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GLYCINEBETAINE SYNTHESIZING TRANSGENIC POTATO PLANTS EXHIBIT ENHANCED TOLERANCE TO SALT AND COLD STRESSES

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Abstract

Abiotic stresses are the most important contributors towards low productivity of major food crops. Various attempts have been made to enhance abiotic stress tolerance of crop plants by classical breeding and genetic transformation. Genetic transformation with glycinebetaine (GB) synthesizing enzymes' gene(s) in naturally non accumulating plants has resulted in enhanced tolerance against variety of abiotic stresses. Present study was aimed to evaluate the performance of GB synthesizing transgenic potato plants against salt and cold stresses. Transgenic potato plants were challenged against salt and cold stresses at whole plant level. Transgenic lines were characterized to determine the transgene copy number. Different parameters like membrane integrity, chlorophyll contents, tuber yield and vegetative biomass were studied to monitor the stress tolerance of transgenic potato plants. The results were compared with Non-transgenic (NT) plants and statistically analyzed to evaluate significant differences. Multi-copy insertion of expression cassette was found in both transgenic lines. Upon salt stress, transgenic plants maintained better growth as compared to NT plants. The tuber yield of transgenic plants was significantly greater than NT plants in salt stress. Transgenic plants showed improved membrane integrity against cold stress by depicting appreciably reduced ion leakage as compared to NT plants. Moreover, transgenic plants showed significantly less chlorophyll bleaching than NT plants upon cold stress. In addition, NT plants accumulated significantly less biomass, and yielded fewer tubers as compared to transgenic plants after cold stress treatment. The study will be a committed step for field evaluation of transgenic plants with the aim of commercialization.

Key words: Transgenic, Potato, Choline oxidase, Glycinebetain, Abiotic stress tolerance.

Introduction

In an era where population and its demand surpasses food supply, crop losses due to environmental stresses such as salinity, extremes of temperature, and drought are adding serious threats to global food security. Environmental stresses are well known to negatively affect crop productivity; these stresses can reduce average yields of major crop plants by more than 50% (Wang *et al*., 2003). The situation becomes worst in the scenario of global warming and climate change. Worldwide, 25% of total irrigated acreage has been adversely affected by salt. This situation is expected to get even worst as, by year 2050, more than 50% of all arable lands is estimated to be affected by salinization (Ashraf, 2004). This daunting situation demands the improvement of environmental stress tolerance of important food crops, especially those having potential to resolve food security issues.

Today, potato is grown in 125 countries and more than a billion people worldwide consume it on daily basis (Mullins *et al*., 2006). Potato is at 4th most important food crop after wheat, maize and rice (Newell *et al*., 1991). Environmental stresses hamper its productivity. The optimum temperature for growth of most potato cultivars is 17-20ºC (Burton *et al*., 1981; Demagante & Vander 1988). Potato is considered salt sensitive plant and presence of 50 mM NaCl can reduce 50% of growth of potato plants (Sayari *et al*., 2005). Similarly, contineous exposure to low temperature also adversely affects the potato plant growth (Evers *et al*., 2007).

Traditional breeding has been extensively used for crop improvement however this approach is too much time consuming; 15 years are required to develop a potato cultivar by classical breeding (Mullins *et al*., 2006). Attempts have been made to develop germplams more tolerant to abiotic stresses by using genetic engineering technology (Sakamoto *et al*., 1998; Sakamoto & Murata, 2002; Park *et al*., 2004; Tang *et al*., 2006; Lim *et al*., 2007). Different genes of varying functions from osmoprotectants to reactive oxygen (ROS) scavenging have been utilized to improve potato plants tolerance (Jeong *et al*., 2001; Sayari *et al*., 2005; Turhan, 2005; Tang *et al.,* 2006; Ahmad *et al*., 2008).

