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Salt Tolerance Enhanced by Transformation of a P5CS Gene in Carrot

Kyu Hyun Han, Cheol Ho Hwang*

School of Bioresource Sciences, Dankook University, Cheonan, Korea

Abstract

Proline is known as an osmoprotectant accumulating in response to salt and dehydration stresses. An increased level of proline is achieved by either an induced synthesis or a reduced degradation of proline. In an attempt to increase salt tolerance in carrot, a P5CS gene from mothbean was introduced via an *Agrobacterium*-mediated transformation. The resulting carrot cells and the regenerated plants containing the transgene showed increased levels of proline compared to nontransgenics. The transgenic cell line, Pj2 showed about 6 times increased degree of tolerance determined by relative growth after a treatment in 250 mM NaCl. In facts, due to the retarded growth shown in non-saline condition, Pj2 cells grow only about 1.2 times better than nontransgenic control under salt stress condition. Taken together, it appears that a P5CS is a key enzyme in proline biosynthesis and the increased accumulation of proline by overexpression of the enzyme is enough to enhance tolerance to salt stress in carrot.

Key words: P5CS, Proline, Carrot, Salt Tolerance

Introduction

Environmental stresses of low temperature, drought, and high salinity limit growth and production of plants. In response to the stress conditions, plants increase the osmotic potential within their cells by synthesizing and accumulating compatible osmolytes such as proline and glycine betaine (Hanson et al. 1980). Proline is known to play an important role as an osmoprotectant in plants subjected to hyperosmotic stresses resulted from drought and soil salinity (Delauney and Verma 1993). It has been proposed that a proline is also able to stabilize subcellular structure and to scavenge free radicals (Smirnoff and Cumbes 1989). The synthesis, accumulation and degradation of proline in plants are highly regulated in order to response to the environmental changes. When a synthesis of proline is induced by osmotic stress, a P5CS has a role as rate-limiting enzyme in the pathway in plants. In addition, a proline catabolism is capable of a high energy output to donate electron to the respiratory electron transport chain and is considered a primary fuel in energy intensive processes such as pollen germination and nodulation (Zhang et al. 1982; Skubatz et al. 1989; Hare and Cress 1997). In plants, proline catabolism may also provide amino nitrogen and reducing power to cell during recovery from stress (Peng et al. 1996; Verbruggen et al. 1996). A correlation shown between transcript levels of P5CS gene and proline contents among Zoysiagrasses indicates that an expression of P5CS gene is a key factor to determine the synthesis of proline in plant (Lee and Hwang 2003). Elevated levels of proline caused by overexpression of mothbean P5CS in transgenic tobacco and rice plants confer enhanced tolerances to salt stress (Kishor et al. 1995; Zhu et al. 1998). Besides, in the light of that an antisense suppression of proline degradation improved tolerance to salinity in *Arabidopsis*, proline is a factor just enough for the tolerance to salinity (Nanjo et al. 1999). All these results together provide strong evidences for a positive correlation between the accumulation of proline and osmotolerance in plants and for the fact that P5CS is a key enzyme to achieve such changes (Hu et al. 1992; Yoshiba et al. 1995; Tomomichi et al. 1998; Stines et al. 1999).

In this study, tolerances to salinity stress in transgenic carrot callus and plant with a mothbean P5CS gene introduced were tested and it appears that a P5CS expression plays a key role in regulation of proline biosynthesis providing an enhanced salt tolerance in carrot.

* Corresponding author, E-mail: sfeho@dankook.ac.kr
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Materials and Methods

Plant materials

Seeds of the carrot (*Daucus carota* L. Jochun5chon) were purchased from Chungang Seeds Company.

P5CS gene construction and transformation

A coding sequence of P5CS gene from *Vigna aconitifolia* (accession number: M92276; Hu et al. 1992) was digested with *Mul* I and *Eco*R I and ligated in between the CaMV 35S promoter and Nos terminator in pGA748 vector. The constructed pGAP5CS vector was introduced into *Agrobacterium tumefaciens* (LBA4404) by freeze-thaw transformation.

