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Isolation of choline monooxygenase (CMO) gene from *Salicornia europaea* and enhanced salt tolerance of transgenic tobacco with CMO genes

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Glycinebetaine (GB) is an osmoprotectant accumulated by certain plants in response to high salinity, drought, and cold stress. Plants synthesize GB via the pathway choline → betaine aldehyde → glycinebetaine, and the first step is catalyzed by choline monooxygenase (CMO). In the present study, by using RT-PCR and RLM-RACE, a full-length CMO cDNA (1844 bp) was cloned from a halophyte *Salicornia europaea*, which showed high homology to other known sequences. In order to identify its function, the ORF of CMO cDNA was inserted into binary vector PBI121 to construct the chimeric plant expression vector PBI121-CMO. Using *Agrobacterium* (LBA4404) mediation, the recombinant plasmid was transferred into tobacco (*Nicotiana tabacum*). The PCR, Southern blot and RT-PCR analysis indicated the CMO gene was integrated into the tobacco genome, as well as expressed on the level of transcription. The transgenic tobacco plants were able to survive on MS medium containing 300 mmol/L NaCl and more vigorous than those of wild type with the same concentration salt treatment. In salt-stress conditions, transgenic plants had distinctly higher chlorophyll content and betaine accumulation than that of the control, while relative electrical conductivity of transgenic plants was generally lower. The results suggested the CMO gene transformation could effectively contribute to improving tobacco salt-resistance.

Keywords: *Salicornia europaea*, Halophyte, Choline monooxygenase, Transgenic tobacco, Salt tolerance, Glycinebetaine

About 20% of the world's cultivated land and nearly half of all irrigated lands are affected by high salinity[1]. Exposure to high saline condition causes ion imbalance and hyperosmotic stress in organisms. Plants employ various specific mechanisms to resist salinity for the adjustment of their internal osmotic status, one of which is the ability to accumulate low molecular weight organic-compatible solutes such as sugars, some amino acids, and quaternary ammonium compounds[2-4]. Glycine betaine (GB) is a major osmolyte, which can effectively stabilize enzymes critical to physiological functions[2-4].

In plants, GB is synthesized in a two-step oxidation of choline via the unstable intermediate betaine aldehyde, by a ferredoxin-dependent choline monooxygenase (CMO) and the NAD^+-dependent betaine aldehyde dehydrogenase (BADH)[2]. Both enzymes are located in the stroma of chloroplasts. Research on genes responsible for betaine synthesis has become attractive because genetic engineering techniques have been applied to improve the salt tolerance of plants[5-7]. CMO was first purified from spinach leaves[8] and identified as an unusual plant oxygenase containing a Rieske-type [2Fe-2S]. Then the cDNA was cloned by RT-PCR using primers corresponding to amino acid sequences in this iron-sulphur centre from spinach[9]. Thereafter, CMO genes have been cloned from *Spinacia oleracea*, *Beta vulgaris*, *Atriplex prostrate* and *Suaeda liaotungensis* [9-11].

Salicornia europaea, a halophyte has been considered as one of higher plants with most salt tolarance. It could tolerate 8% saline exposure[12] and contains significant amount of betaine content. There is a possibility that function of *S. europaea* CMO (SeCMO) gene may be superior to that of other organisms. Thus, in order to further understand the function of CMO gene in betaine synthesis and salt tolerance in plants, in the present study, we have isolated a full-length cDNA from *S. europaea* and overexpressed the SeCMO gene in transgenic tobacco to reveal its possible role in salinity stress.

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Abbreviations: BADH, betaine aldehyde dehydrogenase; CMO, choline monooxygenase; DIG, digoxigenin; GB, glycinebetaine; ORF, open reading frame; RLM-RACE, RNA ligase mediated rapid amplification of cDNA ends; UTR, untranslated region.

