Plant Cell Rep (2008) 27:687–698
DOI 10.1007/s00299-007-0479-4

GENETIC TRANSFORMATION AND HYBRIDIZATION

Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses

Raza Ahmad · Myoung Duck Kim · Kyung-Hwa Back · Hee-Sik Kim · Haeng-Soon Lee · Suk-Yoon Kwon · Norio Murata · Won-Il Chung · Sang-Soo Kwak

Received: 28 September 2007 / Revised: 12 November 2007 / Accepted: 13 November 2007 / Published online: 5 December 2007

Abstract Transgenic potato plants (*Solanum tuberosum* L. cv. Superior) with the ability to synthesize glycinebetaine (GB) in chloroplasts (referred to as SC plants) were developed via the introduction of the bacterial choline oxidase *(codA)* gene under the control of an oxidative stress-inducible *SWPA2* promoter. SC1 and SC2 plants were selected via the evaluation of methyl viologen (MV)-mediated oxidative stress tolerance, using leaf discs for further characterization. The GB contents in the leaves of SC1 and SC2 plants following MV treatment were found to be 0.9 and 1.43 μmol/g fresh weight by HPLC analysis, respectively. In addition to reduced membrane damage after oxidative stress, the SC plants evidenced enhanced tolerance to NaCl and drought stress on the whole plant level. When the SC plants were subjected to two weeks of 150 mM NaCl stress, the photosynthetic activity of the SC1 and SC2 plants was attenuated by 38 and 27%, respectively, whereas that of non-transgenic (NT) plants was decreased by 58%. Under drought stress conditions, the SC plants maintained higher water contents and accumulated higher levels of vegetative biomass than was observed in the NT plants. These results indicate that stress-induced GB production in the chloroplasts of GB non-accumulating plants may prove useful in the development of industrial transgenic plants with increased tolerance to a variety of environmental stresses for sustainable agriculture applications.

Keywords Choline oxidase · Environmental stress · Glycinebetaine · Metabolic engineering

Communicated by J.R. Liu.

R. Ahmad · W.-I. Chung (✉)
Department of Biological Sciences,
Korea Advanced Institute of Science and Technology (KAIST),
Daejon 305-701, South Korea
e-mail: wichung@kaist.ac.kr

R. Ahmad · M. D. Kim · K.-H. Back · H.-S. Kim · H.-S. Lee · S.-S. Kwak (✉)
Environmental Biotechnology Research Center,
Korea Research Institute of Bioscience and Biotechnology (KRIBB), Eoeun-dong 52, Yuseong, Daejon 305-806,
South Korea
e-mail: sskwak@kribb.re.kr

S.-Y. Kwon
Plant Genomics Research Center,
Korea Research Institute of Bioscience and Biotechnology (KRIBB), Eoeun-dong 52, Yuseong, Daejon 305-806,
South Korea

N. Murata
National Institute for Basic Biology,
Myodaiji, Okazaki 444-8585, Japan

Introduction

Plants are frequently exposed to diverse stress conditions, including salt, drought, heat, low temperature, heavy metals, and oxidative stress. Today, approximately 20% of the world's irrigated land is salt-affected (Munns 2005). This high salt level is one of the principal limiting factors in crop productivity. Salt induces several types of plant stresses, including altered nutrient uptake, the accumulation of toxic ions especially Na+, osmotic stress, and oxidative stress (Verslues et al. 2006). Drastic changes in ion and water homeostasis induced by salinity result in molecular damage, growth arrest, and even cell death (Zhu 2001). Drought stress is also a salient factor in reducing the yields of major crops, and continuously reduced water resources have identified it as a serious threat to sustainable food production.

Plants have evolved a variety of mechanisms for adaptation to harsh environments, as the result of the modulation of stress tolerance-related gene expressions and via the synthesis of compatible solutes. Compatible solutes including proline, polyoles, trehalose, and glycinebetaine have been detected in a wide variety of organisms. Glycinebetaine (hereafter GB), a ziwitterionic, fully *N*-methyl-substituted glycine derivative is detected in a wide variety of microorganisms and higher plants and animals (Rhodes and Hanson 1993). Several taxonomically distinct species, including spinach and wheat, are natural accumulators of GB while others, including *Arabidopsis*, rice, tomato, and potato are considered to be non-accumulators (Jones and Storey 1981). GB can accumulate rapidly in GB-synthesizing plants in response to environmental stresses, including salinity, drought, and low temperature (Rhodes and Hanson 1993). Increasing evidence from a series of in vivo and in vitro studies of the physiology, biochemistry, genetics, and molecular biology of plants suggest strongly that GB performs an important function in plants subjected to environmental stresses (Sakamoto and Murata 2000, 2001, 2002). Introducing the GB biosynthesis pathway into non-accumulators induces an increase in tolerance to a variety of abiotic stresses. GB increases salt tolerance in transgenic *Arabidopsis* and rice (Hayashi et al. 1997; Sakamoto et al. 1998), drought tolerance in maize (Shen et al. 2002; Quan et al. 2004), low temperature tolerance in *Arabidopsis* and tomato (Hayashi et al. 1997; Park et al. 2004), freezing tolerance in *Arabidopsis* (Sakamoto et al. 2000), and photoinhibition tolerance (Alia et al. 1999). GB also effectively stabilizes the quaternary structures of enzymes and other complex proteins, and maintains the highly ordered state of membranes at extremes of in vitro temperatures or salt concentrations (Papageorgiou and Murata 1995).

Potato (*Solanum tuberosum* L.) is one of the major food crops in many world regions, and ranks fourth in production after wheat, maize, and rice (Newell et al. 1991). Due to its sparse and shallow root system, the potato plant is vulnerable to a variety of abiotic stresses, including severe temperature changes, drought, and high salinity, and tuber yields can be considerably reduced by such stresses. Increasing salinity can also significantly reduce the total and average yields of different potato cultivars, and the presence of 50 mM NaCl can result in a 50% reduction in the growth of potato plants (Sayari et al. 2005).

The development of germplasms with increased tolerance to abiotic stresses would be an appropriate solution to this problem. Many researchers have employed genetic engineering to introduce desirable genes in the crop plants in order to enhance their tolerance to different stresses (Sakamoto et al. 1998; Park et al. 2004; Tang et al. 2006; Lim et al. 2007). Multigenic traits are involved in salt stress tolerance in plants; thus, success in improving its tolerance via traditional breeding approaches has proven to be very limited (Ashraf 2004). A variety of genes such as the oyster mushroom glyceraldehydes-3 phosphate dehydrogenase, *Arabidopsis thaliana* P5CS1 and barley oxalate oxidase genes have been utilized in the development of transgenic potato plants with enhanced tolerance to salt and oxidative stresses (Jeong et al. 2001; Sayari et al. 2005; Turhan 2005). However, there is still a need to genetically engineer important potato cultivars such as Superior, a worldwide important cultivar for food and industrial use, under the control of a stress-inducible promoter.

The importance of the stress-inducible promoters is overwhelmingly accepted to generate stress-tolerant crops. Previously, transgenic potato plants expressing both Cu/Zn superoxide dismutase (SOD) and ascorbate peroxide (APX) in chloroplasts were developed under the control of an oxidative stress-inducible sweetpotato peroxidase *(SWPA2)* promoter and these plants evidenced enhanced tolerance to oxidative and high temperature stresses (Tang et al. 2006). Moreover, the *SWPA2* promoter evidenced higher expression upon treatment with a variety of stress treatments, including wounding and UV-C, as compared to the 35 S promoter of the Cauliflower mosaic virus (Kim et al. 2003). In addition to this, a variety of studies have been conducted regarding the generation of transgenic crops, including the sweetpotato, potato, and tall fescue (a forage crop) under the control of the *SWPA2* promoter, and the results of these studies have verified its utility in the development of transgenic crops (Tang et al. 2006; Lee et al. 2007; Lim et al. 2007).

