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Research Article

# Overexpression of *Choline Oxidase* Gene in Three Filial Generations of Rice Transgenic Lines

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**Background:** Glycinebetaine (GB) accumulation in many halophytic plants, animals, and microorganisms confers abiotic stress tolerance to salinity, drought, and extreme temperatures. Although there are a few genetic and biochemical pathways to synthesize GB, but isolation of a single gene *choline oxidase (codA*) from *Arthrobacter* spp. have opened a new hatch to engineer the susceptible plants.
**Objectives:** The effects of overexpressed *codA* gene, through multiple copy insertion and GB accumulation on salinity tolerance in rice were studied.
**Materials and Methods:** Seed-derived embryogenic calli of 'Tarom Molaie' cultivar were targeted with two plasmids pChlCOD and pCytCOD both harboring the *codA* gene using the biolistic mediated transformation. The regenerated $T_0$ plants were screened by PCR analysis. A line containing three copies of *codA* gene and harboring pChlCOD and pCytCOD was identified by Southern blot analysis. The expression of *codA* gene in this transgenic line was then confirmed by RT-PCR. The Mendelian segregation pattern of the inserted sequences was accomplished by the progeny test using PCR. The effects of overexpression of *codA* on salinity tolerance were evaluated at germination and seedling stage using $T_2$-pChl transgenic line and control seeds in the presence of 0, 100, 200, and 300 mM NaCl. Finally, leaf growth dynamics of $T_2$-pChlCOD transgenic line and control line under hydroponic conditions in the presence of 0, 40, 80, and 120 mM NaCl were assessed.
**Results:** The seed germination experiment results showed that the transformed seeds had a higher germination rate than the controls under all salinity treatments. But also, the leaf growth dynamics showed that the control plants had a more favorable leaf growth dynamic in all of the treatments. Although, the transgenic lines ($T_0$, $T_1$ and $T_2$) exhibited lower performance than the wild type, the transgenic line varied for GB and choline contents and increasing *codA* gene copy number led to increased GB content.
**Conclusion:** In a salinity sensitive crop such as rice, GB may not significantly contribute to the plant protection against salt stress. Also, insufficiency of choline resources as GB precursor might have affected the overall growth ability of the transgenic line and resulted in decreased leaf growth dynamics.

*Keywords:* Glycine betaine (GB), *Oryza sativa* L, Rice, Salt stress, Transformation

## 1. Background

Many studies conducted on the role of glycine betaine (GB) in stress tolerance have confirmed that GB accumulation in many halophytic plants, animals, and microorganisms occurs in response to abiotic stress conditions such as salinity, drought, and extreme temperatures (1). GB is a dipolar, highly soluble, and typical hydrophilic osmoprotectant which protects macromolecules during abiotic stresses. In addition, it can act as an osmoregulator and scavenger of radical oxygen forms (2). Therefore, it has been assumed that enhanced tolerance against stresses could be achieved

by introducing the GB accumulation capability to stress-sensitive plants.

Although different GB synthesizing pathways such as the direct *N*-methylation of glycine and the two-step conversion of choline to GB pathway had been identified in several plant and microbial species, it was the isolation of a single gene responsible for converting choline to GB in *Arthrobacter glabriformis* which led to the engineering of the pathway *via* transformation (3). Thereafter, GB accumulation in some transgenic organisms such as *Synecochoccus* (3, 4), *Arabidopsis* (5,6), tobacco (7), rice (8, 9), *Brassica juncea* (10), potato (11), chickpea and Indian mustard (12), and tomato (13) was targeted. However, the GB content in these transformed plants was not sufficient to generate a proper tolerance against the stresses. Only one report on engineering of eucalyptus tree with *cod*A gene indicates that GB could be successfully compensated for the negative effects of salinity stress (14).

It then appears that, while GB is accumulated at high concentrations (4–40 μmoL.g$^{-1}$ fresh weight) in naturally-GB accumulating plants like spinach and sugar beet, transgenic plants carrying GB-synthesizing genes reportedly produce much less amounts of GB (0.05–5 μmoL.g$^{-1}$ FW; 15, 16). Moreover, localization of the gene product in chloroplast or cytosol resulted in different GB contents in transgenic lines. More specifically, GB accumulation in cytosol was 3-5 times more than in chloroplast, though the efficiency of the accumulated GB in chloroplast was much higher than the one in cytosolic (8).