Materials and Methods

Plant material and growth conditions

Seeds of *Salicornia europaea* were collected from seashore in Dalian (North-east China) and germinated for 3 days on moistened filter paper in sterile petri dishes. Seedlings were then transferred to plastic pots filled with vermiculite and further grown at 22-25°C under a 16 h photoperiod in a greenhouse. The pots were irrigated every day with 1/2 strength Hoagland solution. One week later, salinity treatment was started by adding 500 mM NaCl to the nutrient solution. Then the plants treated for various times were collected for analysis.

Cloning of SeCMO by RT-PCR and RLM-RACE

The seedlings treated with 500 mM NaCl for 1 week were harvested and total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription was performed with the reverse transcription system (TaKaRa) and adaptor-oligo (dT) primer and 2 micrograms of total RNA were used as template. PCR reactions were performed using ExTaq (TaKaRa) at: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min with 30 cycles.

For cloning SeCMO cDNA, homologous sequences of *Spinacia oleracea* and *Suaeda liaotungensis* were aligned to identify consensus regions. Two primers, C1: 5'-TTTTACAAAGGA TGGCAAGT-3' and C2: 5'-GCAG TGGAAATG GTGGAT-3' were designed based on these consensus regions to amplify a partial length SeCMO. RT-PCR product was purified by S. N. A. P.™ gel purification kit (Invitrogen), cloned on the PMD18-T vector (TaKaRa) and sequenced by TaKaRa biotechnology (Dalian Co., Ltd.)

Invitrogen GeneRacer kit was used for both 5'and 3' RLM-RACE. The RNA for 5' and 3' RACE was prepared according to the GeneRacer protocol. Five micrograms of total *S. europaea* RNA was treated with calf intestinal alkaline phosphate to remove the 5'-terminal phosphates from any RNA that is not full length mRNA. These RNAs were then treated with tobacco acid pyrophosphatase to remove the 5'-cap structure of the full-length mRNA, thus leaving a 5'-PO_4 only on full-length mRNA. The RNA ligase was then used to ligate the GeneRacer RNA-oligo primer to only the full-length mRNA with the 5'-PO_4.

First-strand cDNA synthesis was accomplished with SuperScript III reverse transcriptase, GeneRacer oligo (dT) primer, RNA treated as described above and used as template in 20 µl reaction according to the manufacturer's protocol. Two microlitre of this reverse transcriptase reaction mixture was used as templates for second-stranded cDNA synthesis and amplification. Subsequently, a hot-start PCR was implemented to amplify the 5'-portion of CMO cDNA sequence using the template cDNA generated above. For this purpose, the adapter-specific outer sense primer provided in the kit and CMO5'-specific outer antisense primer, GSPR: 5'-GGTGGCTTTTG TGAGGTCTCCAT CTA-3' were used. The PCR product was further amplified in a nested reaction with the adapter-specific inner sense primer from the RLM-RACE kit and CMO-specific Nest antisense primer GSPRn: 5'-CTCCATCTAAGCCAAACACC CATC-3'.

The same method as 5' RLM-RACE was used to obtain 3'cDNA. GeneRacer™ 3'-primer and CMO 3'-specific outer primer GSPF5'-CGGCAAAGTGGT GATCCAAAGAGT-3 were used in the first round PCR amplification. The PCR product was amplified in a nested reaction with GeneRacer™ 3′ Nest primer and CMO 3'specific Nest primer GSPFn5'-CC AGCCTATCGCAGTGGCAGATA-3'. All the 5' and 3' RACE products were purified by S.N.A.P.™ gel purification kit (Invitrogen), cloned into the PMD18-T vector (TaKaRa) and sequenced by TaKaRa Biotechnology (Dalian Co., Ltd.).

Overexpression of SeCMO in tobacco and salt tolerance analysis of transgenic plants

The ORF of SeCMO was sub-cloned into pUC19 plasmid and sequenced, and then the correct insert of pUC19-SeCMO was selected to construct into binary plant transformation vector pBI121. The plasmid of pBI121-SeCMO was introduced into an *Agrobacterium tumefaciens* strain LBA4404 and transferred into tobacco by leaf discs method. The transformed plants were detected by PCR with a pair of primers (CDSF: 5'-GCGCGGATCCCAA TTATTTTAAGATGGCAG-3' and CDSR: 5'-GCGCGAGC TCTTTGCAAACTAAAATCACTG-3') and further validated by genome blot analysis with a probe containing a fragment of SeCMO ORF.

