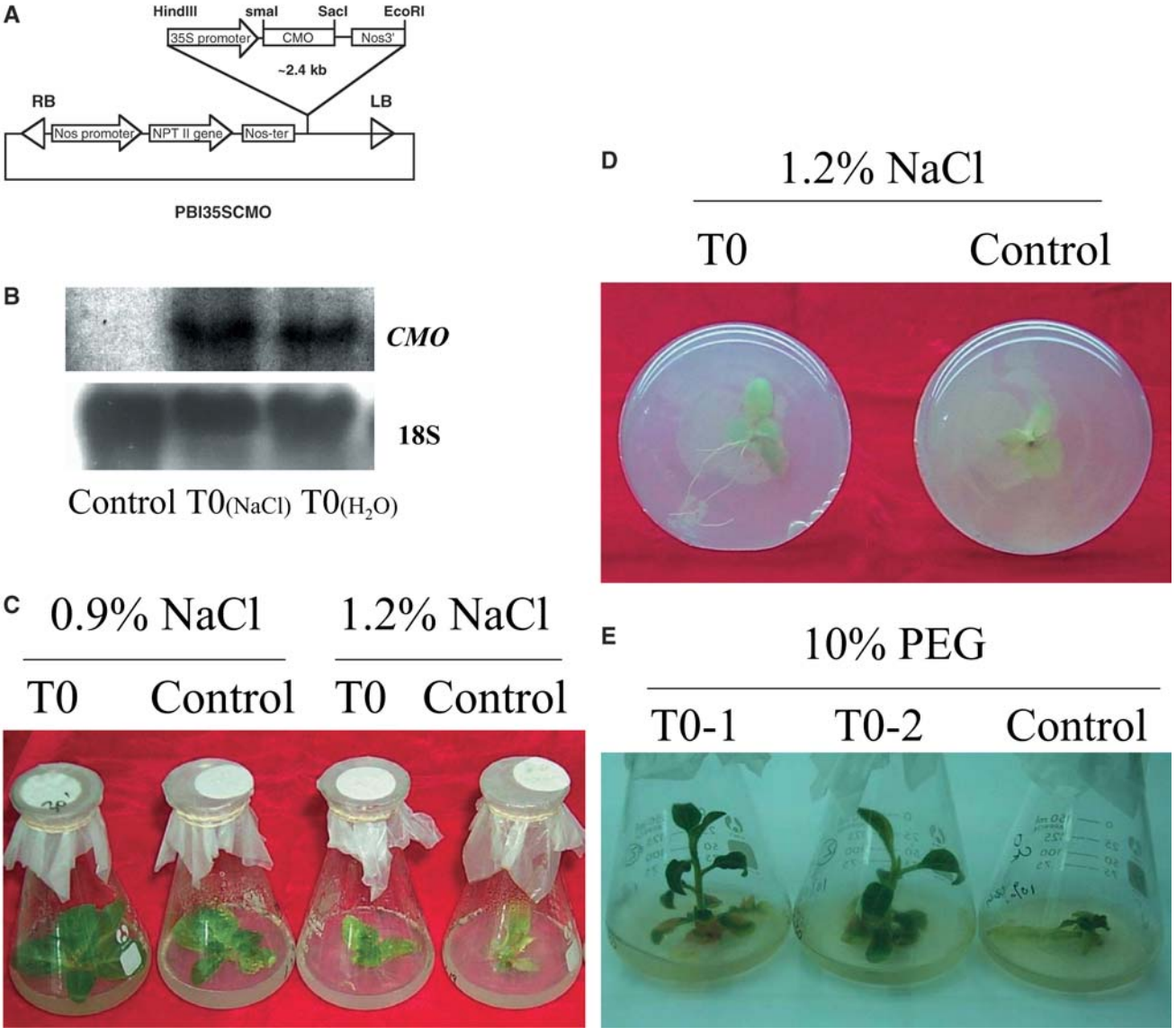

Fig. 5A–E Characterization of transgenic tobacco expressing *AhCMO*. **A** Schematic representation of the *AhCMO* expression cassette. **B** RNA gel-blot analysis of the non-transgenic control and the representative transgenic line stressed with 1.2% NaCl, or not, respectively. **C** The root growth of the representative transgenic line (left) and the non-transgenic control (right) in 1.2% NaCl MS medium. **D** Comparison of the performance of the representative transgenic line and the non-transgenic control in NaCl medium **E** Comparison of the performance of the representative transgenic lines and the control in 10% PEG-6000 medium

dia, or 5% and 10% PEG media, respectively. After 3 weeks, the germination rates in each medium were evaluated. It can be seen from Table 1 that the transgenic seeds had a much higher germination rate under all stress conditions when compared with controls, indicating that the AhCMO gene functioned in the transgenic plants and conferred stress tolerance.

