Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Transgenic *Brassica chinensis* plants expressing a bacterial *codA* gene exhibit enhanced tolerance to extreme temperature and high salinity*

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Received Apr. 15, 2010; Revision accepted May 18, 2010; Crosschecked Sept. 28, 2010

Abstract: Transgenic *Brassica compestris* L. spp. *chinensis* plants expressing a choline oxidase (*codA*) gene from *Arthrobacter globiformis* were obtained through *Agrobacterium tumefaciens*-mediated transformation. In the transgenic plants, *codA* gene expression and its product transportation to chloroplasts were detected by the enzyme-linked immunosorbent assay (ELISA) examination, immunogold localization, and ^{1}H-nuclear magnetic resonance (^{1}H-NMR). Stress tolerance was evaluated in the T_3 plants under extreme temperature and salinity conditions. The plants of transgenic line 1 (L1) showed significantly higher net photosynthetic rate (P_n) and P_n recovery rate under high (45 °C, 4 h) and low temperature (1 °C, 48 h) treatments, and higher photosynthetic rate under high salinity conditions (100, 200, and 300 mmol/L NaCl, respectively) than the wild-type plants. The enhanced tolerance to high temperature and high salinity stresses in transgenic plants is associated with the accumulation of betaine, which is not found in the wild-type plants. Our results indicate that the introduction of *codA* gene from *Arthrobacter globiformis* into *Brassica compestris* L. spp. *chinensis* could be a potential strategy for improving the plant tolerance to multiple stresses.

Key words: *Brassica compestris* L. spp. *chinensis*, *codA*, Stress, Glycine betaine, Net photosynthetic rate (P_n)
doi:10.1631/jzus.B1000137 **Document code:** A **CLC number:** Q943.2

1 Introduction

Glycine betaine (betaine) is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals, and microorganisms. It can stabilize the structure of proteins, and maintain the integrity of cell membrane, thus enhancing the tolerance of organisms to high salt, high temperature, and cold injuries (Gorham, 1995). Many plant species, including rice, tomato, tobacco, and Arabidopsis, are not able to synthesize betaine, but may be engineered to accumulate betaine through a transgenic approach to improve their tolerance to various extreme conditions (Sakamoto and Murata, 2001; Prasad and Saradhi, 2004).

The first successful transformation of a plant for the synthesis of betaine in *Arabidopsis thaliana* was reported by Hayashi *et al*. (1997). Within chloroplasts, choline oxidase encoded by the *codA* gene produces betaine, which provides leaf protection from high temperature and other stresses in *Arabidopsis thaliana* (Alia *et al*., 1998a; Sakamoto and Murata, 2000). Higher betaine accumulation has been reported to endow higher salt tolerance in plants (Saneoka *et al*., 1995). For example, transgenic

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* Project supported by the National Science Foundation of China (No. 30571146) and the National Key Basic Research Special Foundation of China (No. G1999011700)

Arabidopsis expressing *codA* exhibits enhanced germination under high salinity conditions (Hayashi *et al.*, 1998). Transgenic tobacco (*Nicotiana tabacum*) plants show improved tolerance to salt and drought stresses (Holmström *et al.*, 2000; Huang *et al.*, 2000; He *et al.*, 2001). Transgenic rice is advantageous in maintaining stable osmotic pressure and promoting roots growth, thus enhancing the plant tolerance to water deficit (Sakamoto *et al.*, 1998; Sawahel, 2003). In the transgenic *codA* plants, the enhanced performance in germination and tolerance to various stress conditions has been associated with the accumulation of betaine (Alia *et al*., 1998a; 1998b; 1999; Prasad and Saradhi, 2004). Moreover, it has been found that the transgenic *codA* plants show a significantly lower trend of electrolyte leakage, as well as hydrogen peroxide and malondialdehyde contents than wild-type plants (Parvanova *et al.*, 2004).

Brassica compestris L. spp. *chinensis* is a vegetable crop widely cultivated in South China. It does not synthesize betaine in vivo, and is sensitive to salt, drought, and high temperature stresses. Via the *Agrobacterium tumefaciens*-mediated transformation procedure, we have successfully transferred the *codA* gene into the genome of *Brassica compestris* L. spp. *chinensis* var. Aikangqing. In this study, we evaluated the transgenic plants for their tolerance to high and low temperatures and high salinity stresses by examining their photosynthetic performance at the growth stage. We report here the enhanced tolerance observed in these transgenic plants.