Seeds of the carrot (*Daucus carota* L. Jochun5chon) were surface-sterilized in 70% EtOH 4 times and rinsed 3 times with sterile distilled water before being stirred in 5.25% NaOCl for 30 minutes and rinsed 5 times with sterile distilled water. They were germinated in 0.3% Gelrite plate under dark condition at room temperature. Excised fragments of carrot hypocotyls were pre-cultured at 25°C for 2 days in the dark and cocultivated with *Agrobacteria* for 5 minutes and then dried briefly before returning to MS^+ medium and then cultured at 25°C for 3 days. The cocultivated hypocotyl fragments were transferred to MS^+ media including kanamycin at 100 μg/mL and carbenicillin at 400 μg/mL and further incubated at room temperature in the dark for selection. The selected calli were transferred to MS^- regeneration medium to start somatic embryogenesis and regenerated into plants. Regenerated plants were transferred into soil.

PCR analysis

The first set of PCR to amplify a part of the NPT II gene was performed with 1 μg of genomic DNA in a reaction volume of 50 μL with 2.5 units of Taq DNA polymerase. The buffer included 10 mM Tris-HCl pH8.3, 25 mM KCl, 0.01% gelatine, 15 mM $MgCl_2$ and four of each mononucleotide at 0.2 mM. 50 pM of each primer(5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 3′-ATC GGG AGC GGC GAT ACC GTA-5′) were added. The reactions were performed for 30 cycles of 94°C for 1 minute, 67°C for 1 minute, and 72°C for 1 minute with a final extension step of 72°C for 4 minutes in a thermal cycler (GeneAmp PCR system 2400, Perkin Elmer).

The second set of PCR to amplify a part of P5CS gene was performed with 50 pM of primer(5′-GAA TTC GCC GTT AGT ACC AGG AA-3′ and 3′-CTC GAG AAC AGG AAT GCC ACG TTC AG-5′) and reaction was performed in the same condition as used for the first set except the annealing temperature of 53°C. The amplified DNAs were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

Determination of proline concentration and salt tolerance

The concentration of proline was measured according to the method of Bates et al. (1973) as following. A 0.5 g of leaf or callus was frozen with liquid nitrogen and pulverized with mortar and pestle and 1.5 mL of 0.2 N perchloric acid was added to the powdered tissue and pulverized to make a fine suspension. The suspension was centrifuged at 4°C and 10,000 g for 20 minutes. To the supernatant $KHCO_3$ was added to adjust pH 4 and centrifuged again at 4°C, 10,000 g for 20 minutes and the supernatant was saved. To 1 mL of the supernatant, 1 mL of acid reagent (acetic acid 60 mL + phosphoric acid 20 mL + H_2O 20 mL + ninhydrin 1.25 g) were added and 1 mL of acetic acid and put in boiling water bath for 1 hour before an absorption was measured at 520 nm (uv/Vis Spectrophotometer, Unicam) after cooling on ice for 10 minutes.

Measurements of salt tolerance were performed by incubating both nontransgenic and transgenic calli in MS^+ liquid medium supplemented with 250 mM of NaCl and comparing the growth rates shown in increases in cell mass as a dry weight for 7 days after the treatment.

Results and Discussion

Confirmation of the transgenic carrots

The mothbean P5CS cDNA (2061 bp) was constructed under a CaMV 35S promoter (Figure 1) and introduced into carrot cell by way of *Agrobacterium* (LBA4404). The transformed cells were selected by kanamycin and a total of 15 callus lines were stably established. In order to confirm the transformation of the lines, a PCR was performed to amplify a part of NPTII gene introduced along with P5CS gene. The genomic DNA isolated from the transgenic callus showed to amplify a PCR

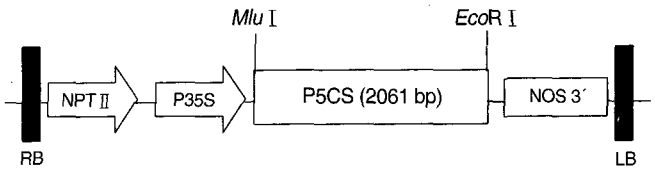

Figure 1. Construction of pGAP5CS by ligation of a *Mlu* I and *Eco*R I fragment of P5CS cDNA from mothbean into a binary vector, pGA748. P5CS coding sequence is under the CaMV 35S promoter. RB: right boader, LB: left boader, NPT II: neomycin phosphotransferase II, P35S: cauliflower mosaic virus 35 subunit promoter, NOS3′: the 3′ terminator region of the nopaline synthase