In this study, we report the successful transfer of choline oxidase *(codA)* cDNA derived from *Arthrobacter globiformis* to the chloroplasts of potato plants (cv. Superior) under the control of the stress-inducible *SWPA2* promoter. The expression of the *codA* gene resulted in GB synthesis in transgenic potato plants, which subsequently enhanced the osmotic and oxidative stress tolerance of the transgenic plants. To the best of our knowledge, this is the first report concerning the engineering of the GB biosynthesis pathway in potato plants.

Materials and methods

Plant material and plasmid construction

Sterile potato plants (*Solanum tuberosum* L. cv. Superior) were utilized in the transformation experiments. The plants were propagated via the sub-culturing of shoot tips and stem nodal sections every 3–4 weeks on MS (Murashige and Skoog 1962) basal medium containing 3% sucrose.

The plant expression vector, pGAH/*codA* containing the *codA*, transit peptide (TP) from a small subunit of Rubisco of tobacco and the nos terminator (T-nos) under the control of the CaMV 35 S promoter (Hayashi et al. 1997) was reconstructed. The TP, *codA* and nos terminator fragments were excised from pGAH/*codA* and ligated to a stress-inducible *SWPA2* promoter (Kim et al. 2003) after which this cassette was loaded into the *Eco*RI and *Hin*dIII sites of the pCAMBIA3300 binary vector, which harbors the *bar* gene as a selection marker. The resultant vector, pScodA, was mobilized into the *Agrobacterium tumefaciens* strain AGL0 via a freeze-thaw method.

Plant transformation and regeneration

For potato transformation, *Agrobacterium* harboring pScodA was grown for 48 h at 28°C in 5 mL of YEP medium (containing 100 mgL^{-1} rifampicin and 100 mgL^{-1} kanamycin). The stem internodes from 3–4-week old plants were cut into 5–10 mm long sections. The axillary buds were removed carefully from the explants. The explants were pre-cultured for 2 days on MS basal medium containing 0.2 mgL^{-1} 2,4-D. These explants were inoculated with *Agrobacterium* for 10 min and co-cultured for two more days in darkness on pre-culture medium. After co-culture, the explants were shifted to regeneration medium (MS medium containing 0.01 mgL^{-1} NAA, 0.1 mgL^{-1} GA3 and 2 mgL^{-1} zeatin) supplemented with 400 mgL^{-1} cefotaxime and 1 mgL^{-1} bialaphos. The tissues were then transferred to fresh medium after each 15 days. The first shoot regenerate was acquired after 4–5 weeks. When the shoots were 10–15 mm long, they were cut and transferred to rooting medium (MS medium containing 3% sucrose, 400 mgL^{-1} cefotaxime and 1 mgL^{-1} bialaphos). Plantlets, which were capable of developing good root systems on selection medium, were screened further for the presence of the *codA* gene via PCR.

PCR analysis

Genomic DNA was extracted using the protocol defined by Kim and Hamada (2005). The PCR reaction was conducted with purified genomic DNA in a PCR premix (Bioneer, Korea) using two convergent primers complementary to the *codA* gene. Primer 1: 5′-GCT GCT GGA ATC GGG ATA-3′ (forward), primer 2: 5′-TGG GCT TAT CGC GGA AGT-3′ (reverse). The amplification reactions consisted of 94°C for 5 min (1 cycle), followed by 30 cycles (94°C 30 s, 62°C 30 s and 72°C 1 min.) and finally an extension cycle of 7 min at 72°C. The PCR products were separated on 1% agarose gel, stained with ethidium bromide, and visualized under UV. All subsequent experiments were conducted on the T_0-generation of transgenic plants.

RT-PCR analysis

Leaf discs from 7-week-old plants grown in soil were treated with 5 μM methyl viologen (MV), 80 mM or 150 mM NaCl solution in order to activate the *SWPA2* promoter and induce *codA* expression. The samples were collected at 0, 24, 48 and 72 h of treatment and immediately frozen in liquid nitrogen to prevent RNA degradation. For drought stress analysis, the leaves were detached from the plants and positioned on moist tissues in a petri dish. The petri dish was sealed with micropore surgical tape and incubated at 25°C in light (150 μmol photons m^{-2} s^{-1}), and the samples were collected at the specified intervals mentioned previously. Total RNA was extracted via the cetyltrimethylammonium bromide (CTAB) method (Kim and Hamada 2005), and treated extensively with RNase-free DNase I in order to remove any contaminating genomic DNA. Two micrograms of RNA was utilized for reverse transcription in accordance with the manufacturer's instructions (Promegea, USA). PCR was conducted with 0.5 μL first-strand cDNA in PCR. The primers for actin (as internal control) were 5′-TGG ACT CTG GTG ATG GTG TC-3′ (forward) and 5′-CCT CCA ATC CAA ACA CTG TA-3′ (reverse), while with *codA*, the same primers were utilized as in the PCR of the genomic DNA. The PCR conditions were identical for both *codA* and actin, as described in the previous section, except for the annealing temperature and extension time for actin, which were 58°C and 30-s/cycle, respectively.

Glycinebetaine analysis

Four week-old-plants grown in soil were sprayed with 400 μM MV solution to induce the expression of *codA*. The samples were collected after 0 and 72 h of MV-treatment. GB extraction was conducted as described by Park et al. (2004). In brief, liquid nitrogen frozen leaves were powdered with a ceramic mortar and pestle. This powder (2 g) was then suspended in 2 mL of ice-cold methanol: chloroform: water (60:25:15) and thoroughly vortexed. An equal volume of distilled water was added to the tubes. The resultant homogenate was shaken gently for 10 min, and then centrifuged for 10 minutes at 570×*g* at room temperature. The upper methanol-water phase was transferred to clean tubes. The extracts were freeze-dried and dissolved in distilled water (2 mL). These extracts were treated with the strong anion exchange resin, AG 1-X8 (Bio-Rad, Hercules, CA, USA), as described by

Bessieres et al. (1999). Micro Bio-spin chromatography columns (Bio-Rad, USA) were packed with AG 1-X8 resin via the addition of 1 mL of resin slurry and centrifuged at 1,000×*g* at room temperature in order to eliminate any water and air bubbles, and to make a tightly uniform resin bed. Afterward, 1 ml of crude GB extract was loaded into the resin bed and centrifuged for 3 minutes at 1,000×*g*. The resin was washed with 0.5 mL of distilled water and mixed with the previous flow-through fraction.

GB was measured via high performance liquid chromatography, as was previously described by Bessieres et al. (1999). In brief, purified GB was detected on an Apollo C_{18} column (250 × 4.6 mm) under isocratic conditions with 13 mM sodium 1-heptanesulphonate and 5 mM Na_2SO_4 solution (pH 3.7) as a mobile phase. The GB peak was monitored using a UV detector (SPD-10A *VP*, Shimadzu, Japan) at 200 nm and quantification was conducted via comparison of the peak surface areas with those obtained using pure GB standard (Sigma, St Louis, USA).