2 Materials and methods

2.1 Construction of vector plasmids

The binary vector pGAH/codA (a kind gift from Dr. Norio MURATA), harboring an expression cassette of the *codA* gene from *Arthrobacter globiformis*, was used for transformation. The construct contains the 35S promoter of cauliflower mosaic virus, the *codA* gene, and the terminator for nopaline synthase gene (Fig. 1). Its T-DNA region also contains genes encoding a neomycin phosphotransferase (NPTII), which confers kanamycin resistance, and a hygromycin phosphotransferase (HPT), which confers hygromycin resistance. In order to direct the expressed choline oxidase to chloroplasts, where betaine can be accumulated to a greater degree, the construct was modified by adding a complementary DNA (cDNA) sequence encoding the transit peptide of a tobacco Rubisco subunit to the *codA* coding region. The resultant binary vector plasmid was introduced into *A. tumefaciens* strain EHA101 containing the Ti plasmid, which was then used for plant transformation.

2.2 Transformation of *Brassica compestris* L. spp. *chinensis*

After germinating for 5–7 d, the hypocotyls or cotyledons of *Brassica compestris* L. spp. *chinensis* were pre-cultured for 1 d on the differentiation medium. *A. tumefaciens* strain EHA101 harboring the pGAH/codA plasmid was grown at 28 °C for 36 h in a 250-ml flask containing 50 ml of yeast extract broth (YEB) liquid medium supplemented with kanamycin 50 mg/L and hygromycin 50 mg/L until the late exponential phase. The explants pre-cultured for 1 d were co-cultured with the *A. tumefaciens* for 5 min, and transferred onto a differentiation Murashige and Skoog (MS) medium supplemented with 0.5 mg/L indole-3-acetic acid (IAA), 0.025 mg/L naphthaleneacetic acid (NAA), 5 mg/L 6-benzyladenine (6-BA), 300 mg/L carbenicillin, and 15 mg/L kanamycin. After co-incubation with *A. tumefaciens* for 2 d at 25 °C (14-h light period) and incubation without *A. tumefaciens* for 4–5 weeks, the shoots of the explants were excised and transplanted to the root induction medium containing kanamycin. The

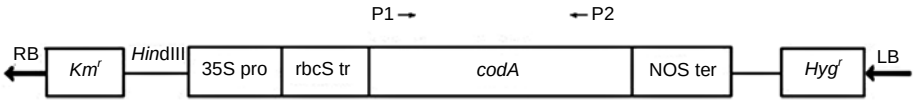

Fig. 1 Structure of T-DNA in binary vector pGAH/codA with the *codA* gene for choline oxidase

RB: right border; Km^r: kanamycin resistance gene; 35S pro: the 35S promoter of cauliflower mosaic virus; rbcS tr: the sequence that encodes transit peptide of subunit of Rubisco from tobacco; *codA*: the gene encoding choline oxidase (COD); NOS ter: NOS terminator; P1 and P2 are upstream and downstream primers, respectively; Hyg^r: hygromycin resistance gene; LB: left border

surviving explants were carefully transferred into plastic pots (10 cm×10 cm×10 cm) loaded with sterilized vermiculite, and grown in a phytotron with fixed temperature (25 °C), 16-h light period (350–400 μmol/(m^2·s)), and a relative humidity of 70%–80%. Transformants were obtained from the selection medium with 60 mg/L kanamycin.

2.3 Development of subsequent T_1, T_2, and T_3 plants

T_1 seeds harvested from the T_0 transgenic plants were first washed in tap water for 3 h, soaked in 1.5% (w/v) Xiaojieling solution for 30 min and in 0.1% (w/v) $HgCl_2$ solution for 2–3 min, and washed repeatedly in sterile water. The treated seeds were sowed in the MS basal (MS_0) medium containing 60 mg/L kanamycin for testing and selecting T_1 plants for antibiotic resistance. The same treatment described above was also applied to T_2 seeds. Those plants without segregation for antibiotic resistance were considered as homozygous. The T_3 seeds harvested from self-crossed T_2 plants were used for subsequent stress experiments.

2.4 Polymerase chain reaction (PCR) examination of the transgenic plants

To detect the presence of the *codA* gene in the transgenic plants, a pair of primers was designed (P1: 5′-AACATCGAGAACCTGAGCGACAGG-3′; P2: 5′-AGCATCAACAGCTTCGGCGTATC-3′) using *codA* as template and used in the PCR. Each 25 μl PCR solution consisted of 10 pmol/L of each primer, 50 μmol/L of deoxyribonucleoside triphosphate, 2.5 μl 10× *Taq* buffer, 1 μl extracted DNA solution, 1 U *Taq* enzyme, 1% (v/v) dimethyl sulphoxide (DMSO), and 5% (v/v) glycerine. Assays were performed on the MJ Research Minicycler (Watertown, MA) with the following thermocycle profile: 94 °C for 5 min, 40 amplification cycles of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min.