Plants respond to abiotic stresses by induction of different transcription factors and various genes including different osmolytes such as glycinebetaine (hereafter GB). GB is potent compatible compound which does not impede normal metabolism of plants. Natural accumulators of GB (spinach, sugar beet andmangroves) are tolerant to different stresses (Ashraf, 2004; Sakamoto

& Murata, 2002). Previously, it has been reported that exogenous application of GBenhanced the activity of various antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), during stress conditions (Banu *et al*., 2009; Sairam *et al*., 2002). Additionally, *In vivo* synthesis of GB, by overexpression of GB-synthesizing enzymes in GB non-accumulator plants, has resulted in GB synthesis albeit less than natural accumulators. Due to its diverse positive effects to ameliorate stresses other than an osmolyte, transgenic GB synthesizing plants showed enhanced tolerance to various abiotic stresses (Ahmad *et al*., 2010; Park *et al*., 2007; Yang *et al*., 2007).

In previous study, we developed transgenic potato plants expressing GB synthesizing choline oxidase (*codA*) gene from *Arthrobacter globiformis*, targeted to chloroplasts (Ahmad *et al*., 2008). The resultant transgenic plants (hereafter referred to as SC plants) showed stable integration, and expression of chimeric gene, resulting in accumulation of GB. Transgenic plants manifested tolerance to oxidative, drought and salt stress in *In vitro* conditions. Here, we report further characterization of SC plants, at whole plants level, as first step towards field evaluation. SC plants were challenged against salt and cold stress to evaluate protective effects of GB on growth and development. Moreover the positive effects of GB accumulation on yield of transgenic plants, exposed to stressful conditions, were also studied.

Materials and Methods

Southern blot analysis of transgenic lines: Genomic DNA was isolated from potato leaves (about 1.0g of fresh weight) as described by Kim & Hamada (2005). Total DNA (30µg each) was digested with *Eco*RI, then subjected to electrophoresis on 0.8% (w/v) agarose gel. DNA was denatured by placing the gel in a bath of 0.4 M NaOH and then blotted to a Zeta-probe GT blotting membrane (Bio-Rad, CA, USA) by capillary transfer. Probe was labeled with [α-32P] dCTP, using a Redi prime kit (Amersham, USA). The membrane was pre-hybridized at 65°C, for 2-4 h, in a buffer containing 0.25 M Sodium phosphate (pH 7.2) and 7% SDS. The membrane was hybridized at 65°C for 18-24 h in the presence of the denatured labeled 1Kb *codA* fragment, and washed twice at room temperature, in 0.02 M sodium phosphate buffer (pH 7.2) and 1% SDS, for 10 min each.

Salt stress analysis: For salt stress analysis, *In vitro* rooted plantlets transgenic and non-transgenic (NT) were transferred to soil-filled pots, and allowed to grow in a growth chamber with a 16 h/8 h photoperiod, at 25ºC, and in 100 µmol photons m^{-2} s^{-1} light conditions, for 4 weeks. Similar amount of soil was added in the pots and two plants from each (NT, SC1andSC2) were planted in one rectangular pot to ensure similar conditions. After 4 weeks of growth in the green house, NaCl (100 mM) was applied on alternate days to the pots by dipping them for 30 minutes in salty solution. The salt treatment was continued until the death of plants (8 weeks). Tubers were collected and yield was compared with the plant grown in normal conditions.

Analysis of membrane integrity: The leaf disc assay for membrane integrity analysis of plants exposed to low temperature was conducted by measuring ion leakage as an indicator of solute leakage. Leaf discs (6 mm in diameter) were prepared from the fifth fully opened leaf from the top of 6 week-old plants. The leaf discs were floated on solution containing 0.4% sorbitol, to avoid any osmotic shock. The samples were incubated in continuous light (100 µmol photon m^{-2} s^{-1}) at 4ºC. Ion leakage of solution was recorded at specified time using an ion conductivity meter. At the end, the samples were autoclaved for 15 min at 121ºC in order kill the leaf tissues to release all solutes. The conductivity of the autoclaved solution was considered 100% ion leakage for calculations of the relative ion leakage.