product of 700 bp as expected with the primer set for NPTII but nontransgenic control (C) showed no band (Figure 2A). Another set of PCR with P5CS primers also showed a presence of the P5CS gene amplified as a band of 297 bp corresponding to a part of P5CS gene from mothbean in transformed callus lines 2, 3, 6, 7, 14 but nontransgenic line marked as C showed a very faint band of the similar size (Figure 2B). The band in nontransgenic line may be amplified from an endogenous carrot P5CS gene. Even though the homology of P5CS between carrot and mothbean is not known yet, however extrapolated from a comparison of P5CS genes in between *Arabidopsis* and mothbean, a low homology of P5CS genes (72%; NCBI) expected in between mothbean and carrot makes more mismatches to mothbean primers and the reduced efficiency of PCR produced only a limited amount of DNA amplified. The plants were regenerated by somatic embryogenesis from the cell lines 2, 4, and 6 and their genomic DNAs were amplified and shown to contain a corresponding band of 700 bp for NPTII gene (Figure 2C). The presences of the transgene in both the selected calli and the regenerated plants were confirmed by PCRs.

Proline accumulation in transgenic carrot callus and plants

To see any biochemical change due to the constitutively expressed P5CS gene introduced, the proline levels in both callus and leaf tissue of plants were measured. Comparing to nontransgenic callus, the transgenic lines Pj1 and Pj2 showed 6 and 5.4 times higher levels of proline accumulated but the lines Pj3 and Pj7 showed only slightly higher (Figure 3A). The levels of proline in the regenerated plants were different with those of corresponding callus. The plants, Pj1-1, Pj1-2, Pj1-3, and Pj1-4 regenerated from a Pj1 line showing the highest level of proline in callus, showed similar levels of proline to that of the nontransgenic plant. This may indicate that there is a discontinuity in proline accumulation between callus and plant. A selection of high proline-accumulating line at callus stage may not be applied to the plant after regeneration. However Pj2 showed the highest levels of proline accumulated in leaf of the plant regenerated as much as the callus did (Figure 3B). Depending on individual transformation event, there may be differences in expression levels of the transgene in callus or plant stage.

Salt tolerance measured by growth of transgenic carrot callus under salt stress condition

Salt tolerance was measured by comparing growth rate of callus cells in 250 mM NaCl relative to that of the same cell without the treatment (Figure 4B). A transgenic line, Pj2

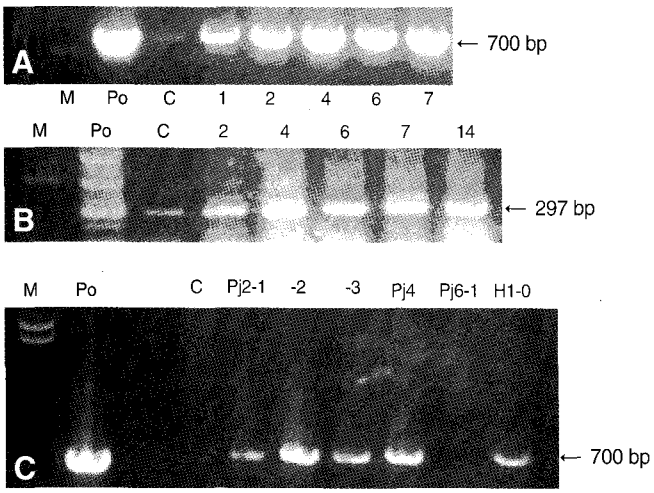

Figure 2. Confirmation of the presence of P5CS gene introduced in transgenic carrot callus cell (A, B) and plants (C) by PCR with NPTII (A,C), P5CS (B) primers.
M: λ *Hind*Ⅲ maker; Po: Positive control (pGAP5CS vector); C: Nontransgenic callus; 1~7 in panel A: Transgenic callus lines Pj1~7; 2~14 in panel B: Transgenic callus lines Pj2, 4, 6, 7, 14; 2-1~H1-0 in panel C: Transgenic plants.

