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BIOTIC AND ABIOTIC STRESS

# Plastid-expressed choline monooxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco

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**Abstract** Glycine betaine (GlyBet), a quaternary ammonium compound, functions as an osmoprotectant in many organisms including plants. Previous research has shown that over-expression of enzymes for GlyBet biosynthesis in transgenic plants improved abiotic stress tolerance, but so far no study on the effects of plastid-expression of choline monooxygenase, the enzyme that catalyzes the conversion of choline into betaine aldehyde, has been reported. In the present study, tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) plants were transformed with a gene for choline monooxygenase (BvCMO) from beet (*Beta vulgaris*) via plastid genetic engineering. Transplastomic plants constitutively expressing *BvCMO* under the control of the ribosomal RNA operon promoter and a synthetic T7 gene G10 leader were able to accumulate GlyBet in leaves, roots and seeds, and exhibited improved tolerance to toxic level of choline and to salt/drought stress when compared to wild type plants. Transplastomic plants also demonstrated higher net photosynthetic rate and apparent quantum yield of photosynthesis in the presence of 150 mM NaCl. Salt stress caused no significant change on the maximal efficiency of PSII photochemistry (Fv/Fm) in both wild type and transplastomic plants, but a decrease in the actual efficiency of PSII (ΦPSII) was observed, and such a decrease was much greater in wild type plants. Our results demonstrate the feasibility of improving salt and drought tolerance in plants through plastid transformation with *BvCMO* gene.

**Keywords** CMO · GlyBet · Plastid transformation · Salt stress · Transplastomic plants

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## Introduction

Glycine betaine (GlyBet) is widely distributed in plants, animals and microorganisms (Sakamoto and Murata 2000). In both prokaryotes and eukaryotes, GlyBet is produced by a two-step oxidation of choline (Ikuta et al. 1977; Hanson et al. 1985; Andresen et al. 1988). In *E. coli*, the first step of the process, oxidation of choline into betaine aldehyde, is catalyzed by a membrane-associated choline dehydrogenase (CDH). CDH also oxidizes betaine aldehyde into GlyBet (Landfald and Strom 1986). The second step of the process, oxidation of betaine aldehyde into GlyBet, is catalyzed by a soluble betaine aldehyde dehydrogenase (BADH). To date, the genes encoding CDH (*betA*) and BADH (*betB*) have been cloned and characterized (Lamark et al. 1991).

In higher plants, GlyBet is also synthesized from the primary metabolite choline (Nuccio et al. 1998). The conversion of choline to GlyBet occurs in the chloroplast (Rhodes and Hanson 1993). The first step of GlyBet synthesis is catalyzed by a ferredoxin-dependent choline monooxygenase (CMO) which catalyzes the conversion of choline into betaine aldehyde (Brouquisse et al. 1989). The second step, the conversion of betaine aldehyde into GlyBet, is catalyzed by a $NAD^+$-dependent betaine aldehyde dehydrogenase (BADH; Weigel et al. 1986).

It has been well documented that GlyBet protects plant by stabilizing the highly ordered structures of complex proteins and membranes in vitro (Gorham 1995; Sakamoto and Murata 2000), as well as functions in the refolding of enzymes as a molecular chaperone (Sakamoto and Murata 2000; Chen and Murata 2002). Accumulation of GlyBet improves tolerance to various abiotic stresses (Rhodes and Hanson 1993). Expression of *betA*, a gene encoding CDH from *E. coli*, provides improved salt tolerance in tobacco and drought tolerance in cotton (Lilius et al. 1996; Holmstrom et al. 2000; Lv et al. 2007). Ectopic expression of a choline oxidase gene (*codA*) from the soil bacterium *Arthrobacter globiformis* increased tolerance to both salt and chilling stress in transgenic *Arabidopsis thaliana* and tomato plants (Hayashi et al. 1997; Sakamoto et al. 2000; Park et al. 2004; Park et al. 2007). Likewise, over-expression of a choline monooxygenase gene (*AhCMO*) from *Atriplex hortensis* led to increased drought tolerance in tobacco plants (Shen et al. 2002), and expression of a gene for CMO from spinach (*Spinacia oleracea*) conferred enhanced tolerance to salt stress and temperature stress on rice (Shirasawa et al. 2006).

Chloroplast genetic engineering has several attractive advantages over nuclear genetic engineering for biotechnological applications; such as high-level foreign protein expression owing to the polyploidy of the plastid genetic system with thousands of genome copies per cell (Daniell et al. 2002), multigene engineering in a single transformation event (DeCosa et al. 2001; Daniell and Dhingra 2002; Ruiz et al. 2003), transgene containment via maternal inheritance (Daniell and Dhingra 2002), lack of gene silencing, position effect due to site-specific transgene integration, and pleiotropic effects due to subcellular compartmentalization of transgene products (Lee et al. 2003). Genetic engineering of carrot chloroplast genome expressing *badh* gene conferred high salt tolerance (Kumar et al. 2004).

Many plant species including *A. thaliana* and rice are unable of synthesizing GlyBet due to functional defects of the corresponding GlyBet biosynthetic genes (Hibino et al. 2002; Luo et al. 2007; Niu et al. 2007). Recent studies have demonstrated that GlyBet accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic plants against abiotic stress (Park et al. 2007), and plants such as tobacco that lack GlyBet ("non-accumulators") show some BADH activity (Holmstrom et al. 1994; Rathinasabapathi et al. 1994). This simplifies engineering to improve the stress tolerance in non-accumulators as it makes it possible to install a GlyBet pathway in chloroplast by expressing CMO alone. In this paper, we demonstrate that transplastomic tobacco plants over-expressing *BvCMO* gene from beet, a halophyte plant that naturally produce GlyBet, accumulate GlyBet and exhibit increased tolerance to salt and drought stress. Our results also show that accumulation of GlyBet in transplastomic plants enhances the net photosynthetic rate and apparent quantum yield of photosynthesis under salt (150 mM NaCl) stress condition.