Given the fact that the GB content in the transgenic lines of the engineered plants was not sufficient to create a proper protection against abiotic stress, we hypothesized that higher GB accumulation by increasing the dosage of the gene in transgenic rice might be a solution. Gene dosage is defined as the number of copies of a gene of interest in a genome (17). Gene dosage is known to be related to the amount of gene product a cell is able to express (18). It should be mentioned that although changes in gene dosage due to gene insertions could be accompanied with significant phenotypic consequences, it is still worth being investigated.

## 2. Objective

The objective of this study was to evaluate the effects of *codA* gene copy number on GB accumulation and its physiological consequences in the transgenic rice lines expressing the gene.

## 3. Materials and Methods

### *3.1. Plant Materials*

An Iranian aromatic rice cultivar, "Tarom Molaie" was used in this study. Callus induction and regeneration ability of the cultivar was confirmed in a previous study (19).

### *3.2. Tissue Culture, Gene Transformation, and Regeneration*

De-husked and surface-sterilized seeds were sown in Petri dishes containing N6 medium (20) supplemented with 2mg.L$^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D) as growth regulator for callus induction. Embryogenic calli were harvested from 5-6-week cultures and were subjected to biolistic gene transformation. Two plasmid vectors comprising pCytCOD and pChlCOD (8) were used in transformation. These plasmids contained *npt*II (as bacterial selectable marker), *hph* (as plant selectable marker), and *choline oxidase A* (*codA*) genes (**Fig. 1**).

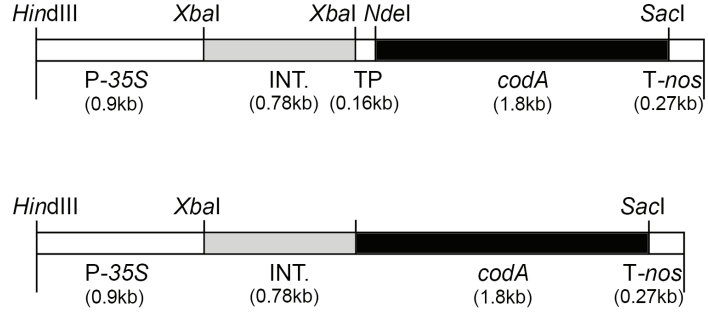


**Figure 1.** The physical map of the *codA* coding region of the pChlCOD (top) and the pCytCOD (bottom) vectors. The difference between the vectors is that the pChlCOD has a transit peptide sequence which targets the produced enzyme toward chloroplast (Sakamoto *et al.* 1998).

The pChlCOD included a leader sequence to localize the gene product in chloroplast (8).

Subsequently, gold particles (1 μm in diameter) were coated with the plasmid DNA and were accelerated towards embryogenic calli originated from mature seeds, arranged in the center of Petri dishes. A Bio-RAD PDS-1000 apparatus and 1100 psi rupture disk in 7 cm distance were used for transformation. Targeted calli were transferred into N6 medium (20) containing 50 mg. $L^{-1}$ hygromycin B one day after transformation, and were subcultured 3 times in the same medium with 20-d intervals. Putative transformed calli were transferred into regeneration medium consisting of MS nutrients (21) supplemented with 50 mg.$L^{-1}$ hygromycin B, 20 g.$L^{-1}$ maltose, 2mg.$L^{-1}$ kinetin, and 3 mg. $L^{-1}$ Naphthalene acetic acid (NAA) as growth regulators, 50 mg$L^{-1}$ tryptophan, and 4 g.$L^{-1}$ agarose. Light intensity for the regeneration was set at about 6000 lux and a 16/8 h day/night photoperiod with 25/20 °C temperature values, respectively, was considered during the regeneration. Regenerated and rooted plants were then transferred into hydroponic medium (22) until flowering and seed production.

*3.3. PCR, Southern Blotting, and RT-PCR Analyses in Putative $T_0$, $T_1$, and $T_2$ Plants*

DNA from the putative transformed plants was extracted according to Dellaporta *et al.* (23). PCR analysis using specific primers for *CodA* and *hph* genes were performed, and a random specific primer, i.e., RG100, which primes a 960 bp sequence in rice genome, was used as internal control.

Southern analysis was conducted using 50 μg of the digested DNA with *Hind* III enzyme (**Fig. 2**). Electrophoresis, blotting with conventional capillary method, probe synthesis, hybridization, and detection were performed according to the standard protocols or supplier recommendations (Roche, Cat No. 1 093 657).