Cold stress treatment and tolerance assay: *In vitro* rooted NT and transgenic plants were transferred to pots and allowed to grow for another 4 weeks in growth chamber as described above. Afterwards plants were challenged to cold stress by putting the pots at 4ºC at 16 h/8 h day/night photoperiod for one week. Top most fully opened leave of each plant was marked for post stress analysis of biomass accumulation and non-destructive chlorophyll content analysis. After the cold stress treatment for one week, plants were shifted back to normal growth conditions for recovery. After 4 weeks of post-cold stress growth, stems were cut from the marked leave. The samples were oven dried for 36 h at 72ºC. The tubers of the plants were also collected and weighed for comparison.

Analysis of changes in chlorophyll contents after cold stress treatment: Chlorophyll contents of marked leave (as described above) were recorded by non-destructive chlorophyll content meter before the onset of cold stress treatment to serve as a reference. Later on chlorophyll contents were measured after 2, 4 and six days post cold stress treatment. Percentage of chlorophyll contents was measured by considering the pre-stress chlorophyll contents as 100%.

Statistical analysis: Assays to record ion leakage, dry weight, yield analysis and chlorophyll contents were repeated five times. Data were analyzed by Student's t-test by using Microsoft Excel 2007. The significance was determined at $p \leq 0.05$ level.

Results

Southern Blot confirms successful integration of chimeric gene in transgenic plants: Transgenic plants were primarily confirmed by PCR amplification of transgene. This was followed by analyzing the expression of transgene through qRT-PCR.[18]However, to further confirm the proper integration of foreign gene in the transgenic plants, southern blot analysis was performed. Genomic DNA extracted from transgenic SC1 and SC2 potato plants was digested with *Eco*R1 and hybridized with the gene specific (*codA*) probe. The SC1 and SC2 lines showed multi copy insertion. The SC1 revealed four copies while the SC2 showed two copies of T-DNA (Fig. 1). This data confirmed the stable integration of transgene in potato genome.

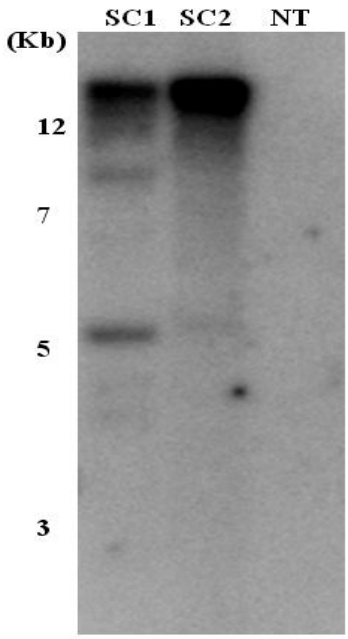

Fig. 1. Southern blot analysis of transgenic SC1 and SC2 lines. Genomic DNA digested with EcoR1 was used in gel and probed with 1Kb fragment of *codA* gene.

Transgenic plants showed normal growth and higher tuber yield in Salt stress conditions: Salt stress causes multiple stresses at a time and plants with shallow rooting system are particularly vulnerable to it. Salt stress analysis was performed to compare the tuber yield of NT and SC plants. Potato plants were transferred to soil and allowed to grow for a period of 4 weeks in growth chamber as described in material and method section. As depicted by representative photograph (Fig. 2A) there was not phenotypic difference between NT and SC transgenic plants, at normal (unstressed) conditions. All the plants were equally healthy at the onset of salt stress treatment. However, clear phenotypic differences between NT and SC plants were observed once plants were exposed to salt stress. NT plants were badly affected by salt stress and showed visible decrease in chlorophyll contents, burning of old leaves and stunted growth, while the SC plants maintained much better health during the stressful conditions (Fig. 2B). After the plants were died, tubers were collected and tuber yield was compared with that obtained in normal conditions (Fig. 3). Tuber morphology of stress-exposed NT and SC plants was also compared. As shown in Fig. 3A, no clear difference was observed between NT and transgenic lines. The tuber yield of NT and SC plants was similar in normal conditions while the yield of NT was decreased by 60% after salt stress. Contrary to NT plants, SC plants showed resistance to salt stress and showed higher tuber yield. The yield of SC1 and SC2 plants were decreased by only 28% and 11%, respectively (Fig. 3B). Transgenic SC plants produced significantly ($p = 0.05$) higher tubers as compared to NT, at salt stress conditions.