## Materials and methods

### Construction of tobacco plastid transformation vectors

The targeting region (*rps*14/*trnf*M) was amplified with primers P1 (5′-GGTTCTTTAAATTCCGTGGGTGGTG-TAGC-3′) and P2 (5′-TCGGGATTGGGCACAGTATG AAAGACCTT-3′) as described previously (Ruf et al. 2001; Fig. 1a). The primers were designed based on the tobacco (*N. tabacum*) chloroplast genome sequence information (accession No. Z000444). The selection cassette, which contains the chimeric *aadA* gene driven by *psb*A promoter and a synthetic *rbc*L-derived Shine-Dalgarno sequence, and terminated by the *psb*A 3′ untranslated region (3′-UTR), was cloned into *Sac*I/*Xba*I-digested pBluescript II KS (Stratagene, LaJolla, CA) to yield the plasmid pKS-AADA. The second expression cassette, which contains the *BvCMO* gene driven by the ribosomal RNA operon promoter and a synthetic T7 gene G10 leader (Ye et al. 2001), and terminated by the *rps*16 3′ untranslated region (3′-UTR), was subsequently cloned into *Xba*I/*Kpn*I-digested plasmid pKS-AADA. The tobacco-specific chloroplast transformation vector pDC-*aadA/BvCMO* (Fig. 1b) was constructed by inserting a blunt-ended *Sac*I-*Kpn*I fragment representing the *aadA-BvCMO* expression cassette into the unique *Spe*I site of the tobacco chloroplast DNA flanking sequence (between *trn*G and *trnf*M) after treated with T4 DNA polymerase. All general bacterial and DNA manipulations

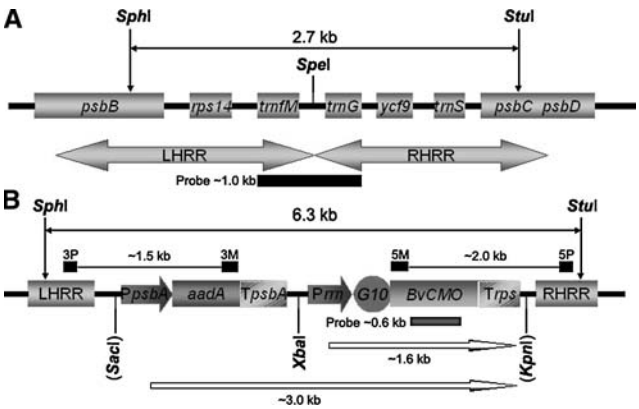


**Fig. 1** Physical map of the targeting region in the plastid genome and structure of chloroplast transformation vector. **a** Map of the plastid-targeting region from which the chloroplast transformation vector was derived. The transgene is targeted to the intergenic region between *trnf*M and *trn*G. **b** Map of the tobacco chloroplast transformation vector pDC-*aadA/BvCMO*. PCR primer landing sites and the probes used for Southern and Northern blotting analysis are shown

were performed as per standard molecular biology protocols (Sambrook and Russell 2001).

### Transformation and regeneration protocol for tobacco

Tobacco axenic seedlings (*N. tabacum* L. cv Wisconsin 38) were raised on MS medium (Murashige and Skoog 1962) containing 3% (W/V) sucrose. Young leaves from 4-week-old tobacco plants were bombarded with DNA-coated 0.6-μm gold particles using a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). The bombarded leaf samples were cut into 5 × 5 mm squares and transplanted to RMOP medium (Svab et al. 1990) containing 500 mg/l spectinomycin. One month later, spectinomycin-resistant shoots appeared. The initial shoots were further screened by double-resistance tests on medium supplemented with 500 mg/1 spectinomycin and streptomycin (Bock 2001). Several independent transplastomic lines were obtained and subjected to two to four additional rounds of regeneration on RMOP/spectinomycin medium to obtain homoplasmic shoots. Then homoplastic shoots were transferred onto rooting medium containing MS salts, 0.1 mg/l NAA, 500 mg/1 spectinomycin and streptomycin. Rooted shoots were finally transferred to potted soil and grown in growth chambers on a 16 h light/8 h dark photoperiod. Homoplasmic seeds were harvested for further examinations.

### DNA extraction and PCR analyses

PCR was carried out to confirm the spectinomycin-resistant chloroplast transformants. Genomic DNA was extracted from leaves of wild type and spectinomycin-resistant plants using CTAB extraction buffer as described previously (Dellaporta et al. 1983). PCR reactions were performed according to standard protocols (5 min at 94°C, then 30 s at 94°C, 45 s at 52°C, 1 min at 72°C; 30 cycles, with a final extension of 7 min at 72°C) using primer pairs specific for the passenger genes (*aadA* and *BvCMO*) and flanking sequence: 3P: 5′-TGGTGGGTATCCTTAATTCTCTCA-3′; 3 M: 5′-ACATTATTTGCCGACTACCTTGG-3′; 5P: 5′-CGTCGATCCT CAAAGAAAGAAAT-3′; 5 M: 5′-ATGGCAGCAAGTGCTACAACC-3′. Primers 5P and 3P were landed on the flanking sequence, 500 bp upstream and downstream of the integration site, respectively. Primer 5 M was landed on *BvCMO* as forward primer. Primer 3 M was landed on the *aadA* gene as a reverse primer.

### Southern and Northern blotting analysis

For Southern blotting, genomic DNA (6 μg) from wild type and PCR-positive plants was digested with restriction enzyme *Stu*I and *Sph*I, separated on a 0.8% agarose gel, and then transferred onto Hybond-N nylon membranes (Amersham, Buckinghamshire, UK). DNA probe (1.0 kb) was obtained from a PCR product with primers 5P and 3P landing on the flanking sequence (Fig. 1a) using Random Primer DNA Labeling Kit (TaKaRa, Shanghai, China). Hybridization was performed at 55°C for 24 h in church buffer at high stringency conditions as per standard molecular biology protocols (Sambrook and Russell 2001).

For Northern blotting analysis, total RNA was extracted from wild type and transformed plants using Trizol Reagent (MRC, USA) following the manufacturer's instruction. Ten micrograms of total RNAs were denatured and separated by electrophoresis as described previously (Hamada et al. 1996). RNAs on the gel were transferred to Hybond-N membranes (Amersham, Buckinghamshire, UK), and hybridized with radiolabeled *BvCMO* specific probe (0.6 kb) generated from a 0.6 kb *Eco*RI restriction fragment (Fig. 1b). Pre-hybridization, hybridization and post-hybridization were performed as per standard molecular biology protocols (Sambrook and Russell 2001).

### GlyBet extraction and quantification

The method developed by Rhodes et al. (1989) was followed with minor revision. Leaf, root and seed samples (1 g) were ground in grinding buffer (1× grinding buffer: 12 ml methanol, 5 ml chloroform, 1 ml water). The aqueous phase was fractioned by ion-exchange chromatograph (AG1,OH−). The GlyBet fraction was dried under a stream of $N_2$ at 60°C, and dissolved in 100 μl of distilled water. The GlyBet content was analyzed by HPLC as described previously (Bessieres et al. 1999).

### Salt stress tolerance tests

Wild type and homogenous $T_2$ transplastomic seeds were surface sterilized with a solution of 10% commercial bleach (0.525% sodium hypochlorite) for 10 min, and washed three times with sterile water. For germination assays, seeds were plated on MS medium supplemented with different concentrations of NaCl (0, 100 and 150 mM). For seedling growth measurements, fresh weight of 13-day-old wild type and transplastomic seedlings cultured on solid MS medium supplemented with different concentrations of NaCl (0,100 and 150 mM) were measured. Three replicates were performed for each experiment.