To study the potential for salinity resistance of transgenic tobacco, the shoots of T0 transgenic and wild type plants were excised and transplanted in the Murashige and Skoog (MS) culture medium supplemented with 0, 100, 200, 250, 300, and 350 mM NaCl for 30 days.

Southern blot analysis

Southern blot was performed according to the previously described procedure[13]. Genomic DNA samples (20 mg) were digested with HindIII enzymes, separated on 1% (w/v) agarose gels and then blotted onto the membranes (Hybond-N+ positively CHGD nylon transfer membrane, Amersham Biosciences) in 10×SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) using a vacuum blotting pump. The filter membranes were then hybridized with a SeCMO-specific probe containing the 5'-UTR labeled with digoxigenin (DIG). The DNA probe preparation, hybridization, washing, and detection were performed according to the instruction manual of DIG high primer labeling and detection starter kit (Roche).

RT-PCR analysis of CMO transcripts

RT-PCR was performed to check whether the CMO gene was expressed in positive plants in the Southern blot analysis. Total RNA was isolated according to the protocol of the Access RT-PCR kit (TaKaRa Dalian CHN). The PCR was carried out for 30 cycles, each consisting of 30 s at 94°C for denaturation, 30 s at 54°C for annealing, and 1 min at 72°C for extension with the primers CDSF and CDSR.

Measurement of glycinebetaine

Glycinebetaine (GB) extraction was done as described earlier[14,15]. Dried ground leaves (1.5 g) used for betaine determination were transferred to vials prepared for methanol extraction. Then the extracts were passed through a 5 ml column of Dowex 1 × 8 (50–100 mesh, OH- form, Sigma) and another 5 ml column of Dowex 50W × 2 (100–200 mesh, H+ form). The column was washed with 3 ml of water and then eluted with 4 ml of 4 M NH_4OH. After filtering, the eluate was evaporated to dryness at 65°C in vacuum. One millilitre of 50 mM KH_2PO_4 (pH 4.45) solution for the HPLC elution was used for dissolving betaine samples before separation on HYPERSIL ODS C_{18} column (5 mm, Φ4.5 mm × 250 mm, Dalian Institute of Chemical Physics, Chinese Academy of Sciences). GB was measured with HPLC (Agilent 1100 Series, USA). The mobile phase was 50 mM KH_2PO_4 (pH 4.45) and flow rate was 0.7 ml min^{-1}. Detection wavelength was set at 192 nm and the injection loop volume was 20 ml.

Relative electrical conductivity

Membrane permeability of the leaves was measured by relative electrical conductivity (REC)[16]. Leaf tissue (1 g) was cut into segments of 1 cm^2, washed with double-distilled water to remove solutes from both leaf surfaces and damaged cell due to cutting and then salt stressed for 12 h with 300 mM NaCl solution. After the stress, the leaf segments were washed quickly three-times with double-distilled water and then put into 20 ml of double-distilled water in test tubes and vibrated slowly using a rotary shaker (100 rpm) at room temperature. Electrical conductivity (EC) was measured 20 min later using a DDS-11A Conductometer (Shanghai Rex Xinjing Instrument Co. Ltd.). Then the tubes containing the segments were immediately placed into boiling water for another 20 min and measured for EC again. REC was expressed as: EC1 (measured before boiled)/EC2 (measured after boiled).