2.5 Enzyme-linked immunosorbent assay (ELISA) examination of the transgenic plants

Fresh leaves of *Brassica compestris* L. spp. *chinensis* were ground in liquid nitrogen. The powder was suspended with a ratio of 3 ml/g fresh weight (FW) in a protein extraction buffer containing 0.1 mol/L Tris-HCl (pH 8.0), 0.01 mol/L $MgCl_2$, 18% (w/v) sucrose, and 40 mmol/L 2-mercaptoethanol, and cell debris were removed by centrifugation at 10000×*g* for 15 min at 4 °C. The antigen supernatant containing the crude protein extraction of choline oxidase was subjected to ELISA examination, as previously described by Kramer *et al.* (1995) with some modification. After 50 μl of antigen solution was moved from the incubated wells and washed three times, nonspecific protein used in blocking buffer was incubated for 2 h at room temperature. After discarding the blocking buffer and triple washing, the 50 μl primary antibody (rabbit immune body of choline oxidase) with a 1/200 (v/v) dilution in blocking buffer was added into each well and incubated for 2 h at room temperature. Following triple washing with sterilized water, 50 μl of goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase (AP) conjugate (Sino-American Biotechnology Co., China) with a 1/1000 (v/v) dilution in blocking buffer was added into each well and incubated for 2 h at room temperature. After triple washing, dye conversion was initiated by charging the wells with 50 μl of the *para*-nitrophenylphosphate (pNPP) at 37 °C for 1 h, to determine if choline oxidase existed in the transgenic plants. The reaction was terminated by adding 25 μl 0.5 mol/L NaOH.

2.6 Immunological examination of the expressed choline oxidase

The localization of the expressed choline oxidase was examined immunocytochemically as previously described by Mustardy *et al.* (1990) with some modifications. For preparation of a soluble fraction, small pieces (2 mm×2 mm) of five-leaf-stage leaves of the transgenic and non-transgenic plants were cut and fixed in 2% (v/v) glutaraldehyde overnight at 4 °C and in 1% (w/v) osmium tetroxide for 2 h at 4 °C. Following triple washing with 5% (w/v) phosphate buffer solution (PBS), 20 min each time, samples were dehydrated in serial concentrations of ethanol (50%→70%→80%→90%→95%→100%) and 100% acetone, infiltrated overnight at room temperature in an acetone-Epon 812 mixture (1:1, v/v), embedded in fresh Epon 812, and subjected to heat-polymerization for 12 h at 37 °C, 12 h at 45 °C, and 24 h at 60 °C. Ultrathin sections (~60 nm) were cut with ultramicrotomy (speed 2–3 mm/s).

Antibodies against choline oxidase raised in a

rabbit were purchased commercially from Sigma (St. Louis, MO, USA). Initially, the ultrathin sections were mounted on uncoated nickel grids, rinsed with 5% H_2O_2 and PBS buffer for three times (5 min per time), subsequently treated for 1 h at 25 °C with a blocking solution consisting of 0.25% (w/v) bovine serum albumin (BSA) (fraction V, Sigma), 0.05% (v/v) Tween 20 (Bio-Rad), 0.05% (w/v) NaN_3, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.17 mol/L H_3BO_4, and 0.12 mol/L NaCl (pH 8.5), and then exposed to the primary antiserum diluted in 1:200 (v/v) in PBS buffer and incubated for 30 min. This was followed by 30 min incubation with the 10 nm-gold-conjugated secondary antiserum (goat anti-rabbit) in a 1:20 (v/v) dilution in PBS containing 1% (w/v) BSA. Samples were required to be vigorously rinsed six times with 1% PBS between the incubations, 5 min per time for optimum labeling, and finally were rinsed in sterilization ddH_2O. After dried in the air, ultrathin sections were stained with 4% (v/v) uranyl acetate for 15 min and 2% (w/v) lead citrate for 5 min, and then examined in a transmission electron microscope (H-600-4, Hitachi Ltd., Japan).

2.7 Betaine measurement

Approximately 5 g fresh leaf materials were ground to a fine powder in liquid nitrogen. The powder was suspended in 25 ml of 1.0 mol/L H_2SO_4 and incubated at 25 °C for 2 h. Cell debris were removed by centrifugation at 1000×*g* for 10 min. The supernatant was incubated in 10 ml of KI-I_2 solution (15.7 g I_2 and 20 g KI dissolved in 100 ml of 1 mol/L HCl) at 0 °C for 2 h, and centrifuged at 1000×*g* for 30 min and the periodide adducts of betaine were collected. The resulting periodide adducts of betaine were then dissolved in 0.5 ml of D_2O, which contained 0.5 mmol 2-methyl-2-propanol (tBA) as an internal standard, and were used for the determination of ^{1}H-nuclear magnetic resonance (^{1}H-NMR). Detailed procedure can be found in Hayashi *et al.* (1997).