For salt stress treatments and growth measurements of plants grown in soil, seedlings of both wild type and transplastomic plants were transferred to pots and grown at 25 ± 2°C with cool white fluorescent light (~125 μmol $m^{-2}$ $s^{-1}$), 70% humidity, under long-day conditions (16 h light/8 h dark). Six-week-old plants were

watered biweekly with 1/2 Hoagland solution with or without 150 mM NaCl for 36 days. Mature plants treated in the same way were also used for physiological tests and photosynthesis analyses.

### Biomass and chlorophyll determination

The above wild type and transplastomic plants were harvested after the 36-day salt treatment. Fresh and dry weights were measured. For dry weight measurements, plants were dried at 120°C for 4 h and 95°C for 48 h. To analyze the chlorophyll contents, tobacco leaf samples (200 mg) were homogenized with 80% acetone (v/v). The homogenate was filtered through filter paper. The total chlorophyll content was determined spectrophotometrically as described by Arnon (1949).

### Measurements of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a FMS-2 pulse modulated fluorometer (Hansatech, UK). The minimal fluorescence (Fo) was determined by a weak modulated light which was low enough not to induce any significant variable fluorescence. A 0.8 s saturating light of 8,000 μmol $m^{-2}$ $s^{-1}$ was used on dark-adapted leaves to determine the maximal fluorescence (Fm). Then the leaf was illuminated by an actinic light of 500 μmol $m^{-2}$ $s^{-1}$. When the leaf reached steady-state photosynthesis, the steady-state fluorescence (Fs) was recorded and a second 0.8 s saturating light of 8,000 μmol $m^{-2}$ $s^{-1}$ was given to determine the maximal fluorescence (Fm′) in the light-adapted state. The actinic light was then turned off; the minimal fluorescence in the light-adapted state (Fo′) was determined by the illumination of the 3 s far red light. The maximal efficiency of PSII photochemistry was determined as the ratio of variable to maximal chlorophyll fluorescence (Fv/Fm), and the actual PSII efficiency (ΦPSII) was determined as $\Phi PSII = 1 - Fs/Fm'$ (Krause and Weis 1991). The measurements were performed on the attached leaves of tobacco plants.

### Measurements of gas exchange

An infrared gas analyzer (CIRAS-2; PP Systems, Hitchin, UK) was used to estimate net photosynthetic rate and stomatal conductance on the attached leaf of each seedling using an open system. These measurements were made at a $CO_2$ concentration of 360 μl l/l. Leaves were illuminated by the CIRAS-2 light source with a PFD of 500 μmol $m^{-2}$ $s^{-1}$. The temperature inside the leaf chamber was maintained at 25°C.

The light response curves of photosynthesis were determined with a range of light intensities at $CO_2$ concentration of 360 μl l/l by the CIRAS-2 light source. The initial slope of the curve was determined as the apparent quantum yield (AQY). The carboxylation efficiency (CE) was determined from the initial slope of the A/Ci curve.

### Whole plant drought tolerance assay

Wild type and transplastomic plant seeds ($T_2$) were surface sterilized as described above. For germination assays, seeds were plated on MS medium supplemented with 300 mM mannitol. For water deficit experiments, plants were grown in the greenhouse under a 16 h light/8 h dark photoperiod. The temperatures were set to 28°C at daytime and 21°C at night. Two transformed lines (M21-6, M11-4) along with wild type plants were chosen and transplanted into nursery plots containing the same type of soil, where a constant supply of a diluted MS solution was added every day. Care was taken to avoid soil loss from pots during the stress regimes. Plastic netting was placed within the pots before filling with potting mix, and the soil level in all pots was kept ~1 cm below the rim. In a typical soil water deficit stress experiment, plants were grown for a period of 4 weeks in soil and watered regularly to field capacity; then the soil was allowed to dry by omitting watering for 15 days. The relative humidity was maintained at ~60%. Photos were taken before stress and after 15, 17, 19 days of stress, and 1, 2 days after re-watering. All experiments were repeated at least three times, and results from one representative experiment are shown.

For water loss experiments, the third leaves from the top of wild type and transgenic lines were detached. Water loss was measured and represented as the percentage of initial fresh weight. The detached leaves were kept under continuous fluorescent cool white light at 24°C. The relative humidity was maintained at 45%.

## Results

### Construction of tobacco plastid transformation vectors

The chloroplast transformation vector pDC-*aad*A/*BvCMO* was constructed to target the expression cassettes to the *rps*14/*trn*fM-*trn*G/*ycf*9 region of the chloroplast genome for integration via homologous recombination. The two expression cassettes which harbor *aad*A and *BvCMO* genes (transgenes) were flanked by two tobacco plastid DNA sequences: left homologous recombination region (LHRR) containing *trn*fM, *rps*14 and 3′ end of *psb*B gene, and right homologous recombination region (RHRR) containing *trn*G, *ycf*9, *trn*S and 5′ part of *psb*C gene (Fig. 1a, b).

## Transformation of tobacco plastid genomes and plant regeneration

For tobacco chloroplast transformation, green leaves of 4-week-old tobacco plants were bombarded with tobacco chloroplast transformation vector pDC-*aad*A/*BvCMO* as described previously (Svab and Maliga 1993). Bombarded leaves were selected on solid RMOP medium supplemented with 500 mg/l spectinomycin. Five transplastomic lines were obtained and subsequently purified to homoplasmy by passing them through additional regeneration cycles on RMOP medium supplemented with 500 mg/l spectinomycin and streptomycin. Transplastomic tobacco was resistant to high concentration of antibiotics in the media (500 mg/l spectinomycin and streptomycin), whereas the wild type tobacco was not viable following the selection (data not shown). Two of the five independently generated homoplasmic lines (M11-4 and M21-6) were characterized in details.

## Confirmation of transgene integration into tobacco plastid genomes

Successful chloroplast transformation was verified by tests for double resistance on medium supplemented with spectinomycin and streptomycin (Svab and Maliga 1993; Bock 2001; Wurbs et al. 2007) and further confirmed by PCR analyses using internal primers 3P (which lands on LHRR, 500 bp upstream of the integration site) and 3 M (which lands on the *aad*A gene) (Fig. 1b). A 1.5 kb PCR product was observed (Fig. 2a). This eliminates mutants that may arise due to a mutation in the chloroplast 16S rRNA gene. In order to confirm the site-specific integration of *BvCMO* gene, primers landed on RHRR (5P), 500 bp downstream of the integration site, and on the *BvCMO* gene (5 M) were also designed to generate a 2.0 kb PCR product (Fig. 2b).

Homoplasmy of transgenic plants was confirmed by Southern blotting analysis. Total genomic DNA isolated from untransformed (wild type) and transformed tobacco plants were digested and hybridized with radiolabeled probe including the *rps*14/*trn*G and *trnf*M/*ycf*9 flanking sequences (Fig. 1a). As shown in Fig. 2c, a 2.7 kb band that corresponds in size to the restriction fragment from the wild type genome was detected in wild type, and a 6.3 kb band that corresponds in size to the transgenes was detected in the transgenic lines (Fig. 2c). No hybridization band that corresponds in size (2.7 kb) to the restriction fragment from the wild type genome was detected in homoplasmic transgenic lines (Fig. 2c).