Methyl viologen (MV) treatment

The leaf disc assay for oxidative stress tolerance was conducted as described by Kwon et al. (2002). In brief, leaf discs (8 mm in diameter) were punched from the fifth to sixth leaf from the top of 7 week-old-plants grown in soil. The leaf discs were floated on solution containing 3 or 5 μM MV in 0.4% sorbitol solution. The samples were incubated in darkness for 12 h to allow for MV diffusion, and then illuminated in continuous light (150 μmol photon m^{-2} s^{-1}) at 25°C. Ion leakage of solution was assessed using an ion conductivity meter (model 455C, Isteck Co., Seoul, Korea) over a time-period ranging from 0 to 72 h. At the end of the specified time-period, the samples were autoclaved for 15 min at 121°C in order to release all of the solutes. The conductivity of the solution was again measured, and this value was considered 100% ion leakage in calculations of the relative ion leakage at different time points.

Salt-stress treatment

For in vitro salt stress treatment, plant shoots (3 cm in height) from the in vitro cultured plants were transplanted into test tubes (3 × 15 cm) containing 20 mL of basal MS medium with or without the addition of 80 mM NaCl. After 4 weeks, sampling was conducted for the analysis of plant growth-related parameters, such as fresh and dry weight of the roots and shoots. The dry weight was assessed after the sample was dried in a drying oven for 48 h at 80°C.

For salt stress analysis at the whole plant level, transgenic SC and non-transgenic (NT) plantlets were permitted to develop roots in MS medium in petri dishes. After rooting, these plants were transferred to soil-filled pots (one plant/pot), and allowed to grow in a growth chamber with a 16 h/8 h photoperiod at 25°C and in 100 μmol photons m^{-2} s^{-1} light conditions. After 6 weeks of growth, plants under similar height and health conditions were selected for NaCl treatment. A volume of 150 mM NaCl solution was supplied via irrigation through the trays underneath the pots on alternate days for 2 weeks. Afterwards, the plants were irrigated only with water for 2 weeks in order to allow the plants to recover. The fresh and dry weights of salt treated plants were determined and compared with the plants grown under normal conditions. After harvesting and measuring the fresh weight, the plant samples were dried for 48 h at 80°C in order to measure the dry weight.

Photosynthetic activity was recorded via chlorophyll fluorescence determinations of photochemical yield (F_v/F_m), which represented the maximum quantum yield of photosystem II, using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 30 min dark adaptation. Measurements were conducted at room temperature (25°C) using saturating light flashes, on the fifth leaves of the plants.

Drought-stress treatment

Plantlets rooted on MS medium were transferred to pots filled with equal quantities of soil, then grown in a growth chamber for 4 weeks under similar conditions as described earlier for salt stress treatment. Drought stress was imposed by withholding the water supply to the plants maintained at 25°C and under light conditions of 100 μmol photons m^{-2} s^{-1}. Before withholding the water supply, the plants were irrigated with similar quantities of water through trays placed underneath the pots for 1 week. After 2 weeks of water withholding, the plants were again irrigated and permitted to recover from the drought conditions. Fresh and dry weight gains were measured after 10 days of recovery, as described in the preceding section, while the pictures of drought-stressed plants shown in the results were obtained after 7 days of recovery.

The degree of drought stress was assessed by the relative water contents of leaves after 10, 12 and 14 days of water withholding. The relative water contents (RWC%) were estimated as described by Ma et al. (2006). In brief, we utilized the following formula: RWC (%) = [(FW−DW)/(TW−DW)] × 100, in which FW = immediate weight of freshly collected leaves, TW = Turgid weight of leaves after incubation in water for 6 h at 20°C in the light and

DW = dry weight of the same leaves after drying at 80°C for 48 h. RWC% was measured from the first fully expanded leaf from the top.

Results

Generation of transgenic potato plants

The *codA* cDNA was sub-cloned from pGAH/*codA* to the pScodA binary vector and was inserted downstream of a stress-inducible *SWPA2* promoter (Fig. 1a). The resultant plasmid was then transferred to the *Agrobacterium tumefaciens* AGL0 strain for potato transformation. In order to express the *codA* gene in the chloroplasts of transgenic plants, a transit peptide (TP) from a small subunit of tobacco Rubisco was utilized. The putative regenerated potato plants were selected on MS medium harboring bialaphos. Seventeen transgenic lines were developed and *codA* cDNA integration was principally verified via genomic DNA PCR (Fig. 1b), and the expected amplification profiles were acquired from all transgenic lines. These transgenic plants were phenotypically indistinguishable from the non-transgenic (NT) plants. The transgenic plants harboring the *codA* gene under the control of the *SWPA2* promoter were designated "SC" plants. Further confirmation of stable foreign gene integration and transcription was achieved via RT-PCR. Four-week-old in vitro grown plants were sprayed with 200 μM methyl viologen (MV), a reactive oxygen species (ROS)-generating non-selective herbicide. Samples were collected after 96 h of MV treatment for the extraction of total RNA. RT-PCR analysis showed the stable integration of the *codA* gene. As expected, the amplified products were acquired, even though the expression levels differed among the transgenic lines (Fig. 1c). This data confirmed the successful transformation and integration of the *codA* gene into the genome of the potato plant.

Line selection for further characterization

All 17 of the transgenic lines were grown in a growth chamber for 6 weeks and utilized to evaluate the enhanced tolerance against MV-mediated oxidative stress using leaf discs. Leaf discs, 8 mm in size were punched from the fifth to sixth leaves from the shoot tip and floated on 0.4% sorbitol solution containing 3 μM MV. Ion leakage was assessed over a time course, and 11 lines evidenced enhanced tolerance, as shown by the lower levels of recorded ion leakage (data not shown). Among these 11 lines, two transgenic lines (6 and 12 in Fig. 1c) with the highest observed tolerance (referred to as SC1 and SC2 plants, respectively) were selected for further characterization.

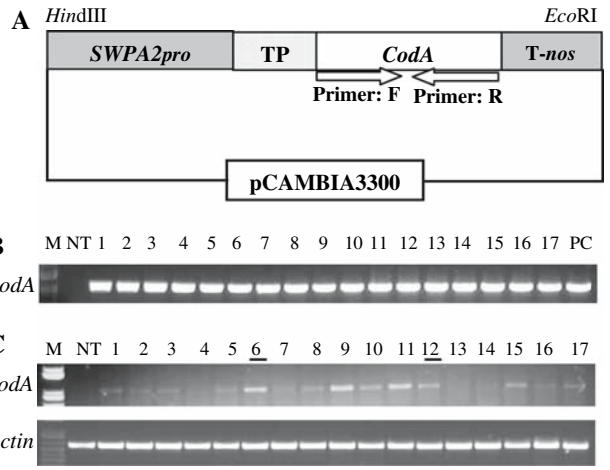

Fig. 1 Development of transgenic potato plants expressing the *codA* gene in chloroplasts. **a** Schematic representation of the T-DNA regions of pScodA used for potato transformation. *SWPA2*pro Sweetpotato peroxidase anionic 2 promoter, *TP* transit peptide from the sequence of the small subunit of Rubisco (tobacco), *codA* choline oxidase cDNA, *T-nos* terminating sequence from the nopaline synthase gene. **b** Genomic DNA PCR analysis of the *codA* gene from transgenic plants. *Numbers* (*1–17*) represent independent transgenic lines, *M* size marker, *NT* non-transgenic plant, *PC* positive control. **c** RT-PCR analysis of 17 lines expressing stable *codA* gene integration in the transgenic plants following MV treatment

Inducible expression of *codA* gene in transgenic potato plants

As the foreign gene is driven by a stress-inducible *SWPA2* promoter, the level of *codA* gene expression was analyzed after MV, salt, and drought stress treatments. It was noted that the expression of the *codA* gene was induced profoundly by MV, salt, and drought stress treatments (Fig. 2). The expression patterns observed during a time course were almost identical in both SC1 and SC2 lines. However, the expression of *codA* gene was slightly lower in mild stress conditions (Fig. 2c). Under non-stressed conditions, the expression of foreign genes was undetectable, but after 24 h of treatment, the *codA* gene was induced profoundly (Fig. 2a–d). As was evidenced by our results, the expression of the *codA* gene was enhanced to a greater degree over time.