2.8 Net photosynthetic rate (P_n) measurement

The plants of 6–8-leaf stage were treated with low temperature (1 °C for 48 h) or high temperature (45 °C for 4 h), then moved into the phytotron to recover for 1 h, and measured for P_n with a portable CO_2 gas analyzer (Model CI-301, CID Inc., Vancouver, WA, USA). The antepenultimate leaves were selected for the P_n measurement. According to the method of Yang (1999), 10 leaf disks from the leaves, which were measured for photosynthesis, were used to analyze the contents of chlorophyll a and b (mg/g FW). All measurements were done with three replicates.

2.9 Salt tolerance

At 8–9-leaf stage, 20 plants of T_3 generation from the transgenic lines 1 and 2 (L1 and L2) and wild-type were subjected to salt treatments by adding NaCl to the growth medium with 50 mmol/L increments every 24 h until a final concentration of 0, 100, 200, and 300 mmol/L, respectively, and maintained for 7 d (Xu *et al*., 2001). The survival rate in each treatment was analyzed to compare the salt tolerance between transgenic and wild-type plants, and the antepenultimate leaves were measured for P_n. The survival rate (*Su*) was evaluated by $Su\ (\%)=N_s/N_a\times 100$, where N_s is the number of survival plants per treatment, and N_a is the number of all plants per treatment.

3 Results

3.1 Transformation with the *codA* gene

We used 769 *Brassica compestris* L. spp. *chinensis* explants (339 cotyledons and 430 hypocotyls) to conduct the transformation experiments with the *codA* gene carried by the vector plasmid shown in Fig. 1, and eventually obtained 129 transgenic plants (T_0), which were derived from 45 cotyledons and 84 hypocotyls. The differentiation rates for the cotyledon and hypocotyl in our experiments were extremely high (13.27% and 19.53%, respectively). We have developed an efficient *codA* transformation system for *Brassica compestris* L. spp. *chinensis* through screening of the compositions of differentiation and selection media (data not shown).

From the 129 transgenic plants (T_0), we obtained 10 transgenic plants (T_1 plants) showing resistance to both kanamycin and hygromycin. Subsequent T_2 and T_3 plants were obtained by the procedure as described in Section 2.3. In each generation from T_0 to T_3, the presence of the transgene (*codA*) was confirmed by PCR (Fig. 2). The band with same molecular weight (M_W) size amplified in L1 and L2 plants was also

amplified from the plasmid pBinMoBc, which was used as the positive control, but no bands appeared in the wild-type plants, suggesting that the *codA* has been successfully inserted into the *Brassica compestris* L. spp. *chinensis* genome by *A. tumefaciens*-mediated transformation.

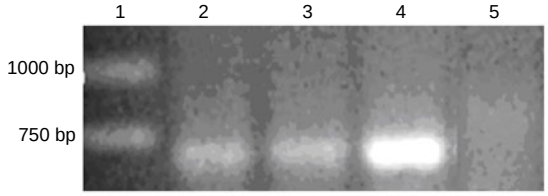

Fig. 2 PCR test of genomic DNA from the transgenic and wild-type plants
Lane 1: DNA marker which has been purchased commercially (TaKaRa); Lane 2: transgenic line 1 (L1); Lane 3: transgenic line 2 (L2); Lane 4: PCR fragment of plasmid pGAH/codA; Lane 5: wild-type plant

We further evaluated the existence of the choline oxidase, the product of the *codA* gene through ELISA examination of the leaves of L1 and L2 plants. The results showed that only L1 and L2 plants had the positive reaction (Fig. 3), indicating that the *codA* gene had been efficiently expressed in each line of the transgenic plants, and that the expressed precursor had been effectively processed to the mature protein.

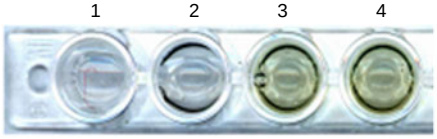

Fig. 3 Immunocytochemical detection of choline oxidase in leaves of the transgenic and wild-type plants
Lane 1: negative control (substrate+buffer+water); Lane 2: non-transgenic wild-type plant; Lane 3: transgenic line 1 (L1); Lane 4: transgenic line 2 (L2)

To determine the localization of the expressed choline oxidase, the immunogold labeling technique was applied in the transgenic and wild-type plants. Many colloid gold particles were observed in the chloroplasts of the L1 and L2 plants with the immunoelectron microscope (Figs. 4b and 4c). In contrast, the density of gold particles in the chloroplasts of the wild-type plants was at the background level (Fig. 4a). The results confirm that choline oxidase was located truly in the chloroplasts of the transgenic plants.

3.2 Betaine accumulation

The results from the ^{1}H-NMR spectrometry experiment showed that there were characteristic peaks of betaine with accumulation levels of 0.224 μmol/g FW and 0.221 μmol/g FW in the transgenic L1 and L2 plants, respectively, but no betaine accumulation in the wild-type plants (Fig. 5). Therefore, the results suggest that the transgene, *codA*, expressed well and the gene product, choline oxidase, functioned properly in the transgenic plants.