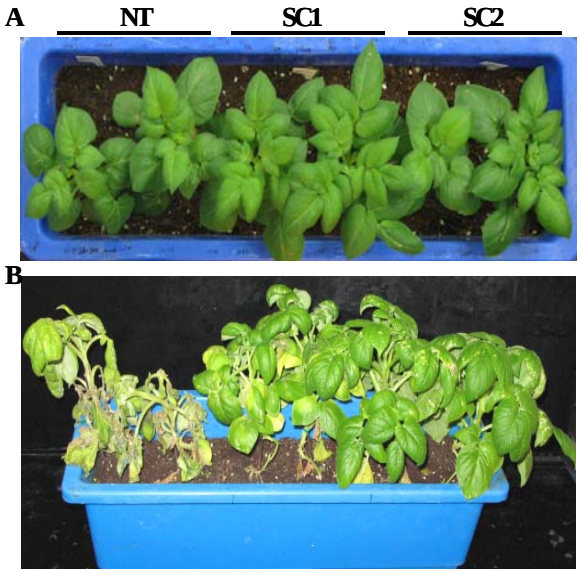

Fig. 2. Phenotype of NT, SC1 and SC2 plants before and after salt stress. (A) Phenotype of NT, SC1 and SC2 plants after 4 weeks of growth in green house before salt (100 mM) stress, which was applied on alternate days by dipping the pots in salty solution for 30 minutes. (B) phenotype of NT and SC plants after 8 weeks of salt stress.

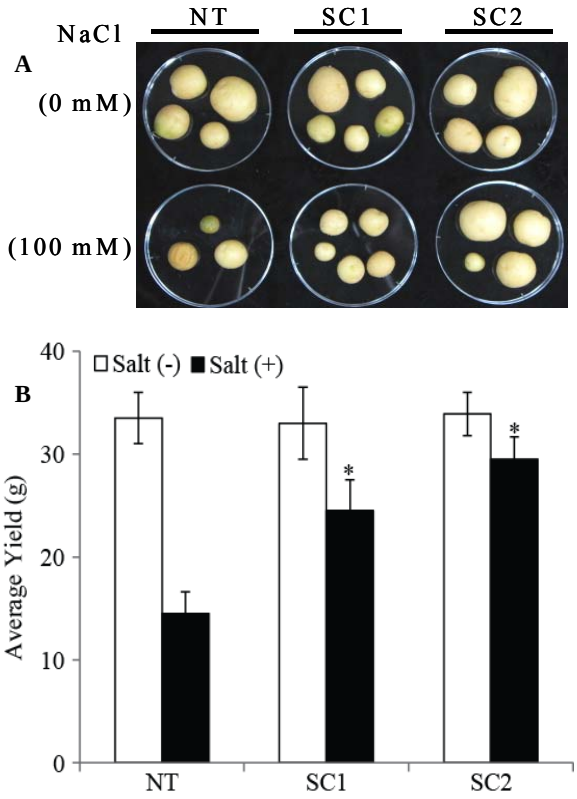

Fig. 3. Effect of salt stress on tuber yield. (A) Morphology of tubers from NT and SC plants grown in normal and in salty conditions. (B) comparison of yield of NT and SC plants after salt stress. Data are expressed as the means ± SD of five replicates. Bars labeled with asterisk show significant differences between NT and SC plants by *t-test* at p =0.05.

GB accumulating plants manifested enhanced membrane stability during the entire course of cold stress treatment: Leaf discs of NT and SC plants were prepared from plants at the same age, floated on 0.4% sorbitol solution and incubated at 4ºC. Membrane stability of NT and SC lines, exposed to cold stress, was assayed, through ion leakage. This parameter is considered to be a good indicator of membrane stability (Tang *et al*., 2006). When both line were exposed to cold stress, SC plants evidenced less ion leakage in comparison with NT counterparts. Moreover, both SC and NT lines showed similar trend throughout the course of treatment. . SC1 and SC2 plants evidenced significantly less (p = 0.05) ion leakage under as compared to NT plants. After 48 h of cold stress treatment, the ion leakage of SC1 and SC2 transgenic lines was 39 and 45%, respectively, while it was 59% for NT plants (Fig. 4).