To test the expression of the introduced *BvCMO* gene, we performed Northern blotting experiments with *BvCMO* gene-specific probe (Fig. 1b). As shown in Fig. 2d, no hybridization signal was detected in wild type, whereas two strong bands were yielded in transplastomic plants (Fig. 2d). The 1.6 kb band corresponds to the expected size of the full length transcript, and the 3.0 kb band corresponds to the size of the co-transcript with the upstream *aadA* marker gene (Fig. 1b). This may due to the co-transcription originated from read-through transcription, which is commonly observed in transplastomic lines that carry selectable marker genes and genes of interest in the same orientation (Zoubenko et al. 1994; Wurbs et al. 2007).

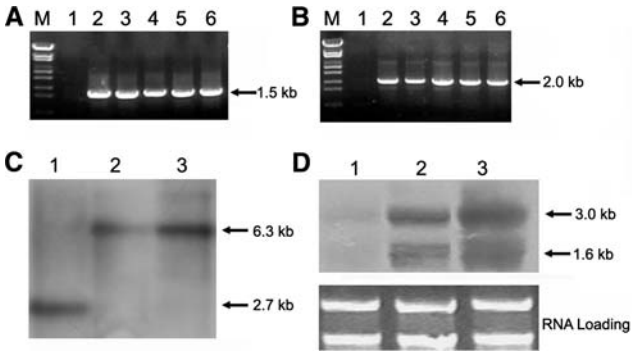


**Fig. 2** Confirmation of transgene integration into the tobacco plastid genome by PCR, Southern and Northern blotting analyses. **a** PCR product (1.5 kb) from internal primers 3P (land on the flanking sequence) and 3 M (land on the *aad*A gene). **b** PCR product (2.0 kb) from an external primer 5P (land on the native chloroplast genome) and an internal primer 5 M (land on the *BvCMO* gene). M, λ-DNA/*Eco*T14 markers; Lane 1, wild type; lanes 2 to 6, different transplastomic lines. All primer landing sites are shown in Fig. 1. **c** Southern blotting analysis of wild type (WT) and PCR positive tobacco plants transformed with the vector pDC-*aad*A/*BvCMO*. Tobacco genomic DNA (6 μg/lane) digested with *Sph*I and *Stu*I was hybridized with the 1.0-kb radioactive-labeled $^{32}$P DNA probe containing the flanking sequence (see Fig. 1 for details). Lane 1, WT; lanes 2 and 3, homoplasmic transgenic plants derived after repetitive subculture under spectinomycin selection. **d** Analysis of RNA accumulation in tobacco chloroplast transformants. Total cellular RNA was hybridized to radioactive-labeled $P^{32}$ DNA probe corresponding to the coding region of the introduced *BvCMO* gene (see Fig. 1 for details). The presence of a second strongly hybridizing band is probably due to read-through transcription generating a dicistronic transcript with the *aadA* as first cistron (see text). The ethidium bromide-stained rRNA bands in the agarose gel are shown as loading control. Lane 1, WT; lanes 2 and 3, homoplasmic transgenic plants derived after repetitive subculture under spectinomycin selection

## GlyBet accumulation in transplastomic tobacco plants

To test whether transplastomic plants producing BvCMO also synthesized GlyBet, the GlyBet concentration in leaves, roots and seeds were measured, respectively. The level of GlyBet found in leaves ranged from 200 to 250 nmol/g FW based on measurements from six seedlings from the BvCMO-producing transplastomic line M11-4, whereas no accumulation of GlyBet could be found in non-transformed wild type plants (<1 nmol/g FW). This is

much higher than the reported level (50–60 nmol/g FW) of GlyBet found in leaves of $CMO^+$ transgenic tobacco plants expressing spinach *CMO* gene (Nuccio et al. 2000). Compared to non-transformed wild type tobacco, transplastomic line M11-4 plants also accumulated high levels of GlyBet in roots and seed (Fig. 3a).

## Resistance of transplastomic tobacco plants to toxic levels of choline

In order to assess if BvCMO produced in transplastomic tobacco retained enzyme activity, we tested the ability of plants to survive toxic concentrations of choline (Lilius et al. 1996). Seeds of wild type and two transplastomic lines (M11-4, M21-6) were germinated on 1/2 MS medium containing different concentrations of choline. The growth yields of wild type and transplastomic plants were similar when cultured on normal 1/2 MS medium (Fig. 3b). However, growth of wild type plants was significantly impaired on 1/2 MS medium supplemented with 30 mM choline, whereas growth of transplastomic plants was less strongly affected. After 28 days on high choline 1/2 MS medium (30 mM), the growth yield of wild type tobacco, measured by fresh weight, reduced to 40% of control plants grown on normal 1/2 MS medium, but the growth yield of transplastomic tobacco producing BvCMO reduced to 55% of control plants grown on normal 1/2 MS medium (Fig. 3b). Furthermore, when cultured axenically for 28 days on 1/2 MS medium supplemented with 5 or 30 mM choline, a five-fold increase in endogenous GlyBet content was seen in transplastomic plants, but no significant increase was observed in wild type plants (Fig. 3c).