Glycinebetaine (GB) accumulation in transgenic potato plants

In order to determine whether the expression of *codA* resulted in GB synthesis in the transgenic plants, the GB was analyzed quantitatively with HPLC. Generally, higher MV concentrations are required for the imposition of stress at the whole plant level and thus transgenic and NT Plants were subjected to 400 μM MV prior to sampling in order to induce the expression of the GB-synthesizing *codA* gene.

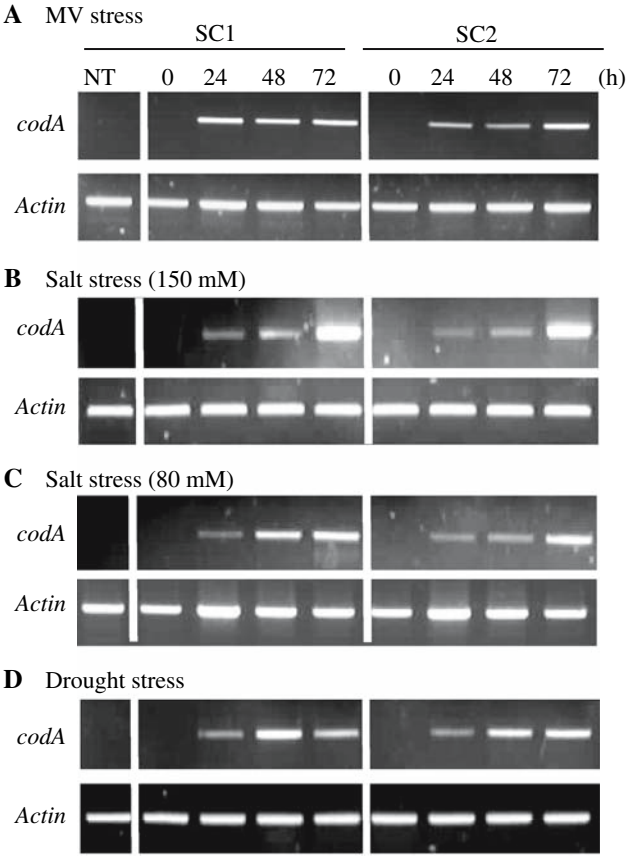

Fig. 2 RT-PCR analysis of *codA* gene expression in the leaves of non-transgenic (*NT*) and transgenic (*SC*) plants subjected to 5 μM MV (**a**), 150 mM NaCl (**b**), 80 mM NaCl (**c**) and drought stress (**d**). Total RNA was extracted after 0, 24, 48 and 72 h of each stress treatment. First-strand cDNA synthesis and PCR were conducted in accordance with the manufacturer's instructions. Actin was utilized as an internal control. Reaction products (8 μl) were analyzed via gel electrophoresis. *NT* non-transgenic potato plants, *SC* transgenic potato plants expressing *codA* gene in the chloroplasts under the control of a stress-inducible *SWPA2* promoter

The potato plant is a natural non-accumulator of GB; we were unable to detect any GB synthesis in the NT plants, whereas the transgenic plants accumulated GB. The GB contents of SC1 and SC2 after 72 h of MV treatment were 0.9 and 1.43 μmol/g fresh weight, respectively, whereas under non-stressed conditions, the GB levels in the leaves of SC1 and SC2 plants were 0.27 and 0.22 μmol/g fresh weight, respectively (Fig. 3).

Oxidative stress tolerance of transgenic potato plants

MV-mediated oxidative stress generates massive ROS bursts, which disrupt the membrane integrity (Bowler et al. 1991). Leaf discs of NT and SC plants were prepared from plants at the same age, and incubated in 5 μM MV. The ion leakage is a good indicator of membrane stability against

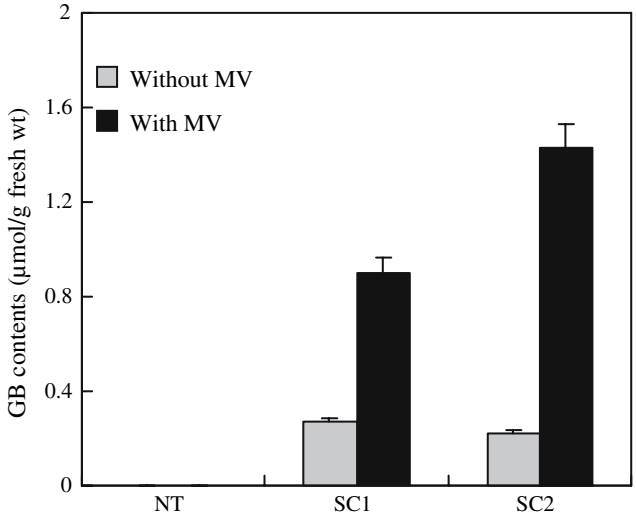

Fig. 3 Analysis of glycinebetaine (GB) levels in the leaves of non-transgenic (*NT*) and transgenic (*SC*) plants treated with 400 μM MV via HPLC. GB was extracted from the non-treated and MV-treated plants. Samples were collected after 72 h of MV treatment. Data are expressed as the mean ± standard deviation (SD) of three replicates

oxidative stress. The protective role of GB against MV-mediated stress proved effective even after 24 h of stress treatment, and the SC plants evidenced less ion leakage until the end of treatment. SC1 and SC2 plants evidenced ($P = 0.05$) significantly less ion leakage under MV-stress as compared to NT plants. The ion leakage of transgenic SC1 and SC2 plants was 25 and 31%, respectively, whereas the NT plants evidenced 60% ion leakage after 72 h of MV treatment (Fig. 4).