Reduced chlorophyll bleaching of transgenic plants was observed after cold stress treatment: The changes in the chlorophyll contents of NT and SC plants were also measured after cold stress (Fig. 5). As mentioned in material and methods, readings were recorded at day 6 after the removal of cold stress treatment. When exposed to cold stress, the chlorophyll content of NT plants started to decrease earlier than that of SC plants. Moreover, statistically significant (p = 0.05) differences were observed in chlorophyll content of NT and SC lines. NT plants exhibited approximately 20% decrease in chlorophyll content, while SC1 and SC2 plants showed 15 and 13% reduction in chlorophyll contents, respectively, after 6 days of post-stress recovery period (Fig. 5).

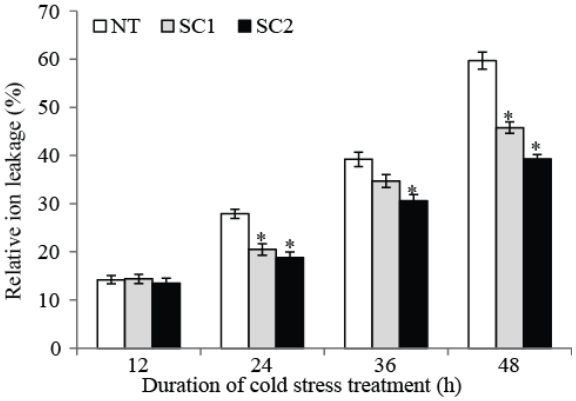

Fig. 4. Effect of cold stress treatment on ion leakage of non-transgenic (NT) and *codA* expressing SC1 and SC2 transgenic plants. Leaf discs were incubated in 0.4% sorbitol solution at 4ºC for 48 h. Ion leakage was measured after 12, 24, 36 and 48 h of cold stress. Percentages of ion leakage were calculated using 100% to represent values obtained after autoclaving. NT corresponds to non-transgenic while SC1 and SC2 are independent transgenic lines. Data are expressed as the mean ± SD of five replicates. Bars labeled with asterisk show significant differences between NT and SC plants by *t-test* at p = 0.05.

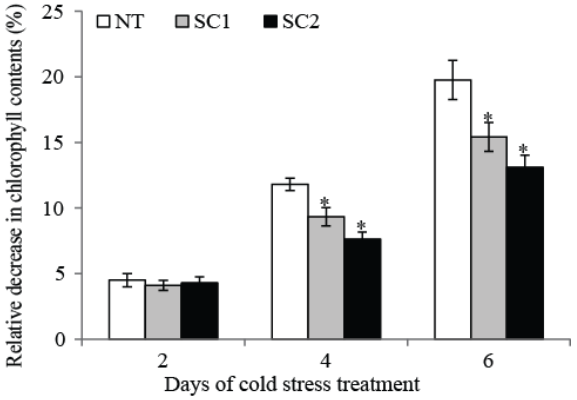

Fig. 5. Effect of cold stress (4ºC) on chlorophyll bleaching of NT and transgenic (SC1, SC2) plants. Soil grown plants were challenged to cold stress for one week time. Effect of cold stress on bleaching of chlorophyll was analyzed after 2, 4 and 6 days post cold stress from the specified leave. Percentage of chlorophyll contents was measured by considering the pre-stress chlorophyll contents as 100%. Data are expressed as the mean ± SD of five replicates. Bars labeled with asterisk show significant differences between NT, SC1 and SC2 plants by *t-test* at p = 0.05.