RNA was extracted according to Chomczynski and Sacchi (24). About 1 μg of the extracted RNA was used in a two-step Reverse-Transcription PCR (RT-PCR) reaction. In the first step, cDNA synthesis using the *CodA* primer was performed, and a simple PCR analysis was carried out in the second step, while RG100 primers was used as internal control. In order to detect any DNA contaminations in the extracted RNA samples, another control reaction for each line was considered in the second step of the analysis where 50 ng RNA was used as template in the reaction.

*3.4. Extraction and Quantification of GB and Choline*

Rhodes and Rich (25) protocol were used for GB and choline extraction. Two gr of sliced dried leaf were flooded into 10 mL methanol in a 50 mL falcon and were refrigerated for 2 d. Then 5 mL of chloroform and 6 mL of distilled $H_2O$ were added to the flask and were shaken gently for 5 min. The upper layer was transferred into a new falcon and was air-dried. The precipitated layer was dissolved in 2 mL of distilled $H_2O$ and filtered through a 0.45 μm filter. GB content was then measured by an HPLC KNAUER apparatus equipped with an Eurokat H 10 μm 300 × 8 mm column and a UV K-2501 detector at 190 nm wavelength. $H_2O$ (pH: 2.0) was used as mobile phase with 0.7 mL. $S^{-1}$ flow rate. $T_0$, selected $T_1$, and respected $T_2$ progeny lines were subjected to GB quantification.

*3.5. Growing Segregation Generations and Analysis*

Seeds of the selected transgenic lines were grown to achieve $T_1$ generation. Dominant homozygous $T_1$ progenies were then selected based on the PCR analysis performed on the $T_2$ progenies for both parental transgenic lines. The seeds of the parental $T_1$ homozygous line for each line were harvested and used in the subsequent analyses.

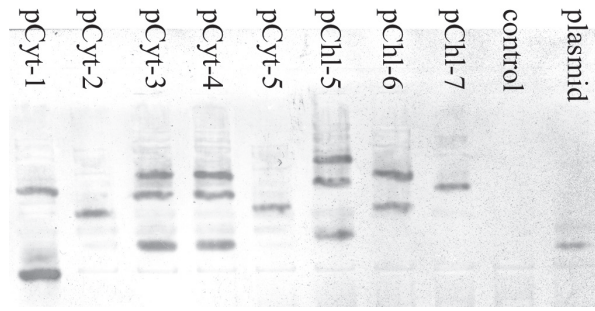


**Figure 2.** The results of the Southern analysis of some ChlCOD and CytCOD-$T_0$ transgenic lines. The results show that there were 1, 2, or 3 inserted copy of the gene in both the ChlCOD and CytCOD-$T_0$ transgenic lines. Moreover, the pCyt-3 and pCyt-4 plants belonged to the same transformation event. The pCyt-1 plant possessed a band located at a lower position than that of the plasmid reflecting a rearrangement during transformation and integration of the desired DNA into the genome.

*3.6. Seed Germination Test in $T_2$ Plants*

Approximately 300 healthy homozygous pChl5-$T_2$ as well as control seeds were surface-sterilized with ethanol 95% and Chlorax 2.5% solutions for 2 and 30 min, respectively. The seeds were rinsed three times with sterile distilled water after each step. Finally, the seeds were sown on a sterile filter paper in 10 cm Petri dishes. A factorial experimental design based on completely randomized design (CRD) was used. The first experimental factor was two kinds of seeds including transgenic and control, while the second factor was four salinity levels, i.e., 0, 100, 200, and 300 mM NaCl. Three replications were considered and 20 seeds were sown in each Petri dish. The Perti dishes were located in a dark incubator at 25 °C temperature and were irrigated every day according to the respective salinity treatment. Irrigation solutions for the transgenic seeds were supplemented with 50mg.$L^{-1}$ hygromycin B. The germinated seeds and plantlets were counted after 14 d. The data were analyzed by using the SAS software (26).

*3.7. Evaluation of Salt Tolerance of pChl5-$T_2$ Plants*

Transgenic $T_2$ and control plants were grown in 20 L containers filled with full strength Yoshida medium (22). A factorial experiment based on CRD was used in which four levels of salinity including 0, 40, 80 and 120 mM NaCl with 3 replications were investigated. Three plants were sown in each container and were grown for 2 weeks in a greenhouse. The greenhouse conditions included 28/20 °C day/night temperatures, 50% relative humidity, and 16 h daylight duration. Cumulative decreases in water weight as amount of water usage by plants, and cumulative increase in leaf length of tube-shaped, unexpanded leaf on the top of the rice shoot as dynamic of leaf growth (27) were daily measured.