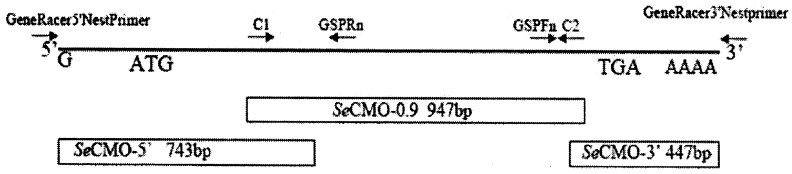
Chlorophyll estimation

Leaf tissue (100 mg) was homogenized in a pre-chilled mortar in 10 ml of 80% solution of acetone. The homogenate was filtered and 500 µl of the supernatant was taken and diluted to 5 ml with 80% solution of acetone. The absorbance of supernatant was taken at 644, 633 and 750 nm for chlorophyll content and was calculated as described previously[17].

Results

Molecular characterization of SeCMO cDNA and sequence analysis

The full-length SeCMO cDNA (GeneBank accession no. AY849925) clone was of 1814 bp and encoded a protein of 442 amino acids with predicted molecular mass of 42.8 kDa. Sequence analysis showed SeCMO contained 3'-untranslated region of 361 bp and 5'-untranslated region 154 bp as illustrated in Fig. 1. At the same time, the transcription start site (TSS) G was identified by RLM-RACE. The predicted amino acid sequence of the SeCMO protein shared high homology with other CMO proteins from *Suaeda liaotungensis* (85% identity), *Beta vulgaris* (75%), *Spinacia oleracea* (78%), *Ophiopogon japonicus* (78%), *Atriplex nummularia* (75%), *Atriplex hortensis* (76%), *Atriplex prostrata* (76%), and *Amaranthus tricolor* (70%) by NCBI Blastp. The SeCMO protein showed the characteristic conserved motifs of the CMO, a Rieske-type [2Fe-2s] cluster motif and a mononuclear



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D V K A H A F D P N L Q F I H R S E
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Fig. 1—Cloning of *S. europaea* CMO gene [A schematic map of the 1844 bp composite cDNA sequence encoding CMO in *S. europaea.* →, Primers used for RT-PCR and RLM-RACE.G is the transcript start site (TSS). The Genebank accession no. is AY849925]