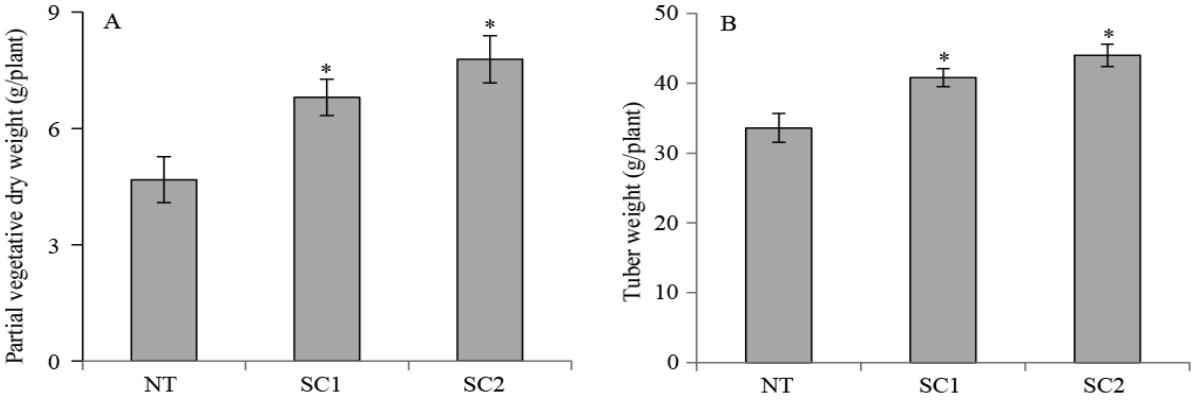

Fig. 6. Effect of cold stress (4ºC) on biomass accumulation of NT and transgenic SC1 and SC2 plants. Four week old plants were stressed for a week. After one of stress, plants were kept in normal green house conditions for 4 more weeks to evaluate aerial parts and underground parts biomass accumulation. (A) Aerial parts dry weight biomass of NT, SC1 and SC2 plants. Top most fully opened leaf was marked before the onset of cold stress treatment. After the completion of recovery period of 4 weeks, plants were cut from the designated leaf and dry weight was measured after drying for 36 h at 72ºC. (B) Tuber weight of NT, SC1 and SC2 plants stressed at 4ºC. Data are expressed as the mean ± SD of five replicates. Bars labeled with asterisk show significant differences between NT, SC1 and SC2 plants by *t-test* at p = 0.05.

SC transgenic lines produced higher biomass during cold stress treatment: Keeping in view the versatile protective role of GB, we analyzed biomass accumulation of transgenic SC plants after cold stress treatment at 4ºC and compared with NT plants. Four weeks old plants were challenged at low temperature for a week and then subjected to a recovery period of 4 weeks. The above ground partial dry weight contents were measured from designated leaf. Transgenic SC plants exhibited significantly ($p = 0.05$) higher partial dry weight after cold stress treatment (Fig. 6A). NT plants accumulated 4.68 g/plant dry weight while SC1 and SC2 depicted 6.8 and 7.78 g/plant dry weight, respectively (Fig. 6A). Tuber yield of NT and transgenic SC plants was also determined after cold stress treatment. SC plants produced significantly ($p = 0.05$) more tubers as compared to NT plants. NT plants produced 33.6 g/plant tubers while SC1 and SC2 yielded 40.8 and 44 g/plant tubers, respectively (Fig. 6B).

Discussion

Glycinebetaine (hereafter GB), a ziwitterionic, fully N-methyl substituted glycine derivative, has been detected in a wide variety of microorganisms, higher plants, and animals (Sakamoto & Murata, 2002). Several species from diverse taxonomic backgrounds, like wheat, sugar beet, spinach and halotolerant cyanobacteria synthesize GB (Jones & Storey, 1981). On the contrary, many other economically important crop plants like rice, potato and tomato do not synthesize GB; therefore, they are called as GB non-accumulators.

Role of GB in to protect enzymes in stress has previously been reported in different studies. GB accumulation prevents 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 *et al*., 1982). Tomato plants expressing the *codA* gene, targeted to chloroplasts, evidenced enhanced tolerance against methyl viologen (MV)-mediated oxidative stress (Park *et al*., 2004, 2007). Moreover, tomato and tobacco transgenic plants with GB synthesizing genes depicted improved antioxidant enzymes activities as compared to the Non-transgenic plants under stress conditions (Park *et al*., 2007; Yang *et al*., 2007).