Salt stress tolerance of transgenic potato plants

GB is primarily found in plants in salt-affected areas and previous transgenic studies evidenced enhanced tolerance to salt stress (Sakamoto and Murata 2001). Keeping this in mind, we tested our SC plants against salt stress. We initially induced salt stresses in test tubes, as an in vitro salt-stress analysis. Shoot apices from the SC and NT plants were transferred to test tubes containing MS basal medium with or without 80 mM NaCl. These plants were permitted to grow for 4 weeks. Severe inhibition of rooting was noted in the salt-containing medium; however, the inhibitory effects of NaCl were lower in the transgenic SC plants as compared to the NT plants. The NT plants could generate either no roots or only 1 to 2 weak roots, whereas the transgenic SC1 and SC2 plants developed good and longer healthy roots. This poor root development in NT plants resulted in poor shoot growth as well; however, the shoot development was far superior in the SC1 and SC2 plants (Fig. 5a). The root and shoot dry weights were determined

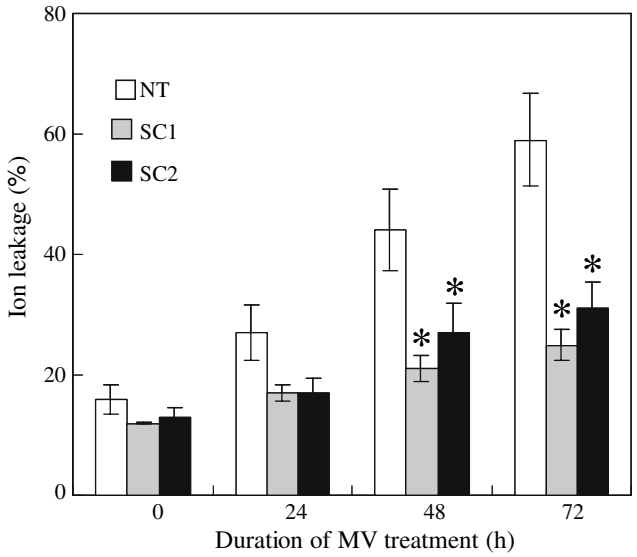

Fig. 4 Effect of MV-mediated oxidative stress treatment on the ion leakage of *codA* transgenic SC1 and SC2 transgenic plants. Leaf discs were treated with 5 μM MV, incubated at 25°C and a light intensity of 150 μmol photons m^{-2} s^{-1}. Ion leakage was measured after 0, 24, 48, and 72 h of MV-treatment. Percentages of ion leakage were calculated using 100% to represent values obtained after autoclaving. *NT* non-transgenic, *SC1* and *SC2* are independent transgenic lines. Data are expressed as the mean ± SD of three replicates. *Bars* labeled with *asterisk* show significant differences between NT and SC plants by *t*-test at $P = 0.05$

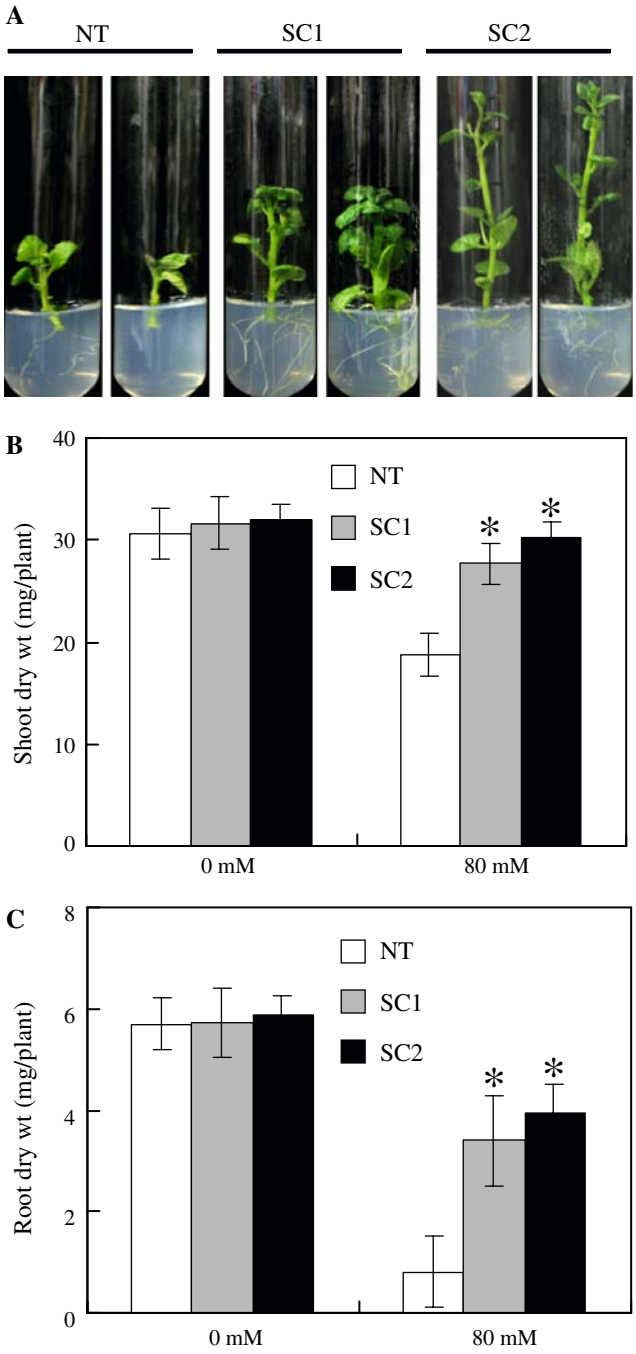

Fig. 5 Effects of salt stress on non-transgenic (*NT*) and transgenic (*SC*) plants grown under in vitro conditions. **a** Effect of NaCl (80 mM) on root and shoot growth of NT and SC plants grown for 4 weeks in test tubes. **b** Shoot dry weight of NT and SC plants after 4 weeks of growth in MS medium with or without 80 mM NaCl. **c** Root dry weight of NT and SC plants after 4 weeks of growth in MS medium with or without 80 mM NaCl. Data are expressed as the mean ± SD of three replicates. *Bars* labeled with *asterisk* show significant differences between NT and SC plants by *t*-test at $P = 0.05$

after 4 weeks of growth in medium with or without 80 mM NaCl. Both the SC1 and SC2 plants maintained significantly ($P = 0.05$) higher root and shoot development as compared to the NT plants at 80 mM NaCl treatment. The dry mass gain in the shoots was reduced by only 12% and 6% in the SC1 and SC2 plants, respectively, whereas the NT plants evidenced a decrease of 39% in shoot dry weight (Fig. 5b). The root dry weights of the SC1 and SC2 plants were 45% and 53% higher than in the NT plants (Fig. 5c).

The GB synthesis rendered the transgenic potato plants tolerant to salt stress in the test tubes, which encouraged us to evaluate the transgenic plants against salt stress at the whole plant level. Six-week-old soil-grown plants were treated with 150 mM NaCl dissolved in water for 2 weeks on alternate days. After 2 weeks of salt treatment, the plants were irrigated with water only to permit recovery. However, the salt absorbed into the soil particles was not washed out. Salt stress-mediated damage to the photosynthetic apparatus was determined at specified intervals via measurements of the fluorescence parameters (*Fv/Fm)* (Fig. 6a). The gradual decrease in *Fv/Fm* was recorded; however, a drastic decrease was noted after two weeks of salt application. Although photosynthesis began to decline after one week of salt stress, both the SC1 and SC2 plants evidenced higher levels of activity than the NT plants during the entire course of salt stress treatment. The *Fv/Fm* of the SC1 and SC2 plants were reduced by up to 42% and 23%, respectively, whereas that of the NT plants declined up to 58% after 4 weeks of growth under salty conditions