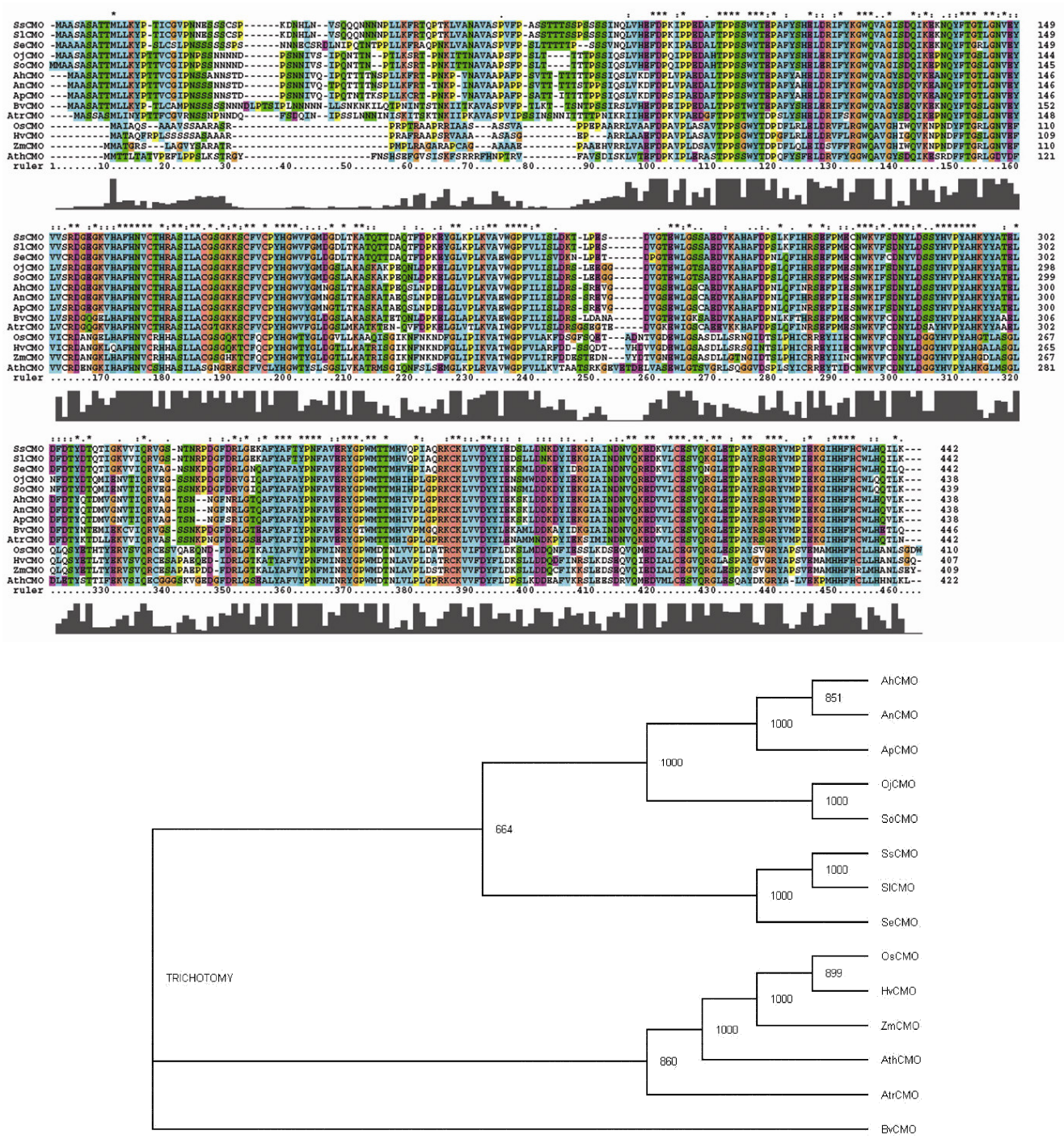

Fig. 2—Phylogenic tree of predicted amino acid sequences of plant choline monooxygenase using the ClustalX of the complete Alignment program [Only complete protein sequences were included in the alignment. SeCMO shared high homology with the protein from *Suaeda liaotungensis* and was classified into the Chenopodiaceae]

non-heme Fe binding motif. As shown in Fig. 2, phylogenetic analysis of deduced amino acid sequences classified SeCMO into the CMO from Chenopodiaceae.

Overexpression of SeCMO in tobacco and the salt tolerance of transgenic plants

To validate the function of SeCMO gene during salt stress, the ORF of SeCMO was constructed into plasmid pBI121 and transferred into tobacco via

Agrobacterium-mediated method. The transformed tobacco was checked by PCR, and DNA gel blot analysis (Fig. 3). The positive transgenic lines were subjected to RT-PCR analysis, and the result indicated that SeCMO was expressed in transgenic tobaccos (Fig. 3).

For salt tolerance experiments, the shoots of 27 transgenic lines and wild-type plants were transplanted into rooting medium containing 100-350 mM NaCl and cultured for 4 weeks in greenhouse under condition of 16 h photoperiod, 25/16°C day/night temperature and 60-70% relative humidity. The result showed that transgenic plants could root in the medium containing up to 300 mM NaCl, while the wild-type plants failed to root in the medium containing higher than 100 mM NaCl and displayed severe chlorosis and wilting (Fig. 4).