3.3 Comparison of photosynthesis under low temperature stress

We measured the net photosynthetic rate (P_n) at the time point of 1 h after the 6–8-leaf stage plants were exposed to low-temperature (1 °C) for 48 h. The transgenic L1 and L2 plants showed a significantly higher P_n (9.61 μmol/(m^2·s) and 9.22 μmol/(m^2·s), respectively) than the wild-type control plants (5.19 μmol/(m^2·s)) (P<0.01), as shown in Table 1.

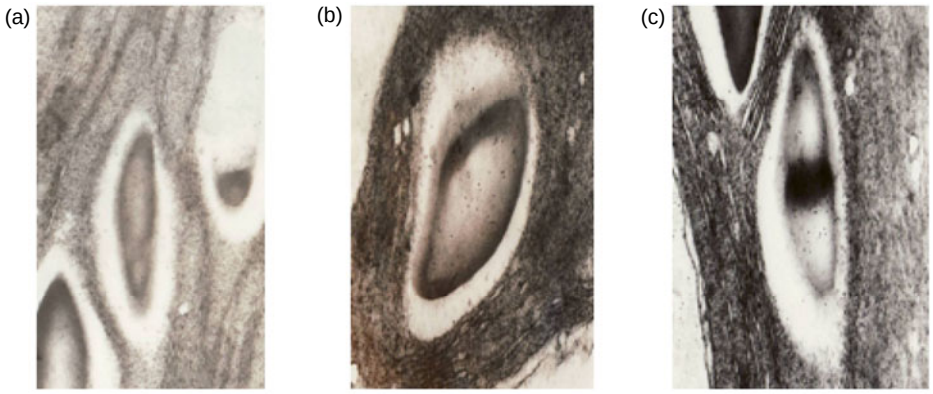

Fig. 4 Immunoelectron microscope of choline oxidase in the chloroplasts of the wild-type and transgenic plants
(a) Wild-type plant; (b) Transgenic line 1 (L1) plant; (c) Transgenic line 2 (L2) plant

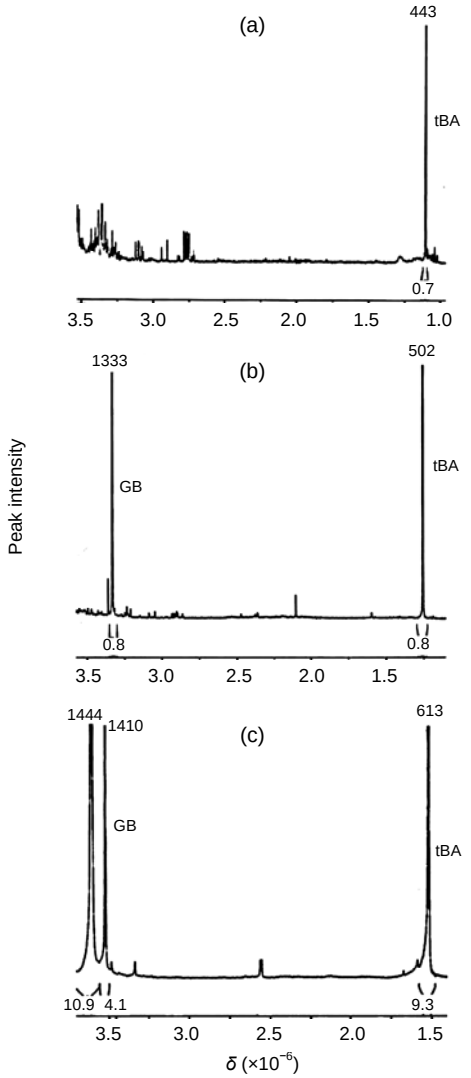

Fig. 5 ^{1}H-NMR spectra of betaine in the wild-type and transgenic plants

(a) Wild-type plant; (b) Transgenic line 1 (L1) plant; (c) Transgenic line 2 (L2) plant. GB: glycine betaine; tBA: 2-methyl-2-propanol or tert-butyl alcohol, which is similar structurally to betaine and used as an internal standard for the estimation of the content of betaine. The distance between the small peaks was indicated by coupling constants. δ: chemical shift

Table 1 Comparison of net photosynthetic rate (P_n), evaporation rate (E), and stoma resistance (Sr) between the transgenic and wild-type plants under low temperature (1 °C for 48 h) treatment

Type	P_n (μmol/(m^2·s))	E (mmol/(m^2·s))	Sr ((m^2·s)/mol)
Wild	5.19±1.07	2.45±0.27	28.18±1.41
L1	9.61±0.83**	2.52±0.26	13.00±1.61***
L2	9.22±0.51**	2.49±0.21	13.08±0.16***

Values were expressed as mean±SD (n=3). **, ***: Significant differences at P<0.01 and P<0.001, respectively; L1, L2: transgenic lines 1 and 2, respectively

Under the same stress condition, stoma resistance (Sr) of the transgenic plants was lower than that of wild-type plants, that is, CO_2 entering into a mesophyll cell through the stomata is reinforced by increased gradient of CO_2 between the external atmosphere and the intercellular space inside the transgenic plant leaves because of the enhanced photosynthesis in the transgenic plants (Table 1). We also measured the chlorophyll contents in the different plants and the results showed that transgenic L2 plants had higher chlorophyll a/b ratio than the wild-type plants (P<0.05) (Table 2).