## 4. Results

*4.1. Characterization of Transgenic Plants*

Twenty-two putative transformed lines were generated through the transformation process using both pChl-COD and pCyt-COD plasmids. **Figure 2** shows the results of the Southern blot analysis for 5 pCyt-COD and 3 pChl-COD plants of the $T_0$ generation as representatives of all the regenerated lines. The analysis confirmed that at least one copy number of the gene was successfully integrated into the transformed rice lines. More specifically, pChl5 and pCyt3 lines were found to harbor three copies of the gene, followed by pCyt1 and pChl6 lines harboring two copy numbers and pCyt2, pCyt5, and pChl7 lines harboring one copy number. Moreover, pCyt3 and pCyt4 lines showed the same digestion pattern indicating that they were originated from one transformation event.

Reverse transcription-PCR analysis of some transgenic $T_0$ lines confirmed the transcription of the gene in some transgenic lines (**Fig. 3**).

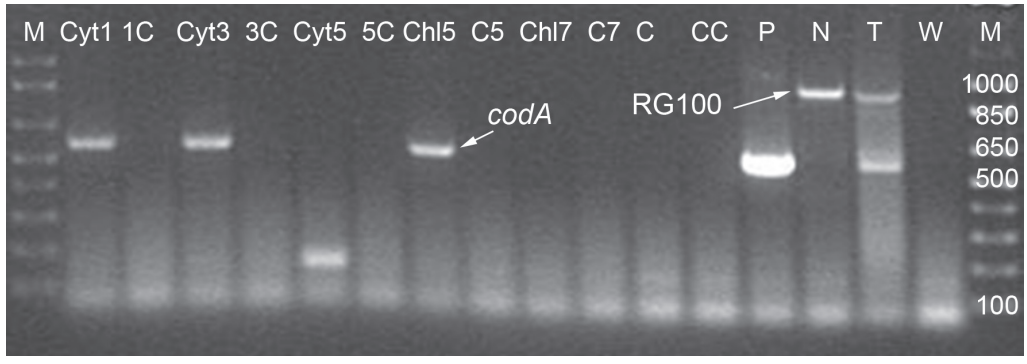


**Figure 3.** The results of the RT-PCR analysis for some transformation events using the RNA extracted from fresh leaves as template as well as CodA and RG100 primers. RG100 primers amplified a 960 bp sequence in rice genome which was used as internal control to detect any DNA contaminations. Abbreviations are as below: L, 1$Kb^+$ ladder, 2-6 and C denote cDNA samples of pCyt1, pCyt3, pCyt5, pChl5, pChl7, and the control lines (non-transgenic) respectively; 2C-6C and CC are the RNA samples without cDNA synthesis step for the above-mentioned lines, respectively (as a second control to detect any DNA contaminations in the RNA samples); P, plasmid DNA; N, DNA from the control plant; T, DNA from the pCyt3 line; and W, negative control.

As presented, the specific primers of the *codA* gene succeeded in priming its specific band in pCyt1, pCyt3, and pChl5 lines, while pCyt5 line showed a different pattern which could be attributed to the rearrangement of the gene during the transformation process. Moreover, pChl7 line did not show the specific band.

*4.2. GB Content*

The GB contents of pCyt1, pCyt2, pCyt3, pChl5, and pChl6 lines were measured at 7.3, 4.2, 14.8, 5.6 and 3.1 $\mu.Mg^{-1}$ dry matter, respectively. Accordingly, pCyt3 and pChl5 lines were selected as high GB content lines in order to grow the next generations.

*4.3. Other Characteristics of Transgenic Lines*

The transgenic lines expressing *codA* gene showed some deformities including shortening, decreased leaf numbers and overall growth, reduced seed production, as well as tendency of the leaves color to yellowish green. It seemed that such effects were further intensified by increasing the GB content as pCyt3 line with the highest GB accumulation was the shortest line and produced the least amount of seeds.

*4.4. Inheritance of CodA Gene*

The results of the progeny test performed for $T_1$ progenies of both pChl5 and pCyt3 lines (**Fig. 4**) revealed a 3:1 Mendelian segregation (**Table 1**). This means that all three copies of the gene were integrated at the same position in each transgenic line.