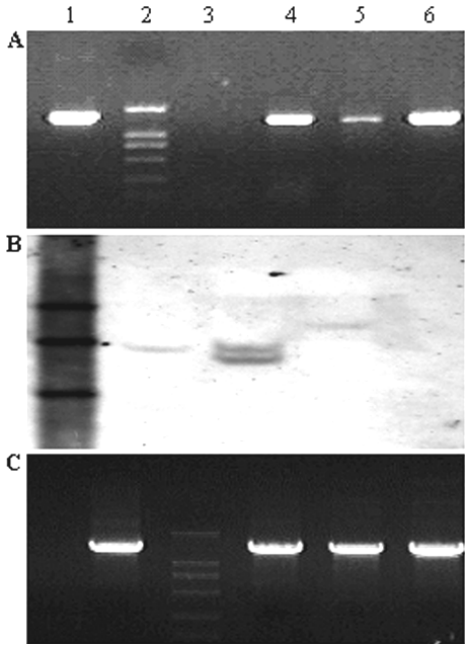

Fig. 3—Analysis of SeCMO gene in transformed tobacco lines [(A): PCR analysis of T0 transformed tobacco. The full-length SeCMO ORF was amplified from the genomic DNA of independent T0 transgenic lines by primers CDSF and CDSR. Lane 1: pBI121-CMO plasmid; lane 2, DNA molecular weight marker (DL 2000); lane 3, wild-type tobacco; lanes 4-6, transformed tobacco lines C9, C24, C27; (B): DNA gel blot analysis of T0 transformed plants. Lane 1, DNA molecular weight marker II, digoxigenin labeled; lanes 2-4, transformed tobacco lines C9, C24, C27; lane 5 wild-type tobacco; and (C): RT-PCR analysis of transgenic samples and wild type using the primers: CDSF and CDSR. Lane 1, wild-type; lane 2, plasmid pBI121-CMO; lane 3, DNA marker DL2000 (TaKaRa); and lanes 4-6, transgenic lines: C9, C24, C27]

Quantitation of GB

The GB concentration in the wild-type tobacco was 2.32 μg/g fresh wt, whereas the transformed plants (C9, C24, C77) had 19.37 to 21.75 μg/g fresh wt under 300 mM NaCl stress. Transgenic plants accumulated 8.36- to 9.37-fold more GB in their leaves than wild-type plants. Analyses of variance indicated that differences between transgenic plants and wild-type were significant in terms of GB accumulation (Table 1).

Electrolyte leakage measurement

The experimental method is based on electrolyte leakage measurement in leaf tissues that are immersed in double-distilled water after salt stress. As electrolyte leakage is greatly influenced by the age of both plants and leaf, as well as the sampling position

Table 1—Glycinebetaine (GB), relative electrical conductivity (REC) and chlorophyll content of wild-type and transgenic tobacco plants overexpressing SeCMO under 300 mM NaCl stress

[Each value is the mean ± S.D of three independent experiments]

Tobacco line	GB (μg/g fresh wt)	REC	Chlorophyll (μg/g fresh wt)
Wild-type	2.31 ± 0.1	0.795 ± 0.05	0.115 ± 0.03
C9	19.37 ± 0.3	0.653 ± 0.04	0.756 ± 0.05
C24	20.10 ± 0.5	0.612 ± 0.03	0.831±0.04
C77	21.75 ± 0.4	0.638 ± 0.02	0.781 ± 0.05