Table 1 Comparison of the germination rates of the non-transgenic control seeds and the representative T1 seeds under various stresses. Freshly harvested seeds of the non-transgenic control and the representative transgenic line were plated on various stress media, respectively. Plates were incubated directly at 23 °C with constant light. After 3 weeks, the numbers of germinated seeds were recorded and expressed as the percentage of the total number of seeds plated (200–300). Data of the T1 seeds were normalized with the parallel germination rate of T_1 seeds on MS media containing 50 mg/l of kanamycin

Stresses	Control	T1
0.9% NaCl	12.0%	40.8%
1.2% NaCl	6.2%	23.0%
5% PEG-6000	15.7%	48.7%
10% PEG-6000	1.6%	16.5%
Kanamycin	0	40%
MS	100%	100%

Discussion

We isolated a full-length cDNA of *AhCMO* from *A. hortensis*. The deduced amino-acid sequence of AhCMO showed a high similarity to those of the other two CMO proteins from spinach and sugar beet. No other oxygenases from plants showed a similarity to this CMO family. This is different from the situation of BADH from *A. hortensis* which we isolated before, and which showed a high similarity to many other dehydrogenases from plants and bacteria. It is thus possible that the existence of CMO, but not BADH, determines GlyBet biosynthesis in plant species. Southern-blot analysis indicated only one *AhCMO* copy in the *A. hortensis* genome. A conserved amino-acid sequence (ASATTMLLKYPT) in the targeting peptide of CMO supports the observation that mature CMO activity was concentrated in the chloroplast (Hanson and May 1985; Weigle et al. 1986). This chloroplast-targeting peptide will be very useful in future genetic engineering of the plant chloroplast.

Plenty of genes are regulated or influenced by the Circadian rhythm in higher plants and many of them are associated with a light rhythm (Schaffer et al. 2001). We detected the Circadian rhythm in *CMO* expression of GlyBet natural-accumulating plants. The rhythm of *AhCMO* expression is possibly associated with the physiological fluctuation of osmotic pressure, temperature and photosynthesis. After sunrise, the osmotic pressure in the cytoplasm rises because active transpiration brings an influx of ion from the soil solute and makes the water content lower. At around the noon, the high light-intensity and temperature become most unfavorable to metabolism especially with respect to Photosystem II in the chloroplast. During this period, high *AhCMO* transcription can enrich high levels of GlyBet to rescue the cell by protecting enzymes, regulating the osmotic pressure, stabilizing the cell membrane, and promoting the recovery of the PSII complex from photo-induced damage in the chloroplast (Sakamoto and Murata 2001). After sunset, the *AhCMO* transcript degraded rapidly and the synthesis of GlyBet declined. The circadian rhythm of *AhCMO* expression in the betaine natural accumulator may be a conserved self-protection mechanism acquired during long-term evolution and should facilitate the survival of the plants under unfavorable osmotic conditions.

A low level of GlyBet cannot adjust the osmotic pressure in vivo. Under salt stress, young plants of *A. hortensis* have to accelerate the synthesis of GlyBet in many organs including the root, stem and leaf. Therefore the expression of *AhCMO* in these organs was raised about 3-fold in response to NaCl treatment. In mature leaves, drought and ABA induced the *AhCMO* transcription. ABA also induced *BADH* transcription (Ishitani et al. 1995). These facts probably indicated that GlyBet synthesis in higher plants was regulated through an ABA-dependent pathway. Under normal conditions the transcription of *CMO* can be detected in mature leaves but not in expanding leaves of *A. hortensis*, suggesting that the high level of GlyBet is unfavorable to seedling growth. The growth of BADH transgenic rice was also inhibited by a high level of endogenous GlyBet (Kishitani et al. 1998).

Seven *AhCMO* transgenic lines were obtained in the present study. In the salt tolerance test, the transgenic plants survived better and longer on MS medium containing NaCl than the control plants. T1 seeds also showed better salt tolerance than control seeds. We performed osmotic stress with 5% and 10% PEG-6000 on transgenic tobacco and found that primary transgenic plants and T1 seeds showed a considerable improvement in drought tolerance. Although the transgenic plants performed better under both salt and drought stresses, the phenotypes obtained after these stresses are different. As shown in Fig. 5C, D and Table 1, salt stress inhibited the shoot-apex growth and germination in both transgenic and control plants, and transgenic plants increased biomass a little more than the controls. However, under drought stress, the elongation of the transgenic shoots was not influenced (Fig. 5E), and the germination rate and biomass increases were much more than those in control plants. This difference may reflect different effects of the salt and drought stresses exerted on plants. For salt stress, it causes both osmotic stress and ion toxicity, whereas PEG treatment causes only osmotic stress. Therefore, AhCMO transgenic plants were more tolerant to drought stress, possibly by releasing the damage caused by water deficiency, through covering the protein complex and the cell membrane in transgenic tobacco.

It is important to note that, so far, all plant metabolic engineering of GlyBet suffered from one feature: the GlyBet level in transformants is lower than that in the natural accumulator to adjust the osmotic pressure in vivo (Sakamoto and Murata 2001). Recently it has been proposed that the transport of choline into the chloroplast constrains GlyBet accumulation in CMO^+ tobacco (McNeil et al. 2000). If supplied with extraneous substrate, transgenic plants would then synthesize enough GlyBet and greatly promote stress tolerance (Nuccio et al. 1998; Huang et al. 2000). It is then necessary to characterize the regulation of the methylation network that controls choline production in higher plants.

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