*4.5. Seed Germination Test in $T_2$ Generation*

Analysis of variance for seed germination test showed that both cultivars and salinity effects were significant. **Figure 5** summarizes the mean comparison for cultivar×salinity interactions. As presented, the seed germination rate of the transgenic line was significantly higher than those of the control in 100 and 200 mM NaCl treatments.

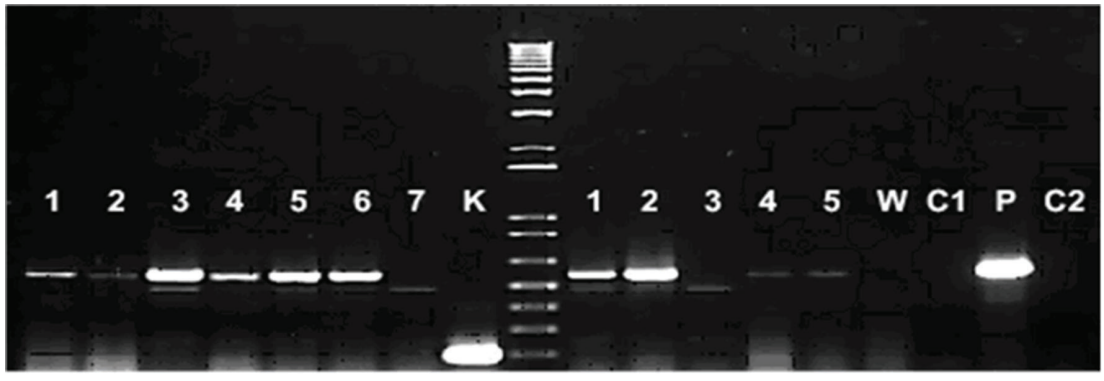


**Figure 4.** A part of the results obtained using the RT-PCR analysis for the $T_1$-pChl (right) and the $T_1$-pCyt plants (left) using the extracted RNA from the respective plants. Numbers denote plant No.; K, positive control containing 18s rRNA primers; C1, RNA sample without reverse transcriptase enzyme; P, sample with plasmid as template; C2, sample with RNA as template and *Taq* polymerase. The absence of the relevant band in C1 and C2 samples confirmed that the cDNAs were generated the expected band for *CodA* mRNA.

**Table 1.** Results of $\chi^2$ analysis for classic Mendelian segregation ratios in $T_1$-pCyt3 and $T_1$-pChl5 progenies (df=1).

| Segregation Ratios | pCyt3 Progenies | pChl5 Progenies |
|---|---|---|
| 3:1 | 0.098 $^{ns}$ | 0.057 $^{ns}$ |
| 15:1 | 8.297 $^{**}$ | 3.918 $^{*}$ |
| 63:1 | 58.879 $^{***}$ | 24.975 $^{***}$ |

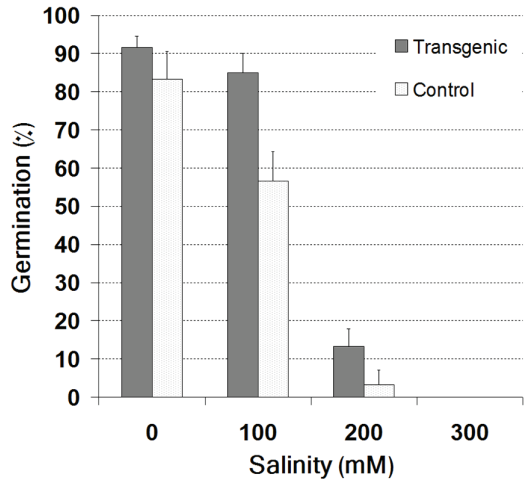


**Figure 5.** Mean comparison for cultivar * salinity interaction of seed germination test. The result shows that the germination rate of the transgenic pChl-$T_2$ line under all saline conditions was higher than that of the control line.