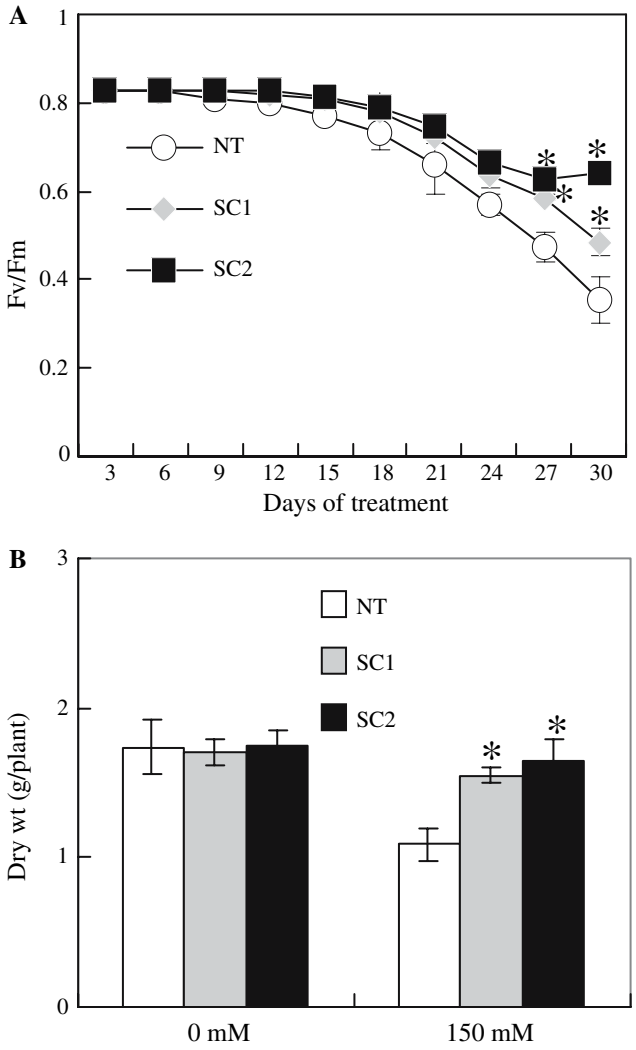

Fig. 6 Effect of salt stress (150 mM) on 6 week-old non-transgenic (*NT*) and transgenic (*SC*) plants grown in soil. Salt was applied for 2 weeks on alternate days and over the next 2 weeks, recovery was allowed via the application of water only. **a** Effects of salt stress on photo-inhibition measured by means of the *Fv/Fm* values. **b** Dry weight of NT and SC plants after salt stress (150 mM) under greenhouse conditions. Data are expressed as the mean ± SD of three replicates. *Bars* labeled with *asterisk* show significant differences between NT and SC plants by *t*-test at $P = 0.05$

(Fig. 6a). The reduction in the photosynthetic activity of SC plants at the end of treatment was significantly ($P = 0.05$) less than that of the NT plants.

After the two-week recovery period, the upper portions of the plants were harvested in order to determine the fresh and dry weights. The dry weight accumulation of the NT plants was decreased by 37%, whereas that of the SC1 and SC2 plants was reduced by only 9% and 4%, respectively (Fig. 6b). The recorded dry weight was significantly ($P = 0.05$) higher in the SC1 and SC2 plants than in the NT plants. In addition to this, transgenic SC plants generated higher tuber yields (wt) in our preliminary pot experiments, which were conducted to study the tuber yield parameters under salt stress conditions (data not shown).

Drought stress tolerance of transgenic potato plants

Glycinebetaine (GB) is generally believed to be an effective osmoprotectant. In order to evaluate whether or not GB synthesis enhanced the drought tolerance of transgenic potato plants, 4-week-old soil-grown plants were subjected to drought stress by withholding their water supply for 2 weeks. It was noted that NT plants began wilting after 12 days of water withholding, while the transgenic plants evidenced vigorous health. At the end of the drought treatment, the NT plants were severely wilted, whereas the transgenic plants evidenced less wilting than the NT plants (Fig. 7a). After 2 weeks of water withholding, the plants were again watered and permitted to grow for 10 days in order to recover from drought stress. Upon watering, the SC1 and SC2 plants re-assumed a turgid state after 24 h and continued to grow, whereas the NT plants evidenced negligible recovery, exhibiting severe bleaching of the younger leaves and wilting of the lower leaves after 7 days of recovery (Fig. 7a), which resulted in a failure to survive after 10 days of recovery.

The relative water contents of drought-stressed plants were measured over a specified time-period during drought stress. After 10 days of water withholding, we noted no significant reduction in the water contents of the NT and SC plants; however, the water contents of the SC plants were higher at that time as well (data not shown). The water contents of the NT plants were drastically reduced over the next 2 days. After 14 days of drought stress, the reductions in the water contents of the SC1 and SC2 plants measured at only 43 and 33%, respectively, and in the NT plants a 62% reduction was observed (Fig. 7c). The SC1 and SC2 plants maintained significantly ($P = 0.05$) higher water contents than the NT plants after 12 and 14 days of water withholding. The vegetative biomass of the drought-stressed NT plants was also adversely affected as compared to the SC1 and SC2 plants. The gain in dry weight of the SC1 and SC2 plants was decreased by 11 and 6%, respectively, while it was reduced by 55% in the NT plants (Fig. 7b). SC1 and SC2 plants accumulated significantly ($P = 0.05$) higher dry weight during drought stress than the NT plants.

Discussion

In the present study, we have demonstrated the successful *Agrobacterium*-mediated transformation of an elite potato cultivar, "Superior", via the introduction of the

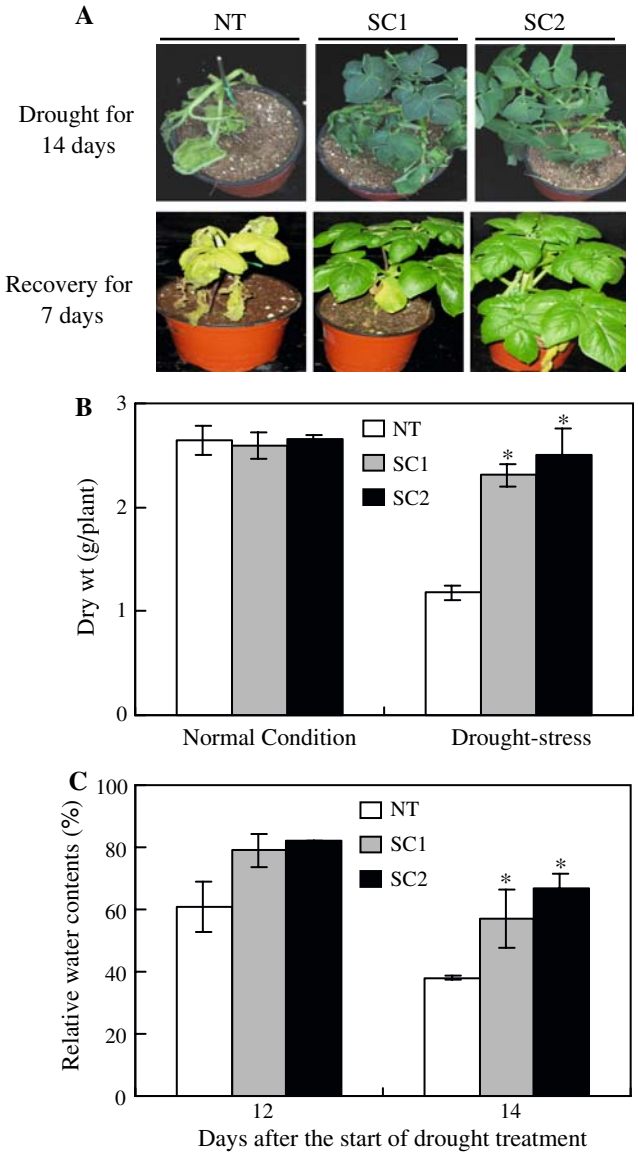

Fig. 7 Drought stress analysis of non-transgenic (*NT*) and transgenic (*SC*) plants. **a** Representative figure taken during the treatment, *upper panel* shows the plant growth condition after 2 weeks of water withholding and the *lower panel* shows the recovered state of plants after one week of water supply. **b** Dry weight of NT and SC plants after drought stress. Dry weights were measured after 10 days of recovery. Data are expressed as the mean ± SD of five replicates. **c** Relative water contents of NT and SC plants after 12 and 14 days of water withholding. The relative water contents were measured from the first fully expanded leaf from the shoot tip. The data are expressed as the mean ± SD of three replicates. *Bars* labeled with *asterisk* show significant differences between the *NT* and *SC* plants by *t*-test at $P = 0.05$