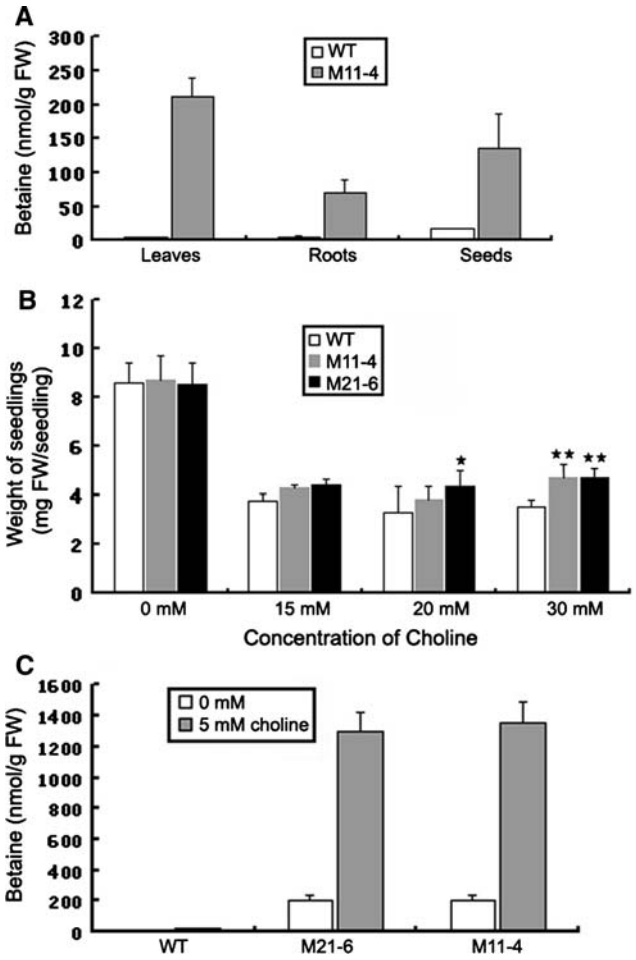


**Fig. 3** Quantification of glycine betaine (GlyBet) in tobacco plants and resistance of transplastomic tobacco plants to toxic levels of choline. **a** GlyBet levels in wild type (WT) and transplastomic tobacco (M11-4) leaf, root, and seed. **b** Growth of WT and transplastomic tobacco on choline. **c** The effect of choline supplementation on the level of GlyBet. Plants were grown on MS medium supplemented with various concentration of choline for 28 days and their fresh weights were measured. *Error bars* represent standard deviation based on the growth of 30 plants in three independent experiments. *Significant differences at $p < 0.05$ when compared with WT plants. **Significant differences at $p < 0.01$ when compared with WT plants. The GlyBet data are means and SE for three replicate plants of each genotype

## Plastid-expression of *BvCMO* increases salt tolerance of transplastomic tobacco plants

We first studied the salt tolerance of *BvCMO* transplastomic plants during germination and early seedling development. Seeds ($T_2$) of wild type and a homoplasmic line (M11-4) were germinated on MS medium containing different concentrations of NaCl. Germination rates of wild type and transplastomic seeds were similar when cultured on normal MS medium (Fig. 4a, b). However, germination of wild type seeds was significantly impaired on MS medium supplemented with 100 or 150 mM NaCl, whereas germination of transplastomic seeds was less strongly affected. After 6 days on high salt MS medium (100 mM NaCl), only ~30% of the wild type seeds, but more than 80% of the seeds obtained from *BvCMO* transplastomic plants, germinated (Fig. 4b). When sown on MS medium supplemented with 150 mM NaCl, only ~20% of the wild type seeds, but ~80% of transplastomic seeds germinated after 13 days (Fig. 4c). To further test the effect of salt on growth, seeds of wild type and transplastomic plants were sown on MS medium supplemented with NaCl at different concentrations. After 13 days, strong growth retardation was observed in wild type seedlings. Inhibition of growth was less evident in transplastomic lines (Fig. 4a, c).

We also performed salt tolerance experiments with wild type and two transplastomic lines grown in the greenhouse in soil. The plants were watered bi-weekly with 1/2 concentrated Hoagland salt solution supplemented with or without 150 mM NaCl. In the absence of salt stress, no overt morphological difference was observed between wild type and transplastomic plants. However, growth of wild

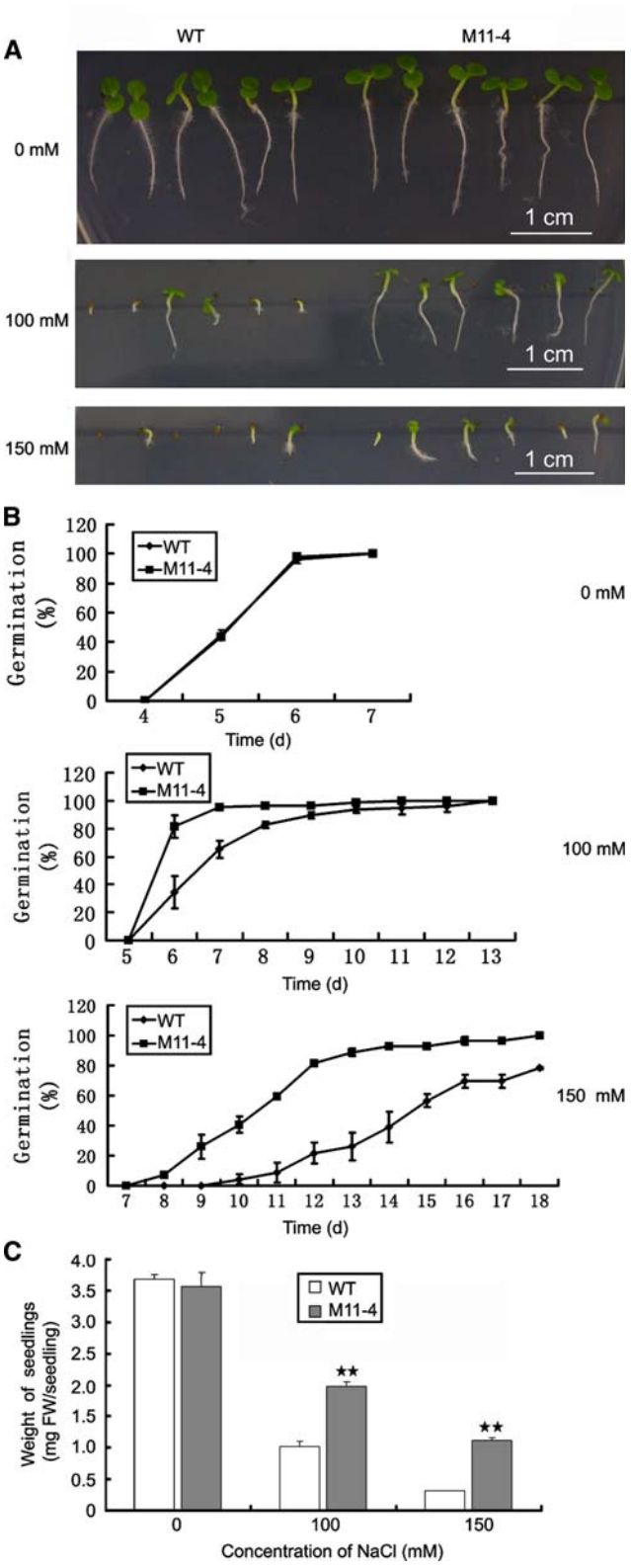


**Fig. 4** Effect of salt stress on germination and early growth of wild type (WT) and transplastomic tobacco plants (M11-4). **a**, **b** Germination in the presence of different concentrations of NaCl. Seeds were germinated at 26°C on MS medium supplemented with 0, 100 or 150 mM NaCl. The photos were taken 13 days after the sowing of seeds. Results are presented as means and standard errors from three experiments. **c** Effect of salt stress on biomass accumulation (fresh weight of plants). *Error bars* represent standard deviation from eight plants from each line. **Significant differences at $p < 0.01$ when compared with WT plants

type tobacco was severely inhibited in the presence of 150 mM NaCl (Fig. 5a), leading to a depression of leaf fresh weight by about 25% in comparison to *BvCMO* transplastomic plants after a 36-day stress treatment (Fig. 5b). Root fresh weights of wild type and transplastomic plants were similar in the presence of 150 mM NaCl (Fig. 5c). This may be attributed to the lower GlyBet content in roots of transplastomic plants (Fig. 3a). Moreover, in the presence of salt stress, chlorophyll loss was significantly delayed in *BvCMO* expressing plants compared to that of the wild type (Fig. 5d). All these results indicated that plastid-expression of *BvCMO* alleviated the negative effects imposed by salt stress on plant growth.