3.4 Comparison of recovery of net photosynthetic rate (P_n) under high temperature stress

Under non-stressed condition, the P_n in the transgenic line L1 (5.36 μmol/(m^2·s)) was almost the same as the wild-type control plants (5.34 μmol/(m^2·s)). However, after 4 h exposure to high temperature (45 °C) and 1-h restoration in the phytotron, the P_n of the transgenic line L1 dropped to 4.01 μmol/(m^2·s), which is significantly higher than that of the wild-type plants (1.87 μmol/(m^2·s)) (Table 3). Nevertheless, after 25-h restoration, there is no significant difference for the P_n between them (Table 3). The results suggest that the transgenic plants can recover much faster from the stress than the wild-type plants (74.9% vs. 35.0% with 1-h restoration).

3.5 Comparison of survival rate and photosynthesis under salt stress

The survival rate for the transgenic L1 plants was higher than that of wild-type plants under all the three levels of NaCl concentration (Table 4). For example, under 100 mmol/L NaCl, 94.4% of L1 plants vs. 65.4% of wild-type plants survived (Fig. 6). While most of the wild-type plants (16.7% survivals) died under 200 mmol/L NaCl, more than half (53.3%) of the transgenic L1 plants were still alive. Though most died among both types of plants under 300 mmol/L NaCl, there were still 27.8% transgenic L1 plants that survived.

Similar P_n were observed between the transgenic line L1 plants (5.75 μmol/(m^2·s)) and wild-type plants (5.71 μmol/(m^2·s)) under non-stress condition. However, like the situation discussed above in high temperature stress, less reduction of P_n was also observed in the transgenic L1 plants than in the wild-type plants

Table 2 Comparison of chlorophyll values between the transgenic and wild-type plants under low temperature (1 °C for 48 h) treatment

Type	Chlorophyll value (mg/g FW)			
	a	b	ab	a/b
Wild	0.4251±0.0245	0.1765±0.0113	0.6016±0.0351	2.408±0.1134
L1	0.4154±0.0211	0.1677±0.0089	0.5831±0.0255	2.477±0.1225
L2	0.4469±0.0231	0.1487±0.0067	0.5956±0.0348	3.005±0.1317*

Values were expressed as mean±SD (n=3). * Significant difference at P<0.05. L1 and L2 are the transgenic lines 1 and 2, respectively

Table 3 Comparison of recoveries of net photosynthetic rates (P_n) of 1-h and 25-h restoration after exposure to high temperature (45 °C for 4 h) stress

Type	P_n (μmol/(m^2·s))			Recovery of P_n (%)	
	Pre-exposure	1 h after exposure	25 h after exposure	1 h after exposure	25 h after exposure
Wild	5.34±0.37	1.87±0.1	4.02±0.38	35.0±1.0	75.2±12.8
L1	5.36±0.42	4.01±0.30**	4.85±0.55	74.9±11.0**	90.5±3.7*

Values were expressed as mean±SD (n=3); A total of 15 plants per treatment were tested. *, ** Significant difference at P<0.05 and P<0.01, respectively. L1: transgenic line 1

Table 4 Comparison of some photosynthetic characteristics and survival rate between the transgenic L1 and wild-type plants under the stress of different NaCl concentrations

NaCl (mmol/L)	Types	P_n (μmol/(m^2·s))[a]	E (mmol/(m^2·s))	Sr ((m^2·s)/mol)	Su (%)
0	Wild	5.71±0.67	1.28±0.11	27.72±2.74	100±0
	L1	5.75±0.68	0.95±0.14	29.71±3.01	100±0
100	Wild	1.50±0.53	0.61±0.12	61.66±3.87	65.4±8.9
	L1	4.12±0.75**	0.87±0.13	42.21±3.65*	94.4±8.0**
200	Wild	0.27±0.41	0.40±0.09	84.11±3.23	16.7±7.8
	L1	2.66±0.52***	0.74±0.13*	47.75±3.90*	53.3±9.1***
300	Wild	−0.10±0.12	0.37±0.08	90.93±4.22	13.6±6.0
	L1	1.27±0.21***	0.58±0.11*	72.45±4.43*	27.8±5.7***