**Table 2.** GB and choline contents (μ$Mg^{-1}$ dry matter) in different generations of the transgenic rice lines harboring the *codA* gene and the parental wild type (WT).

| **Generation (pChl5 line)** | **WT** | **$T_0$** | **Selected $T_1$** | **$T_{2-1}$** | **$T_{2-2}$** | **$T_{2-3}$** | **$T_{2-4}$** |
|---|---|---|---|---|---|---|---|
| **GB Content** | - | 5.6 | 5.8 | 5.8 | 4.7 | 5.8 | 5.2 |
| **Choline content** | 0.1 | - | 0.09 | 0.1 | 0.1 | 0.09 | 0.09 |
| **Generation (pCyt3 line)** | **WT** | **$T_0$** | **Selected $T_1$** | **$T_{2-1}$** | **$T_{2-2}$** | **$T_{2-3}$** | **$T_{2-4}$** |
| **GB Content** | | 14.8 | 12.0 | 12.6 | 12.1 | 13.3 | 10.0 |
| **Choline content** | 0.1 | - | 0.05 | 0.05 | 0.05 | 0.5 | 0.6 |

The highest germination rate was observed under the non-saline condition (82.5 %), and when the salinity level was increased, the rate of seed germination decreased rapidly reaching zero at 300 mM. The transgenic and non-transgenic seed germination means (47.5 and 33.33%, respectively) showed that the transgenic lines had better germination rates in response to the salinity treatments. Similar results were also reported by Sakamoto *et al.* (8) and Su *et al.* (9).

*4.6. GB Accumulation and Choline Content in $T_1$ and $T_2$ Generations*

**Table 2** summarizes the results of the GB and choline quantification in different generations of the selected transgenic lines. As presented, the GB content in CytCOD-$T_0$ line (14.8 μ.$Mg^{-1}$) was about three times higher than that of ChlCOD-$T_0$ line (5.6 μ.$Mg^{-1}$). Also, choline content in wild type, pCHl5 and pCyt3 lines were achieved as 0.1, 0.09 and 0.05 μ.$Mg^{-1}$ dry matter, respectively. In a study, Sakamoto *et al.* (8) claimed that GB content in transgenic Japonica CytCOD lines was 5 times higher than the ChlCOD ones, 5.7 *vs.* 1.1 μ.$Mg^{-1}$, respectively.

*4.7. Evaluation of Salt Tolerance of $T_2$ Plants*

**Figure 6** illustrates the leaf growth rates of the transgenic and control lines under different saline conditions. As shown, the growth rate of the control line in all salinity treatments was higher than that of the transgenic line in the respective salinity level. Moreover, the control plants were higher in height than the transgenic lines.

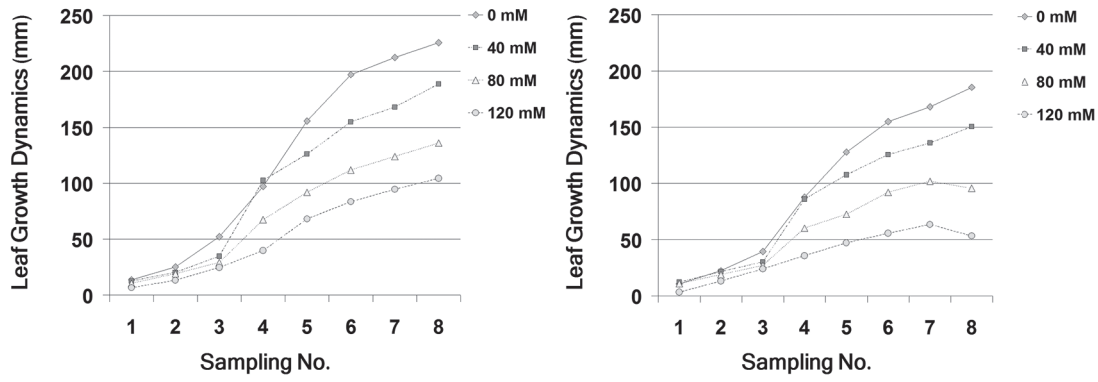


**Figure 6.** Leaf growth dynamics of the pChl-$T_2$ (left) and control (right) lines in the presence of 0, 40, 80 and 120 mM NaCl. As shown, the leaf growth rate of the control line under all saline and normal conditions was better than that of the transgenic line.

## 5. Discussion

Biolistics gene transformation has been criticized because of the risk of multiple gene copy insertion into plant genome. In this study, this approach was used successfully to introduce multiple copies of the *codA* gene into rice genomes in order to investigate their impacts on GB accumulation as well as on the other physiological characteristics of the recipient line.