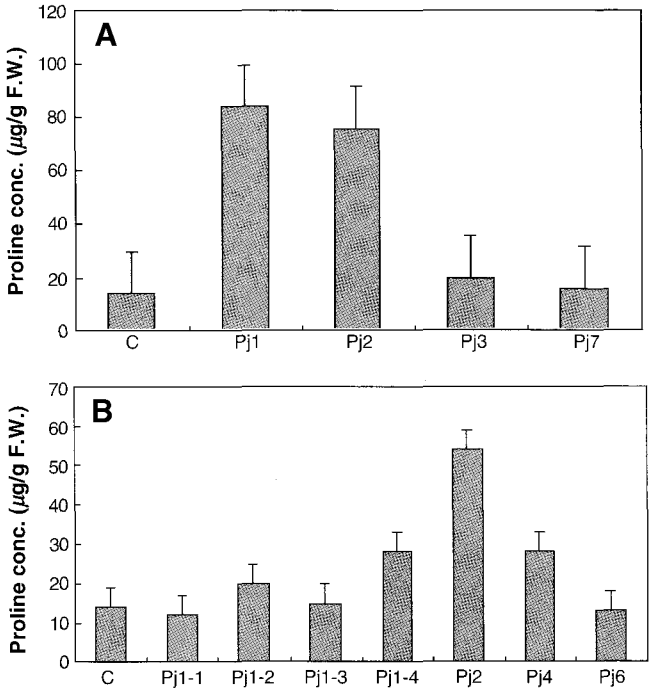

Figure 3. A. Proline concentrations of four independent transgenic carrot calli (Pj1, 2, 3, 7) and nontransgenic control (C). B. Proline concentrations of leave from transgenic carrot plants (T_1) and nontransgenic (C). Pj1-1, 2, 3, 4 were plants regenerated from the same line, Pj1.

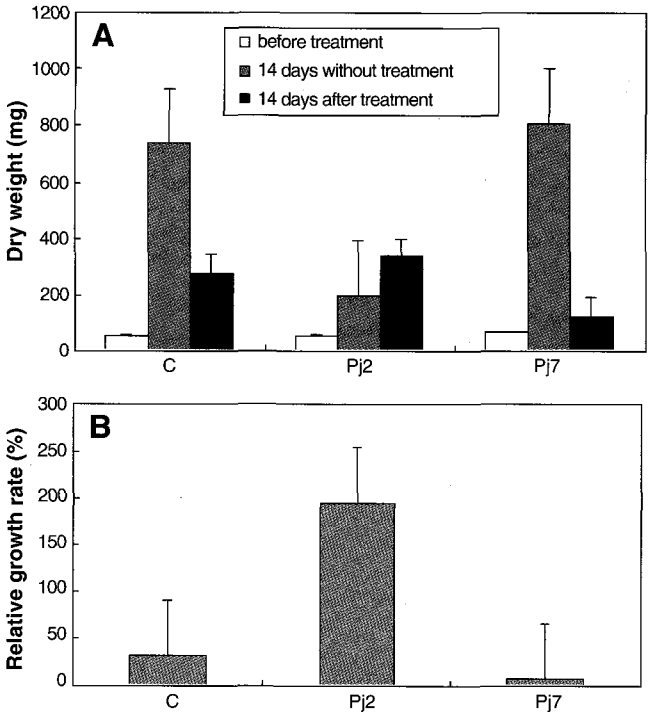

Figure 4. Effect of a mothbean P5CS gene expression on relative growth rate of the callus cells. Cell masses of the transgenic lines (Pj2, Pj7) and nontransgenic line (C) were measured in dry weight before (0 day) and 14 days after incubating in 250 mM NaCl (A). Relative growth rate were calculated by dividing the cell mass increased for 14 days' incubation with the salt by the cell mass increased for 14 days' incubation without the salt (B). Values presented are the average of five independent samples.

showed a higher level of tolerance than nontransgenic line only in presence of the salt stress but a lower growth without salt (Figure 4A). In the light of the fact that Pj2 line showed higher levels of proline accumulated in both callus and plant, an excess amount of proline in cells may inhibit their growth but allow them to survive better under salt stress. However the transgenic tobacco plant with the mothbean P5CS gene introduced showed normal growth in non-saline condition and better growth in saline condition than nontransgenic (Kishor et al. 1995). This discrepancy may be due to difference in sensitivity between carrot cells and tobacco plants. It was reported that age or stage in development of the plant is quite critical to determine sensitivity to salt stress (Tarczynscki et al. 1993). In case of the line Pj7 showing similar levels of proline accumulated in callus cell to the control, their growth was similar in growth to the control in either absence or presence of salt treatment (Figure 4A). An excess amount of proline accumulated may disturb the cell not to grow to the normal. Based on the observations with the line Pj2, it suggests that a constitutive expression of P5CS transgene leads to an overproduction of the P5CS enzyme, an increased accumulation of proline and an enhanced tolerance to salt stress in transgenic carrot. However an inhibition of normal growth as observed with Pj2 should be solved by selecting a transgenic line with a fine tuning to achieve optimal level of proline accumulated to guarantee a normal growth as well as a salt tolerance. A finding of the best compromise between growth and salt tolerance will allow molecular breeding of salt tolerance in plants using a P5CS gene to be fruitful.

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