Values were expressed as mean±SD (n=3). *, **, *** Significant differences at P<0.05, P<0.01, and P<0.001, respectively. P_n: net photosynthetic rate; E: evaporation rate; Sr: stoma resistance; Su: survival rate

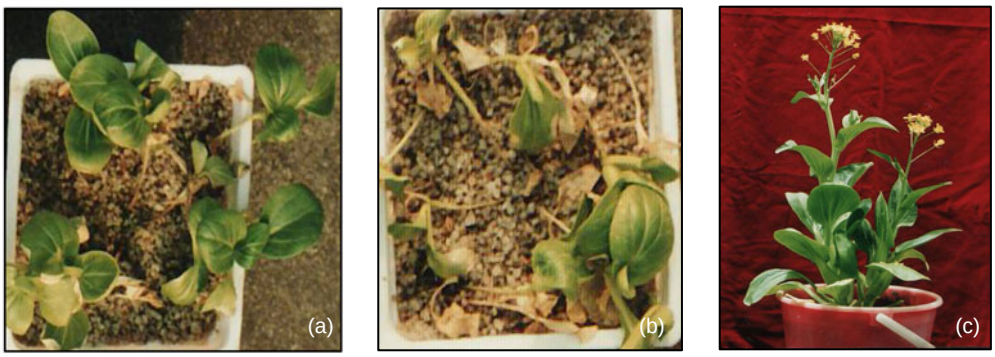

Fig. 6 Comparison between the transgenic L1 and the wild-type plants grown under salt stress of 100 mmol/L
(a) Transgenic L1 plants; (b) Wild-type plants; (c) Transgenic plants with flowering

under all the three NaCl concentrations (Table 4). For example, under 100 mmol/L NaCl, the P_n of the transgenic L1 plants was close to three-fourths of that observed under non-stress condition, but that was only one-fourth for the wild-type control plants (1.50 μmol/(m^2·s)).

As for the evaporation rate (*E*), no differences were observed between the transgenic and wild-type plants under non-stress condition. However, the evaporation rate was higher in the transgenic plants than in the wild-type plants under both 200 and 300 mmol/L NaCl ($P<0.05$) (Table 4).

Under high salinity stress, the transgenic plants showed a lower stoma resistance (*Sr*) than the wild-type plants (Table 4), indicating that the high photosynthesis for transgenic plants is due to less reduced stomata opening from stress, i.e., the stomata opening of transgenic plants is less affected by salt stress than that of wild-type plants, which facilitates CO_2 entering the leaf and increases photosynthesis. Therefore increased photosynthesis is the result of more CO_2 entering inside the leaf through stomata.

4 Discussion

Brassica compestris L. spp. *chinensis* is a very important vegetable crop, commonly grown in South China. It is not able to synthesize endogenously betaine, and therefore is very sensitive to salt, drought, high temperature, and other environmental stresses. In this study, we obtained transgenic plants expressing *codA* gene from *Arthrobacter globiformis* and demonstrated enhanced tolerance to extreme temperature and high salinity in these transgenic plants.

The content of betaine in the transgenic plant is influenced directly by its subcellular location. In tomato, there are reports that the transcript of *codA* could be targeted to the chloroplasts (Chl-codA), cytosol (Cyt-codA) or both compartments simultaneously (ChlCyt-codA). A comparison between these three types of transgenic plants showed that Chl-codA plants with the lowest amounts of betaine exhibited equal or higher degrees of enhanced tolerance to various abiotic stresses, suggesting more effectiveness of chloroplastic betaine in protecting plant cells (Park *et al*., 2007). Our results showed that the *codA* gene product in *Brassica compestris* L. spp. *chinensis* was targeted to chloroplasts, which agreed with the results from both *A. thaliana* (Alia *et al*., 1998b) and *Brassica juncea* (Prasad *et al*., 2000). The contents of betaine in chloroplasts of the *codA* transgenic plants varied generally due to the different plant species, such as 0.30 μmol/g FW in transgenic *Diospyros kaki* and *Lycopersicon esculentum*, and 1.43 μmol/g FW in transgenic *Solanum tuberosum* (Chen and Murata, 2008). Moreover, the expression level of *codA* is affected by the cultivation conditions and the tested tissues or organs for the transgenic plants. In our experiment, all the plants were cultured in water, and thus had much higher water content (~90%) than that cultured in soil. Therefore, our transgenic plants had low measured values of betaine (~0.22 μmol/g FW). However, they still showed high tolerance to extreme temperature and high salinity.