## Effects of salt stress on photosynthetic gas exchange and PSII photochemistry

Under salt stress condition, leaf photosynthetic rate (Fig. 6a) and stomatal conductance (Fig. 6b) in both wild type and transplastomic plants decreased significantly. However this decrease was much greater in wild type plants than in transplastomic plants. Similar results were also observed with the apparent quantum yield (AQY) (Fig. 6c) and the carboxylation efficiency (CE) of photosynthesis (Fig. 6d). The changes in the maximal efficiency of PSII photochemistry (Fv/Fm) in non-salt-stressed and salt-stressed plants showed that there were no significant changes in Fv/Fm in salt-stressed plants. Also, no significant changes in Fv/Fm were observed between wild type and transplastomic plants (Fig. 6e). Salt stress also caused a decrease in the actual efficiency of PSII (ΦPSII), and such a decrease was much greater in wild type plants than in transplastomic plants (Fig. 6f). These results indicated that photosynthesis was improved in transplastomic plants under salt stress and such an improvement was associated with an improvement in stomatal conductance and the actual PSII efficiency.

## Transplastomic plants showed increased drought tolerance

In order to determine whether plastid expression of *BvCMO* affects drought tolerance in plants, $T_2$ seeds of wild type and homoplasmic line M11-4 were germinated on MS medium containing 300 mM mannitol. Germination rates of wild type and transplastomic seeds were similar when cultured on normal MS medium (data not shown). However, germination of wild type seeds was significantly

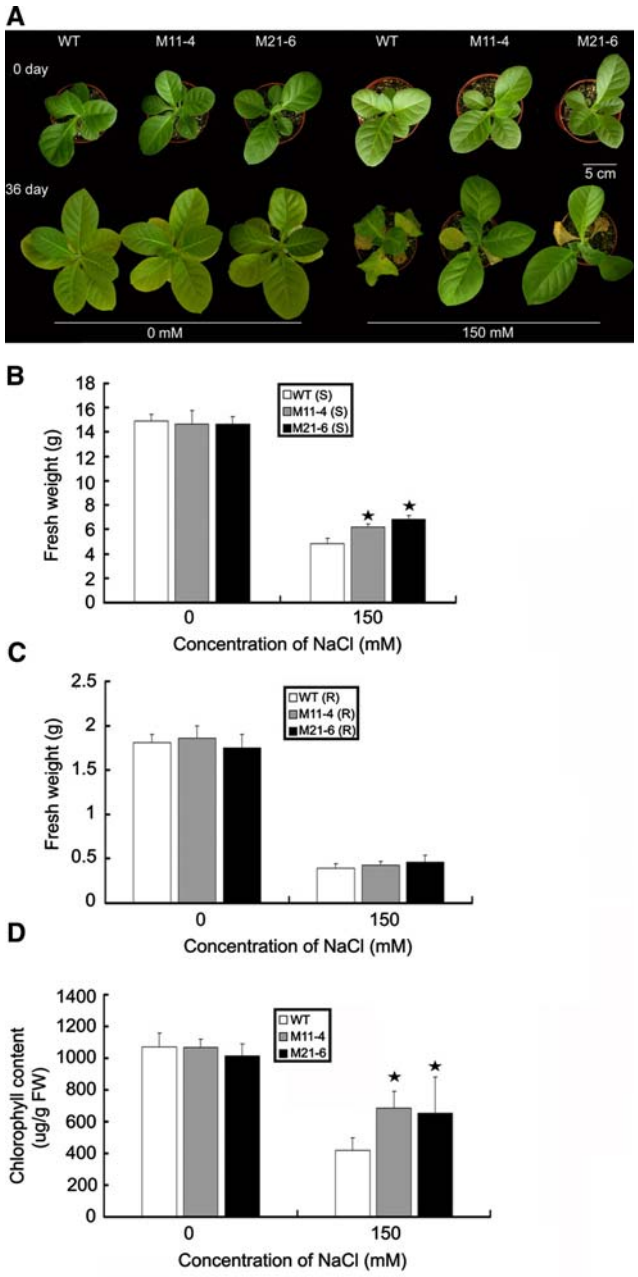


**Fig. 5** Enhanced salt stress tolerance in greenhouse grown transplastomic tobacco plants. **a** Effect of salt on growth of wild type (WT) and transplastomic tobacco plants. Seeds were germinated in vitro, and seedlings were transferred to greenhouse and grown in soil pots. Plants were subsequently salinized stepwise to a final level of 150 mM NaCl and subjected to salt stress by watering twice a week with 150 mM NaCl. The photos were taken 36 days after the initiation of the stress treatment. **b** ,**c** Shoot and root fresh weight of WT and transplastomic (M11-4, M21-6) tobacco plants after treatment in (**a**). **d** Chlorophyll content of WT and transplastomic (M11-4, M21-6) tobacco plants in (**a**). *Error bars* represent standard deviation from 5 plants from each line. *Significant differences at $p < 0.05$ when compared with WT plants

impaired on MS medium supplemented with 300 mM mannitol, whereas germination of transplastomic seeds was less strongly affected (Fig. 7a). After 24 days on high mannitol MS medium (300 mM), only ~20% of the wild type seeds, but more than 70% of the seeds obtained from *BvCMO* transplastomic plants, germinated (Fig. 7a).

We also tested the effect of water shortage on plant growth. After a 15-day drought period, wild type plants completely wilted, while transplastomic plants (M11-4 and M21-6) were not affected (Fig. 7b). At the end of the drought tolerance test, the plants were re-watered (Fig. 7b). Growth damage was less severe in transplastomic plants compare to wild type plants. Then we measured water loss by using detached leaves from wild type and homoplasmic line M11-4 plants. Water loss in M11-4 plants was less than that in the WT (Fig. 7c). These results indicate that transplastomic expression of *BvCMO* is responsible for drought tolerance observed in homoplasmic plants.

## Discussion

To date, most previous work on engineering of the GlyBet biosynthesis pathway in plants was conducted by expressing transgenes from the nuclear genome. In spinach, both CMO and BADH are targeted to chloroplasts (Weretilnyk and Hanson 1990; Rathinasabapathi et al. 1997). The cDNA for spinach CMO used in the previous study encoded the precursor to mature CMO, and included a transit peptide, namely, chloroplast stromal targeting peptide (Rathinasabapathi et al. 1997). Although CMO was targeted to chloroplast in these transgenic plants (Nuccio et al. 1998), the expression level and enzyme activity of CMO were not higher than those transit peptide was replaced with *Arabidopsis* RuBisCo small subunit (Nuccio et al. 2000). These researches indicate that native transit peptide is not very effective for transgenes. Thus, a possible way to overcome this obstacle is to express transgenes from the chloroplast genome.