chloroplast-targeted *codA* gene under the control of a stress-inducible *SWPA2* promoter. The introduction of the *codA* gene enabled the transgenic potato plants (SC plants) to synthesize GB, and rendered these plants increasingly tolerant to MV, as well as salt and drought stresses. This work, together with the earlier reports of Hayashi et al. (1997) for *Arabidopsis*, Mohanty et al. (2002) for rice and Park et al. (2004, 2007) for tomato, conclusively demonstrates that engineering for GB synthesis is an effective way to impart stress tolerance to non-accumulators, such as the potato plant. Moreover, this work was conducted using the popular and economically important *Solanum tuberosum* cultivar, "Superior".

The transgenic lines harboring the *codA* gene evidenced enhanced tolerance to a variety of stresses. As the *codA* gene was driven by a stress-inducible promoter, the induction pattern of the *codA* gene under different stress conditions, including MV, salt, and drought verifies the stable integration and transcription of foreign genes in SC plants (Fig. 2). Interestingly, Su et al. (2006) compared the tolerance of transgenic rice plants expressing stress-induced and constitutive GB-synthesizing genes, indicating that the accumulation of biomass tends to be greater in the case of stress-induced GB producers than in constitutively GB-producing plants during salt stress. In addition to this, the transgenic potato plants expressing NDPK2 under the control of the stress-inducible *SWPA2* promoter evidenced significantly enhanced tolerance as compared to constitutively NDPK2-expressing plants (Tang et al. 2007). This evidence allows us to suggest the potential usefulness of inducible promoters for the development of stress-tolerant transgenic crops.

Although the GB level observed (0.9–1.43 μmol/g fresh weight) for SC plants was lower than that of natural accumulators, including spinach and the sugarbeet, it was more or less similar to those described in earlier reports regarding transgenic plants such as *Arabidopsis*, rice, and tomato (Sakamoto and Murata 2000, 2001; Mohanty et al. 2002; Park et al. 2007). Due to the stress-inducible promoter, the GB levels observed after MV treatment were enhanced by four to sixfold as compared to those without MV treatment (Fig. 3). Holmström et al. (2000) reported that GB levels as low as 0.035 μmol/g fresh weight were capable of enhancing the salinity and low-temperature tolerance of transgenic tobacco plants.

ROS have been implicated in all types of stresses, and if not scavenged well in the proper amount of time, they may induce senescence and cell death. The function of GB in protecting the enzymes during stressful conditions has previously been documented in a variety of studies. GB accumulations prevent membrane damage from a variety of environmental stresses (Deshnium et al. 1997; Chen et al. 2000), via direct membrane stabilization (Rudolph et al. 1986) and the maintenance of the water shell that surrounds the surface-exposed membrane proteins (Coughlan and Heber 1982). Transgenic tomato plants expressing the *codA* gene targeted to chloroplasts evidenced enhanced tolerance against MV-mediated oxidative stress (Park et al. 2004,

2007). Moreover, the GB-synthesizing transgenic plants, such as tomato and tobacco, evidenced more profound antioxidant enzyme activity as compared to the NT plants under stress conditions (Park et al. 2007; Yang et al. 2006). Our results are consistent with the findings of previous reports, in which enhanced tolerance has been measured by lower levels of ion leakage under the challenging conditions imposed by MV-mediated oxidative stress (Fig. 4).

Natural GB accumulators are generally salt tolerant. Our transgenic SC plants also evidenced enhanced growth rates under salt stress conditions as compared to NT plants, both in vitro and at the whole plant levels (Figs. 5, 6). It has been determined, via transgenic approaches, that different non-accumulators including *Arabidopsis*, rice, and tomato not only accumulated GB, but also evidenced enhanced tolerance against salt stress (Hayashi et al. 1997; Mohanty et al. 2002; Su et al. 2006; Park et al. 2007). Transgenic tomato plants that synthesize GB in the chloroplasts evidenced higher levels of photosynthetic activity than wild-type plants under salt stress conditions (Park et al. 2007). We assume that the *codA* gene in SC plants is targeted successfully to the chloroplasts, as the same transit peptide was utilized as described by Hayashi et al. (1997). The SC plants maintained higher *Fv/Fm* values over the entire period of salt stress (Fig. 6a). Nishiyama et al. (2006) have presented evidence to suggest that the primary sites at which photo-damage occurs are the oxygen-evolving complex and the D1 proteins. Thus, the inhibition of PSII repair is the principal cause of poor photosynthetic activity occurring under stressful conditions. The protective effect conferred by GB has been shown to guard the machinery required for the degradation and synthesis of the D1 protein under stress exerted by high salt conditions (Ohnishi and Murata 2006). Therefore, it may be concluded that the enhanced photosynthetic activity of SC plants was the result of GB synthesis in the chloroplasts.

GB is a compatible solute, also known as an osmolyte, which performs an important function in plants evidencing enhanced tolerance to osmotic stress. However, the low level of GB accumulation in transgenic plants has raised some questions as to its role as an osmolyte. It has been demonstrated in a variety of previous studies that GB exerts protective effects, and stabilizes macromolecules, enzyme activities, and membranes under stressful conditions (Papageorgiou and Murata 1995; Chen and Murata 2002; Sakamoto and Murata 2002). Quan et al. (2004) reported that GB-synthesizing transgenic maize withstands drought conditions better than wild-type (WT) plants, even though the GB contents were significantly lower than those of the natural accumulators. Moreover, during drought stress, transgenic maize plants accumulated higher levels of soluble sugars and free amino acids than were observed in WT plants, which ensured the enhanced osmotic protection of transgenic plants. The high levels of soluble sugars and free amino acids might result from the protective effects of GB on enzyme activities under stress conditions (Quan et al. 2004). Similarly, transgenic tobacco plants evidencing GB synthesizing abilities also evidenced enhanced tolerance to polyethylenglycol (PEG)-mediated osmotic stress conditions (Shen et al. 2002). According to our data, GB-synthesizing potato plants evidenced enhanced tolerance to drought stress via the maintenance of higher relative water contents than the NT plants, and these plants recovered normal growth after irrigation (Fig. 7). Keeping in view the lower GB levels for osmotic adjustment, we assume that GB may play a role similar to that observed in other transgenic plants, and may perform some additional functions as well, which remain to be elucidated in potato plants.

In conclusion, we successfully developed transgenic potato plants to synthesize GB in chloroplasts under the control of a stress-inducible *SWPA2* promoter. Our results indicated that the successful integration of a chimeric gene resulted in GB synthesis, which subsequently enhanced the tolerance of transgenic potato plants against a variety of abiotic stresses including MV, salt, and drought. This tolerance was assessed by the reduction in the inhibitory effects of salt against photosynthesis efficiency and the maintenance of higher relative water contents under drought stress conditions. Transgenic plants also evidenced enhanced membrane stability against oxidative stress conditions. However, the evaluation of SC plants with regard to yield parameters under different stresses and in field conditions remain to be conducted. In addition, we are attempting to introduce the *codA* gene to transgenic potato plants expressing both CuZnSOD and APX in their chloroplasts (Tang et al. 2006) or in transgenic potato plants expressing NDPK2 in the cytosol (Tang et al. 2007) in order to evaluate the possible synergistic effects of both the transgenic plants. We anticipate that the study of the genetic engineering of the GB biosynthesis pathway in economically important crops, including the potato plant, may hold great promise for the development of a breeding program for plants resistance to multiple abiotic stresses.