Environmental stresses such as salt and extreme temperature will eventually cause yield losses for plant production. During adaptation, plant may develop tolerance to environment stresses by accumulating some small organic solutes, including the polyhydroxylated compounds, carbohydrate, amino acid, betaine, and related compounds (Bohnert and Jensen, 1996; Hayashi *et al.*, 1998) known as compatible materials (Bohnert *et al.*, 1995). In plants, the major compatible osmoprotectant solutes include betaine, proline, and polyols (Rontein *et al.*, 2002). In this study, transgenic plants expressing the *codA* gene were able to accumulate betaine in vivo, which may have possible protective effects on the biological macromolecules (Schobert, 1977). In the chloroplast of spinach and sugar beet plants, betaine could be synthesized and accumulated naturally, but not in that of most other plant species. However, we could engineer theses plants to accumulate betaine through a transgenic approach (Sakamoto and Murata, 2001; Prasad and Saradhi, 2004). The strategy for engineering betaine synthesis was employed by transformation with the *codA* gene which encodes choline oxidase and offers an attractive conversion from choline to betaine under the enzymatic catalysis. Transgenic *A. thaliana* plants with the *codA* gene significantly enhanced the tolerance to low temperature and high-salt stress (Hayashi *et al.*, 1998). Under low temperature, the transgenic *A. thaliana* with *codA* gene had an obviously higher biological output than the wild-type control (Hayashi *et al.*,

1997; Alia *et al.*, 1999).

The *codA*-mediated tolerance to salt stress has been reported in other field crops, including corn, (Saneoka *et al.*, 1995), rice (Mohanty *et al.*, 2002; Sawahel, 2003), *Brassica juncea* (Prasad *et al.*, 2000), and tobacco (He *et al.*, 2001). Here we reported that transgenic *Brassica compestris* L. spp. *chinensis* seedlings expressing a *codA* gene from *A. globiformis* could accumulate betaine in vivo and showed significant tolerance to high and low temperatures and high salinity stresses, compared with wild-type plants. Moreover, the wild-type plants had more difficulty surviving compared to the transgenic plants under the 300 mmol/L NaCl condition, suggesting that betaine may be important for osmotic adjustment under salinity stress in the transgenic plants.

It has been demonstrated that the biosynthesis of betaine is stress-inducible (Sakamoto and Murata, 2002). The direct protective effects of betaine on macromolecules and membranes may be not only osmotic, but also via the mechanism of compatible solutes and oxygen radical scavenging (Blumwald and Grover, 2006). This point may be supported by our results that the transgenic seedlings of *Brassica compestris* L. spp. *chinensis* showed a higher P_n after the low temperature treatment and a higher recovery rate of photosynthesis after exposure to high temperature (45 °C for 4 h). The role of betaine in stress tolerance could be protecting the oxygen-evolving PSII complex, stabilizing the protein structure of PSII complex, and maintaining ATP synthesis under stress conditions (Sakamoto and Murata, 2001; Rahman *et al.*, 2002).

In this study, we found that the transgenic plants with *codA* gene showed higher P_n and lower stoma resistance accompanying with higher evaporation rate than the wild-type plants under the salt stress condition. In contrast, under temperature stress, an insignificant difference of evaporation rate but higher P_n was observed in the transgenic plants, compared with the wild-type plants. This may indicate that the action mechanisms for increasing P_n were different in the transgenic plants under the two different stress conditions. The stomatal resistance may be involved in the salt stress, but not in the temperature stress. Our results are consistent with the experiment of Mäkelä *et al.* (1998), who showed that application of exogenous betaine can significantly increase stomatal conductance of plants grown in saline conditions. An increase in stomatal conductance was related to the maintenance of higher turgor pressure or water potential in plant leaf cells (Cushman *et al.*, 1989). We found that the transgenic plants with *codA* did not show significantly higher transpiration rates than the wild-type plants in the temperature stress experiments, indicating a less or insignificant effect of stomatal opening on the P_n. Actually, the P_n can be affected by a number of factors, including a variety of antioxidant enzymes in vivo, such as superoxide dismutase, catalase, peroxidase, ascorbic acid oxidase, and glutathione reductase. The activity of those enzymes may be well conserved by the presence of betaine. Some experiments showed that the increase of the above antioxidant enzymes could efficiently eliminate active oxygen and oxygen free radicals, and thus maintain the structural stability and integrity of the cell membrane and chloroplast membrane under stress conditions (Hayashi *et al.*, 1997). It appears that the remaining higher stomatal conductance under salt stress and protecting integrity of photosynthetic apparatus under temperature stress might be two reasonable explanations for increased photosynthesis observed in our transgenic plants.

5 Acknowledgements

We thank Dr. Norio MURATA (National Institute for Basic Biology, Okazaki, Japan) for his kind gift of the binary vector pGAH/codA and are grateful to Mr. Fang-zheng WANG, Prof. Da-quan XU, Ms. Ya-fang ZHU, and Mr. Ji-hu SU (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, China) for technical assistance. We also thank Dr. Neng-yi ZHANG (Cornell University, USA) for English language correction.

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