Tobacco plastid engineering has been conducted to confer herbicide resistance (Daniell et al. 1998), insect resistance (DeCosa et al. 2001), disease resistance (DeGray et al. 2001), drought tolerance (Lee et al. 2003), phytoremediation of organomercurial compounds (Ruiz et al. 2003), and salt tolerance (Kumar et al. 2004). The genetic engineering of carrot chloroplast genome expressing *badh* gene conferred high salt tolerance (Kumar et al. 2004). The enzyme encoded by *badh* catalyzes the second step in the GlyBet biosynthesis pathway, converting betaine aldehyde into GlyBet. In this work, we have studied the other GlyBet biosynthesis gene encoding the enzyme CMO, which catalyzes the first step of the pathway (oxidation of choline in to betaine aldehyde), as plastid transgene in tobacco. The transplastomic line was compared, in their ability to produce GlyBet and in their stress resistance, to the wild type plants.

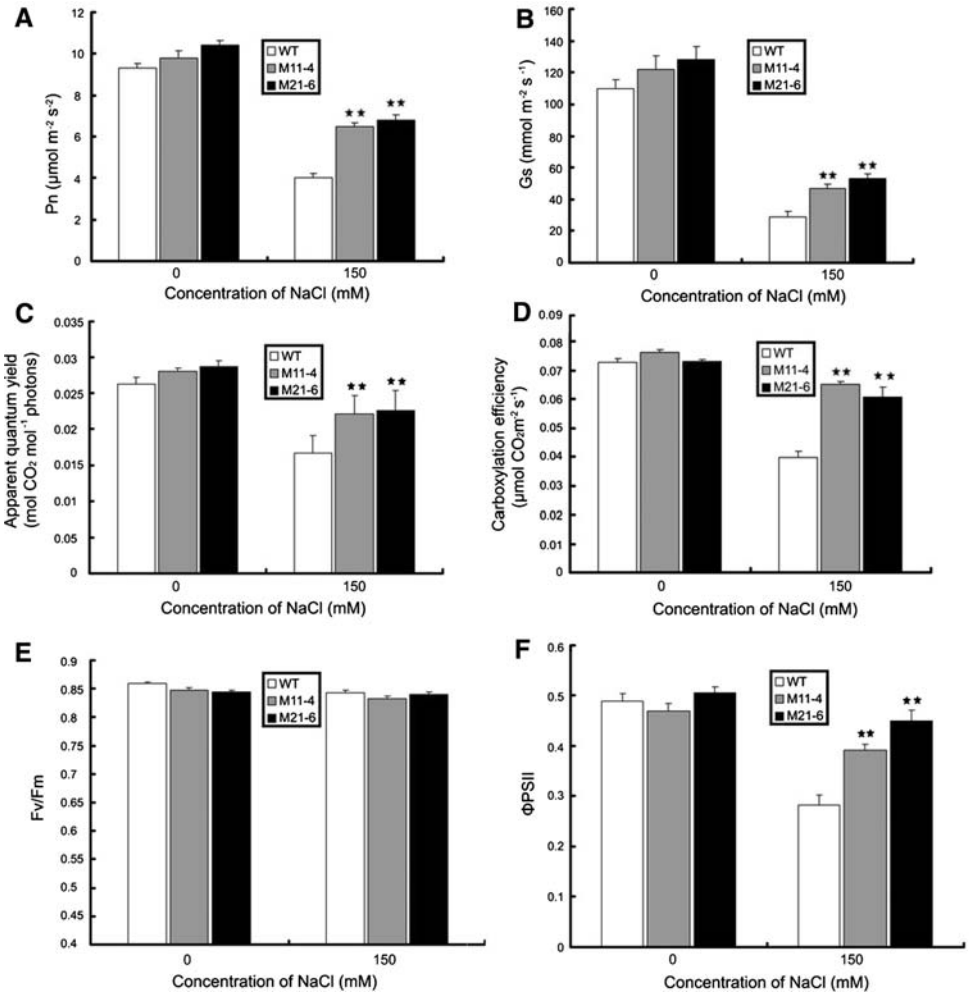


**Fig. 6** Photosynthetic performance of wild type (WT) and transplastomic (M11-4, M21-6) tobacco plants after treatment with 150 mM NaCl for 4 weeks; *Error bars* represent standard deviation from five plants from each line. **a** Net photosynthetic rate. **b** Stomatal conductance. **c** Apparent quantum yield. **d** Carboxylation efficiency (CE) of photosynthesis. **e** Maximal efficiency of PSII photochemistry (Fv/Fm). **f** Actual efficacy of PSII (ΦPSII) of WT and transplastomic tobacco under non-salt-stressed and salt-stressed condition. **Significant differences at $p < 0.01$ when compared with WT plants

Previously, Nuccio et al. generated transgenic tobacco plants constitutively expressing a spinach CMO gene. However, the GlyBet content was extremely low (max. 0.06 μmol/g FW) in $CMO^{+}$ transgenic tobacco and the effects of stress on transgenic plant growth was not reported (Nuccio et al. 1998, 2000). In our study, transplastomic plants expressing *BvCMO* produced four-fold more GlyBet (max. 0.25 μmol/g FW) (Fig. 3a), suggesting that plastid-expression of *BvCMO* in transplastomic plants generates an active enzyme capable of oxidizing choline. However, the GlyBet level in CMO transplastomic tobacco is still far lower than that in species naturally accumulating GlyBet, such as spinach and beet or those plants transformed with *BADH/CodA* gene. Furthermore, choline import into chloroplast and the native BADH activity are also essential for GlyBet synthesis (Nuccio et al. 2000). Transplastomic plants are more tolerant to toxic level of choline and accumulate higher level of GlyBet than wild type (Fig. 3b), indicating that the majority of supplemented choline was converted into betaine aldehyde and subsequently into GlyBet in the transplastomic plants. The conversion from betaine aldehyde to GlyBet could be catalyzed by the native BADH in tobacco (Holmstrom et al. 1994; Rathinasabapathi et al. 1994). When exogenous choline was added, GlyBet content increased by five-fold in transformed plants but no increment in wild type plants (Fig. 3c), indicating that a limitation on GlyBet synthesis was imposed by inadequate endogenous choline. The large increase in choline (substrate) supply may lead to the flux from choline via betaine aldehyde to GlyBet.