Acknowledgments This work was supported by grants from the Biogreen 21 program (Code # 20070301034015), Rural Development Administration, Korea, from the Environmental Biotechnology National Core Research Center, KOSEF/MOST, and from the Korea Foundation for International Cooperation of Science and Technology (KIOCS).

References

Alia, Kondo Y, Sakamoto A, Nonaka H, Hayashi H, Saradhi PP, Chen THH, Murata N (1999) Enhanced tolerance to light stress of transgenic *Arabidopsis* plants that express the *codA* gene for a bacterial choline oxidase. Plant Mol Biol 40:279–288

Ashraf M (2004) Some important physiological selection criteria for salt tolerance in plants. Flora 199:361–376

Bessieres MA, Gibbon Y, Lefeuvre JC, Larher FA (1999) Single-step purification of glycine betaine determination in plant extracts by isocratic HPLC. J Agric Food Chem 47:3718–3722

Bowler C, Slooten L, Vandenbranden S, De R, Botterman JR, Sybesma CJ, Montagu MV, Inze D (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J 10:1723–1732

Chen THH, Murata N, (2002) Enhancement of tolerance to abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol 5:250–257

Chen WP, Li PH, Chen THH (2000) Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. Plant Cell Environ 23:609–618

Coughlan SJ, Heber U (1982) The role of glycinebetaine in the protection of spinach thylakoids against freezing stress. Planta 156:62–69

Deshnium P, Gombos Z, Nishiyama Y, Murata N (1997) The action in vivo of glycine betaine in enhancement of tolerance of *Synechococcus* sp. strain PCC 7942 to low temperature. J Bacteriol 179:339–344

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Holmström KO, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51:177–185

Jeong MJ, Park SC, Byun MO (2001) Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. Mol Cells 12:185–189

Jones RGW, Storey R (1981) Betaines. In: Paleg LG, Aspinal D (eds) The physiology and biochemistry of drought resistance in plants. Academic Press, New York, pp 171–204

Kim SH, Hamada T (2005) Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas* (L) Lam. Biotechnol Lett 27:1841–1845

Kim KY, Kwon SY, Lee HS, Hur Y, Bang JW, Kwak SS (2003) A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol Biol 51:831–838

Kwon SY, Jeong YJ, Lee HS, Kim JS, Cho KY, Allen RD, Kwak SS (2002) Enhanced tolerance of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. Plant Cell Environ 25:873–882

Lee SH, Ahsan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J Plant Physiol doi: 10.1016/j.jplph.2007.01.003

Lim S, Kim YH, Kim SH, Kwon SY, Lee HS, Kim JS, Cho KY, Paek KY, Kwak SS (2007) Enhanced tolerance of transgenic sweetpotato plants that express both CuZnSOD and APX in chloroplasts to methyl viologen-mediated oxidative stress and chilling. Mol Breeding 19:227–239

Ma QQ, Wang W, Li YH, Li DQ, Zou Q (2006) Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum*) by foliar-applied glycinebetaine. J Plant Physiol 163:165–175

Mohanty A, Kathuria H, Ferjani A, Sakamoto A, Mohanty P, Murata N, Tyagi AK (2002) Transgenics of an elite *indica* rice variety Pusa Basmati 1 harboring the *codA* gene are highly tolerant to salt stress. Theor Appl Genet 106:51–57

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473–497

Newell C, Rozman R, Hincee MA, Lawson EC, Haley L, Sanders P, kaniewski W, Tumer NE, Horsh RB, Fraley RT (1991) *Agrobacterium*-meidated transformation of *Slonaum tuberosum* L.cv. Russet Burbank. Plant Cell Rep 10:30–34

Nishiyama Y, Allakhverdiev SI, Murata N (2006) A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. Biochem Biophys Acta 1757:742–749

Ohnishi N, Murata N (2006) Glycinebetaine counteracts the inhibitory effects of salt stress on the degradation and synthesis of D1 protein during photoinhibition in *Synechococcus* sp. PCC 7942. Plant Physiol 141:758–765

Papageorgiou GC, Murata N (1995) The unusually strong stabilizing effects of glycinebetaine on the structure and function of the oxygen-evolving photosystem II complex. Photosyn Res 44:243–252

Park EJ, Jeknic Z, Pino MT, Murata N, Chen TH (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. Plant Cell Environ 30:994–1005

Park EJ, Jeknic Z, Sakamoto A, Denoma J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. Plant J 40:474–487

Quan R, Shang M, Zhang H, Zhao Y, Zhang J (2004) Engineering of enhanced glycine betaine syntesis improves drought tolerance in maize. Plant Biotechnol J 2:477–486

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in the higher plants. Annu Rev Plant Physiol Plant Mol Biol 44:357–384

Rudolph AS, Crowe JH, Crowe LM (1986) Effects of three stabilizing agents-proline, betaine, and trehalose-on membrane phospholipids. Arch Biochem Biophys 245:134–143

Sakamoto A, Murata N (2000) Genetic engineering of glycinebetaine synthesis in plants: Current status and implications for enhancement of stress tolerance. J Exp Bot 51:81–88

Sakamoto A, Murata N (2001) The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiol 125:180–188

Sakamoto A, Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ 25:163–171

Sakamoto A, Alia, Murata N (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Mol Biol 38:1011–1019

Sakamoto A, Valverde R, Alia, Chen THH, Murata N (2000) Transformation of *Arabidpsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. Plant J 22:449–453

Sayari AH, Bouzid RG, Bidani A, Jaoua L, Savoure A, Jaoua S (2005) Overexpression of Δ^1-pyrroline-5-carboxylazte synthetase increase proline production and confers salt tolerance in transgenic potato plants. Plant Sci 169:746–752

Shen YG, Du BX, Zhang WK, Zhand JS, Chen SY (2002) AhCMO regulated by stresses in *Atriples hortensis*, can improve drought tolerance in transgenic tobacco. Theor Appl Genet 105:815–821

Su J, Hirji R, Zhang L, He C, Selvaraj G, Wu R (2006) Evaluation of the stress-inducible production of choline oxidase in transgenic rice as a strategy for producing the stress-protectant glycine betaine. J Exp Bot 57:1129–1135

Tang L, Kwon SK, Kim SH, Kim JS, Choi JS, Cho KY, Sung CK, Kwak SS, Lee HS (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and

ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. Plant Cell Rep 25:1380–1386

Tang L, Kim MD, Yang KS, Kwon SK, Kim SH, Kim JS, Yun DJ, Kwak SS, Lee HS (2007) Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses. Transgenic Res doi: 10.1007/s11248-007-9155-2

Turhan H (2005) Salinity response of transgenic potato genotypes expressing the oxalate oxidase gene. Turk J Agric 29:187–195

Verslues PE, Agarwal M, Agarwal SK, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect water status. Plant J 45:523–539

Yang X, Wen X, Gong H, Lu Q, Yang Z, Tang Y, Liang Z, Lu C (2006) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225:719–733

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66–71