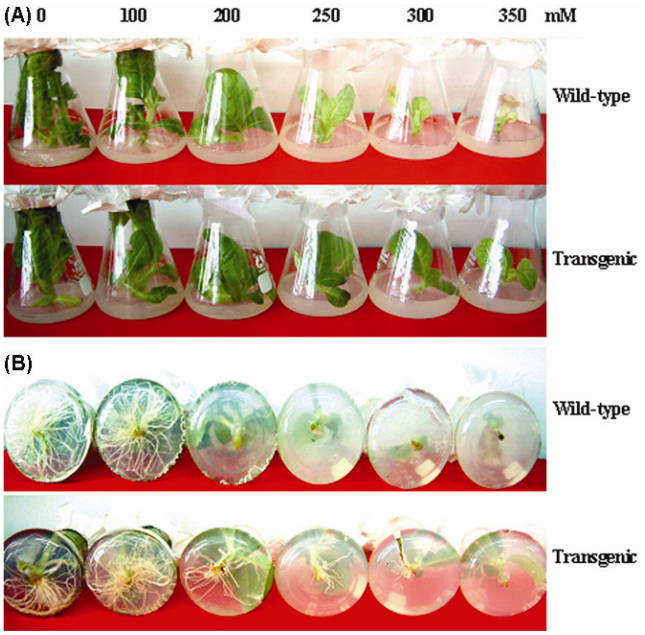

Fig. 4—Salt tolerance of wild-type (WT) and transgenic plants overexpressing SeCMO grown at various salt concentrations [(A): The shoots of wild-type and T0 transgenic lines rooted in MS culture medium containing 0-350 mM NaCl for 3 weeks; and (B): The roots of wild-type and T0 transgenic lines rooted in MS culture medium containing 0-350 mM NaCl for 3 weeks]

of the leaf, all experiments were conducted at the seven leaf seedling stage and using the fully expanded leaf blade from the same position. The result showed that difference of REC between the transgenic (C9, C24, C77) and wild-type plants was significant ($P < 0.05$) (Table 1).

Chlorophyll content

The CMO transgenic plants (C9, C24, C77) were analyzed for their chlorophyll content and the data were analyzed by variance. Compared with the wild-type, all of the transgenic plants had higher chlorophyll content (Table 1).

Discussion

Some plants accumulate a lot of GB under salt stress. GB protects plants by acting as an osmolyte, maintaining water balance between the cells and the environment, and stabilizes macromolecules under cellular dehydration and high salt concentrations[18]. Some economically important crops, including rice (*Oryza sativa*), potato *(Solanum tuberosum*), and tomato *(Solanum lycopersicum*) are unable to accumulate GB; therefore, these species are potential targets for engineering of betaine biosynthesis[19]. With increasing knowledge of genomics and proteomics coupled with gene engineering technologies, several plant species have been engineered with genes of the GB biosynthetic pathway, which confer tolerance to several abiotic stresses in these plant species[20].

In this work, by cloning the CMO gene from *S. europaea* and transforming into tobacco, we found that the GB concentration of transgenic tobacco plants showed about 9-fold increase, as compared with wild type plants. But, earlier studies[6,7] demonstrated that GB content increased only slightly in transgenic tobacco. These studies suggested that short supply of endogenous choline limited the betaine synthesis in transgenic tobacco and the GB content increased about 30-fold, when 5 mmol/L choline was provided to transgenic tobacco.

The issue of endogenous choline short supply was solved after McNeil et al[21] enhanced synthesis of choline and GB in transgenic tobacco plants that overexpressed phosphoethanolamine N-methyl-transferase (PEAMT). The overexpression of PEAMT gene in transgenic tobacco increased the GB levels to 30-fold[21] via betaine synthesis gene engineering. The genes of PEAMT, CMO and BADH are needed to synthesize GB. As cereal, such as rice (*Oryza sativa*) with only BADH gene[22] can not accumulate GB, it would be interesting to co-overexpress PEAMT and CMO for gene engineering of betaine synthesis to enhance salt tolerance in cereal.

The salt tolerance of transgenic tobacco which harbored SeCMO gene was also studied. The REC was much lower, but the chlorophyll content was significantly higher in transgenic tobacco than that of the control. These results suggested that GB could prevent membrane damage in transgenic plants under salt stress and transgenic tobacco could grow well on medium containing 250-300 mmol/ L NaCl. In summary, SeCMO gene was effective for increasing salt tolerance of transgenic plants.

As salt stress tolerance is the result of polygenic expression in nature, for salt stress tolerance, increasing efforts are required to combine different strategies involving multiple genes. The co-expression of anti-porters with genes of the GB biosynthetic pathway has the potential to increase salt tolerance by many times. Recently, co-transformation of the Na^+/H^+ anti-porter gene (SeNHX1) and BADH was reported in tobacco[23]. The resulting transgenic plants accumulated more GB and showed higher biomass than either the single-gene transgenic plants or untransformed controls. Thus, future research into genetic engineering of economically important crop plants should be focused on enhanced and durable tolerance to multiple abiotic stresses.

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