It has been well documented that low GlyBet accumulation in some transgenic plants is adequate to confer stress tolerance. For example, a low level GlyBet accumulation ($35 \pm 15$ nmol/g FW) improved salt tolerance in transgenic tobacco expressing *betA*, a gene encodes CDH from *E. coli* (Holmstrom et al. 2000). And, an accumulation of 290–430 nmol/g DW GlyBet conferred enhanced tolerance to salt and temperature stress on rice expressing a gene for CMO from spinach (Shirasawa et al. 2006). Similarly, 100 nmol/g FW GlyBet content was apparently sufficient to confer high levels of stress tolerance in tomato (Park et al. 2004). Although the GlyBet content was much lower

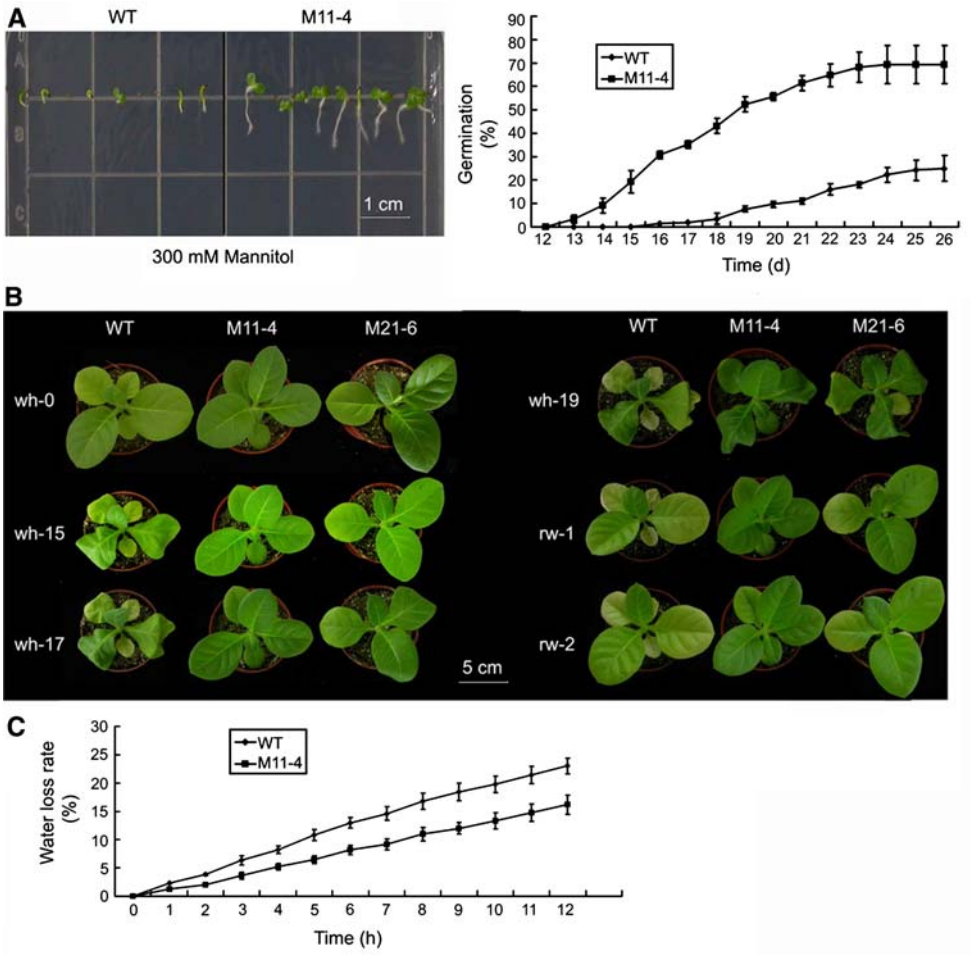


**Fig. 7** Enhanced drought tolerance of transplastomic tobacco plants. **a** Effect of mannitol on germination and early growth of wild type (WT) and transplastomic tobacco plants. Seeds were germinated at 26°C on MS medium supplemented with 300 mM Mannitol. The photos were taken 26 days after the sowing of seeds. Results are presented as means and standard errors from three experiments. **b** Phenotypes of WT and transplastomic tobacco plants after they were withheld (wh) from water. Seeds were germinated in vitro, and seedlings were transferred to greenhouse and grown in soil pots. Photos were taken 15 (wh-15), 17 (wh-17) and 19 (wh-19) days after the initiation of the water stress treatment, and 1 (rw-1) and 2 (tw-2) days after the re-watering treatment. **c** Water loss test of wild WT and transplastomic tobacco plants. Water loss is least and greatest from detached leaves of WT and transplastomic plants, respectively. Water loss is expressed as the percentage of initial fresh weight. *Values* indicate a mean of three measurements with standard deviations, each with a sample size of five to eight leaves. One of the triplicate trials is shown. Regression analysis confirmed that the WT curve differs significantly from the transplastomic M11-4 response. **Significant differences at $p < 0.01$ when compared with WT plants

than other reported transgenic plants that express *BADH* or *CodA* (Sakamoto and Murata 2000), our data demonstrate that the accumulation of GlyBet in transplastomic plants could lead to enhanced salt (150 mM) and drought tolerance.

The enhanced salt and drought tolerance in BvCMO transplastomic plants may be due to the compartmentation of plastid-expressed BvCMO. The sub-cellular localization of BvCMO expressed from chloroplast genome may be more suitable for assembling foreign, soluble Rieske-type [2Fe-2S] proteins which are required for CMO catalytic activity. Compared with nuclear transformation, plastid-expression of CMO omits the trans-membrane procedure directed by a transit peptide of N-terminal of CMO protein. Furthermore, GlyBet accumulation in chloroplasts may be more effective than in other organelle for protecting plants against abiotic stress (Park et al. 2007). It helps protect the stabilization of chloroplast proteins, membrane and photosynthesis systems under abiotic stress condition.

The finding that transplastomic plants expressing *BvCMO* gene were more salt and drought resistance than wild type plants may be associated with improved photosynthetic $CO_2$ assimilation. We observed that salt stress induced a significant decrease in leaf photosynthetic rate and stomatal conductance. However, the decrease was much less in transplastomic plants (Fig. 6a, b). The improved photosynthetic $CO_2$ assimilation in transplastomic plants under salt stress condition may be associated

with improved stomatal conductance (Brugnoli and Bjorkman 1992).

Salinity and water deficit are two major factors limiting plant growth and development. The biosynthesis of osmoprotectant GlyBet is one of the metabolic adaptations to salt and drought stress in higher plants. It has been well known that GlyBet accumulates in response to osmotic stress and protects plants from osmotic stress (Robinson and Jones 1986), and GlyBet-synthesized plants have improved drought tolerance (Shen et al. 2002; Quan et al. 2004). Our results, together with other independent studies (Holmstrom et al. 2000; Nuccio et al. 2000; Shirasawa et al. 2006; Yang et al. 2008), imply that chloroplast expression of CMO could be a promising approach to enhance crop tolerance to different abiotic stresses.

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