Rice is a naturally non- GB-accumulator plant. Introduction of three gene copy numbers into the plant genome in the present study resulted in an enhanced GB accumulation in both pChl and pCyt lines, up to 5.6 and 14.8 $\mu.Mg^{-1}$, respectively. These GB amounts were much greater than any reported accumulation rates in transgenic lines so far. Such increases in GB content could be attributed to the increases in integrated gene copy numbers.

Seed germination as a complex process involving various physical and biochemical cues has been frequently reported a sensitive stage of plant life to various abiotic stresses including salinity. Hence, germination test has been widely used to evaluate salt tolerance in different plant species including rice varieties (28, 29). In the present study, seed germination test was used to evaluate effect of the accumulated GB in the seeds against salinity. The results of the seed germination test in the selected transgenic $T_2$-pChl line showed that the germination rate of the transgenic line at 100 and 200 mM NaCl was more than that of the control line. This finding may indicate that the accumulation of GB in the seeds increased their salt tolerance.

The uptake of water by dry seeds also known as imbibition is the main step of seed germination which is essential for activating hydrolytic enzymes to digest starch and to consequently supply the energy and carbon skeleton required for the biosynthetic pathways. At salinity levels of 200 and 300 mM NaCl, osmotic pressure would be obviously too high making it hard for the seeds and seedlings to absorb water. Moreover, the sodium ions introduced into the cells could have exerted their toxic effects. Since GB has been considered as an osmoprotectant rather than an osmoregulator (30), the accumulated GB in the transgenic lines could not have affected water absorption into seeds. It should be highlighted that although seed germination under the salinity level of 200 mM NaCl occurred in both the transgenic and controls lines, but the germination rate achieved in the transgenic seeds was higher than that of the control line. Therefore, it could be concluded that GB must have been involved in the protection of enzymes structure, photosynthetic apparatus, and membrane properties by alleviating the toxicity of the sodium ions leading to improved salt tolerance during the seed germination phase. These results were in line with those of Sakamoto *et al.* (8, 31) who studied transgenic *Arabidopsis* and rice species. They showed that the transgenic plants expressing *codA* gene had improved seed germination rates under saline and cold conditions.

In this study, the physiological evaluation of salt tolerance of the $T_2$-pChl transgenic line was investigated by observing the growth rate of the first young, tube-shaped, and unexpanded leaf on the top of the rice shoot. During the vegetative growth stage, the growth pattern

is represented by leaf growth dynamics which follows an accumulative trend. Upon salt stress, the osmotic pressure inside the plant cells, which is an engine for plant growth and development, decreases. As a result, cell division and expansion in the meristematic regions decreases and thereafter, leaf initiation and growth follow a downward pattern. The analysis of the growth pattern during salt stress showed that leaf growth rate was higher in the control line compared with the transgenic line. Moreover, the control plants were higher in height than the transgenic line. Therefore, it could be concluded that the GB content of the $T_2$ lines was not sufficient to induce a suitable tolerance against salinity, and on the other hand, their lower growth rate even under normal condition could suggest the occurrence of a transformation-oriented disadvantage in terms of total growth rate in these lines.

The Southern Analysis showed that the selected transgenic lines harbor three copies of the transgene. Also, the segregation analysis in $T_1$ generation showed a simple 3:1 Mendelian pattern strongly suggesting that all copies of the transgene were incorporated in to one locus. This usually happens when more than one plasmid is wrapped around a gold particle (a usual event in biolistic approach) and after crossing over they are inserted together in one single locus (32).

The GB contents varied among different transgenic lines and was proportional to the *cod*A gene copy number. In better words, GB increased by increasing the gene copy number. For instance, GB content in the pCyt transgenic lines was much higher than in the pChl transgenic lines. Moreover, the transgenic lines in this study showed more or less some signs of deformity in plant style including shortening of plant height, reduction in seed production, and yellowish appearance (**Fig. 7**). The pCyt transgenic lines were affected to a higher extent than the pChl lines (**Fig. 7**). As an example, the pCyt3 line harboring 3 gene copy numbers was 30-40 centimeters in height and produced only four seeds. Since the production of a high GB content line was one of the objectives of the present study, the accumulative effect of three gene copy numbers assisted in achieving the mentioned goal.