Previously, we reported successful development of potato plants expressing bacrerial *codA* gene, targeted to chloroplasts, under the control of stress inducible SWPA2 promoter (Ahmad *et al*., 2008). These transgenic plants successfully synthesized GB and showed inducible expression pattern of chimeric genes. Resultantly, transgenic plants designated as SC plants showed enhanced tolerance to various environmental stresses such as salt, drought and oxidative stress in in-vitro conditions. To extend our experiments, we conducted salt and cold stress analysis to evaluate the protective effects of GB on biomass accumulation of transgenic plants.

Salt stress poses various threats to plants such as; a) drought-like conditions, b) ionic toxicity and c) oxidative stress. GB is a potent osmolyte and ameliorates negative effects of salt stress in natural accumulators such as spinach and sugar beet. Our transgenic SC1 and SC2 potato plants exhibited better growth when exposed to salt stress. The NT plants showed retarded growth and died before the SC plants. SC plants showed better tuber production after salt stress (Fig. 3). Few studies have been conducted on yield trials of GB synthesizing transgenic crop plants exposed to salt stress, such as cotton (Zhang *et al*., 2009), rice (Mohanty *et al*., 2002), tomato (Zhou *et al*., 2007) and wheat (He *et al*., 2010). All these transgenic crop plants exhibited enhanced yield as compared to NT plants. Our study also depicted improved yield of transgenic potato plants after salt stress. GB is well known to function like a chaperone to protect enzymatic activities during stress, as it has been reported to protect Rubisco and malate dehydrogenase activities during salt stress (Aran *et al*., 2986). It is also involved to help in refolding and stability of proteins in stressful environment (Diamant *et al*., 2003). In our previous study, SC plants maintained higher photosynthetic activity as compared to NT plants during salt stress treatment (Ahmad *et al*., 2008). Improved yield of SC plants under salt stress can be attributed to the synthesis and subsequent protective actions of GB towards enzymes involved in photosynthesis

Various studies have shown that the GB synthesis in naturally non-accumulators such as brassica, persimmon, carrot, rice, tomato, potato etc resulted in enhanced tolerance to various stresses such as drought, salt, and chilling (Ahmad *et al*., 2013 and references therein). Although potato plant tolerate cold environment very well, but continuous low temperature coupled with frost initiate ice nucleation in the cell, which results in severe damage to crop. Potato cultivars respond differently to cold stress, particularly *Solanum tuberosum* were found to be less tolerant to cold stress (Evers *et al*., 2007). This variation in tolerance to cold stress was mainly due to differential gene expression (Oufir *et al*., 2008).We comprehensively studied the effects of cold stress on potato plants by evaluating the membrane stability to cold stress-mediated stress. Various stresses coupled with oxidative stress result in membrane damage which can be effectively monitored my measuring the ion leakage. SC plants showed less ion leakage as compared to NT plants. GB synthesis play diverse role to impart stress tolerance. It was reported that GB synthesis in potato plants stabilizes the activities of reactive oxygen (ROS) scavenging enzymes such as superoxide dismutase and ascorbate peroxidase (Ahmad *et al*., 2010). GB synthesizing transgenic tomato plants also exhibited enhanced tolerance to chilling stress and enhanced fruit setting (Park *et al*., 2004). In addition to improved membrane stability, SC plants maintained higher chlorophyll contents and produced more aerial and underground biomass as compared to NT plants, after cold stress treatment. The improvement can be due to the synthesis of GB, which play a significant role in imparting stress tolerance against various stresses.

Conclusion

GB synthesis in naturally non-accumulator potato plants by transformation of *codA* gene improved salt and cold stress tolerance by various ways such as, stabilizing photosynthetic activities, improved ROS scavenging and reduced chlorophyll bleaching. This enhanced tolerance was translated to improved tuber yield of SC plants in stress conditions. This study prompts us to evaluate performance of SC plants in the field as well to make advancements for commercialization.

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