Overall, in spite of the fact that the GB content achieved herein was the highest compared with those reported in the published literature (**Table 2**), but the side effects of such high GB accumulation on plant performance was too severe to induce an efficient salt tolerance. The emergence of these side effects could be explained by the findings of McNeil *et al.* (33) who showed that the rate of production and accumulation of GB in transgenic tobacco was limited by the GB precursor, i.e., choline. Along with our result on choline contents of studied lines (**Table 2**), they showed that choline content in transgenic tobacco (*codA* gene) has been dropped dramatically. It should be highlighted that choline is a

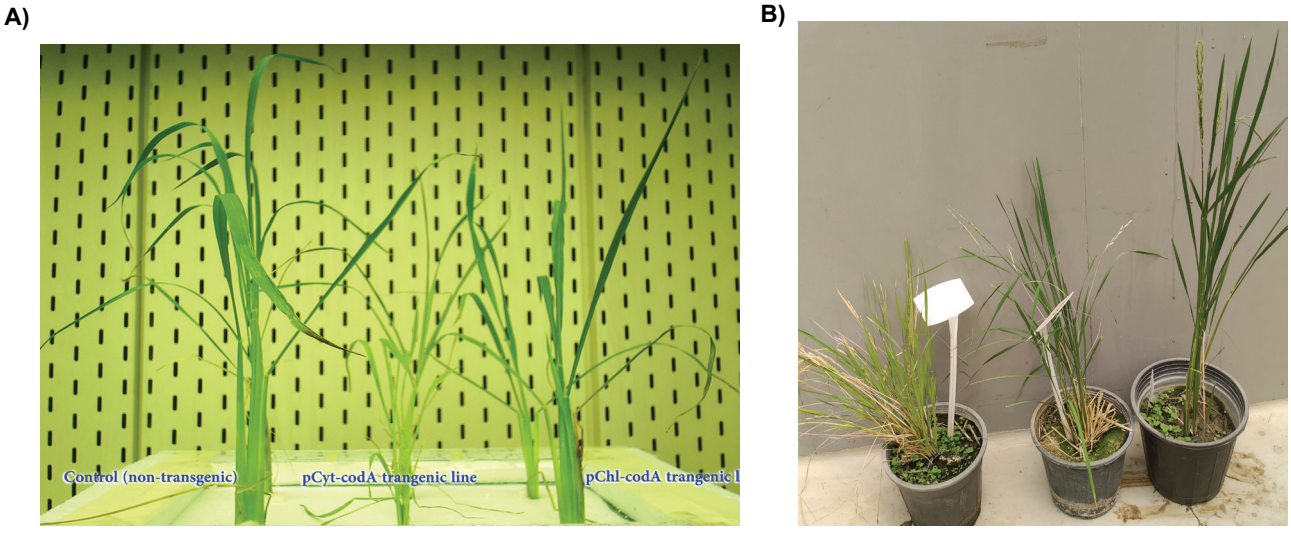


**Figure 7. A)** Seedlings of the selected transgenic and non-transgenic lines in $T_1$ generations. As shown, pCyt-*codA* transgenic line growth was severely affected (shorten and yellowish green in color) compared with the other transgenic (pChl-*codA*) and control lines in the same hydroponic medium. **B)** A $T_1$ pCyt-*codA* transgenic line (left), $T_1$ pChl-*codA* transgenic line (middle) and control plants (right) in the greenhouse. As shown, pCyt-*codA* line has a grass-like shape, yellowish green color leaves and empty spikelets.

precursor of many vitamins as well and is also involved in many metabolic pathways in the cells. Therefore, considerable decreases in choline content could severely influence these pathways and consequently the overall plant growth. Such justification was supported by the results of the study conducted by Huang *et al.* (34) who claimed that spraying a choline solution on transgenic rapeseed leaves could compensate for its deficiency in the cells, restore production and accumulation of GB, and promote plant growth. In a different investigation, McNeil *et al.* (33) also found that choline treatments used on transgenic *Arabidopsis*, tobacco, and mustard plants increased the amount of GB accumulation up to 250, 650, and 750 μmoL.g$^{-1}$ dry matter, respectively. According to these findings, it could be deduced that the limiting factor for GB accumulation for stress tolerance is choline deficiency.

## 6. Conclusion

The results revealed that increasing *cod*A gene copy number led to increased GB content. However, since the limiting factor for GB accumulation is choline as its precursor, and on the other hand deficiencies in choline content could negatively impact the other important pathways in the cell, therefore, normal growth pattern of the transgenic line was jeopardized. On such basis, it could be suggested that the growth rate of transgenic lines be restored by introducing genes for enhancement of choline biosynthesis. An alternative to this approach would be the introduction of the genes of the other pathways which GB synthesis could be initiated with glycine.

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