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Salt Tolerance in Transgenic Pea (*Pisum sativum* L.) Plants by P5CS Gene Transfer

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Abstract

Slices of embryonic axis of mature pea (*Pisum sativum* L. cv. Green Arrow) seeds were used as explant. Transformation of explants was done via *Agrobacterium tumefaciens* bearing vector pBI-P5CS construct. The best results for inoculation of explants were obtained when they were immersed for 90 s at a concentration of 6 × 10^8 cell ml^{-1} of bacterial suspension. Transformed pea plants were selected on 50 mg l^{-1} kanamycin and successful transformants were confirmed by PCR and blotting. Transgenic plants were further analyzed with RT-PCR to confirm the expression of P5CS. Transgenic plants and non-transgenic plants were treated with different concentrations of NaCl 0 (control), 100, 150 and 200 mM in culture medium. Measurement of proline content indicated that transgenic plants produced more amino acid proline in response to salt in comparison with non-transgenic plants. Photosynthetic efficiency in transgenic plants under salt-stress was more than that of non-transgenic plants.

Key words: Δ^1- pyrroline-5- carboxylate synthetase, P5CS, Pea, Salt Tolerance

Introduction

Salinity in soil or water is one of the major stresses and, especially in arid and semi-arid regions, can severely limit crop production (Shannon 1998). The deleterious effects of salinity on plant growth are associated with (Grime 1979) low osmotic potential of soil solution (water stress), (Shannon 1998) nutritional imbalance, (Ashraf 1994) specific ion effect (salt stress), or a combination of these factors (Ashraf 1994; Marschner 1995). All of these cause adverse pleiotropic effects on plant growth and development at physiological and biochemical levels (Levitt 1980; Munns 2002) and at the molecular level (Winicov 1998; Tester and Davenport 2003). In recent decades, considerable improvements in salinity tolerance have been made in crop species through conventional selection and breeding techniques (Shannon 1998; Ashraf 1994; Noble et al. 1984; Ashraf 2002).

Transgenic plants allow the targeted expression of drought-related genes in vivo and are therefore an excellent system to assess the function and tolerance conferred by the encoded proteins. The accumulation of low- molecular weight metabolites that act as osmoprotectants is a widespread adaptation to dry, saline, and low-temperature conditions in many organisms (Ingram and Bartels 1996). In engineering plants that synthesize protective osmolytes, microorganisms appear to be useful sources for genes. Transgenic tobacco plants that synthesize and accumulate the sugar alcohol mannitol have been obtained by introducing a bacterial gene that encodes mannitol 1-phosphate dehydrogenase. Plants producing mannitol showed increased salt tolerance (Tarczynski et al., 1993).

Proline accumulation is a widespread response of higher plants, algae, animals and bacteria to low water potential (Delauney and Verma 1993; Samaras et al. 1995). In plants, proline is synthesized from glutamate during osmotic stress and nitrogen limitation. The biosynthetic pathway from glutamate is thought to be a reaction in which glutamate is converted to Δ^1-pyrroline-5-carboxylate (P5C) via glutamic-γ- semialdehyde (GSA) by P5C synthetase (P5CS), and P5C is converted to proline by P5C reductase (P5CR)

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(Delauney and Verma 1993). Proline accumulation is achieved by upregulating the gene for P5CS, a key enzyme in proline biosynthesis (Delauney and Verma 1993; Savouré et al. 1995 ; Peng et al. 1996; Igarashi et al. 1997; Yoshiba et al. 1995, 1997). Proline overproduction, induced by overexpression of the P5CS gene of *Vigna aconitifolia*, conferred osmotolerance in transgenic tobacco (Kavi Kishor et al. 1995; Verma and Hong 1996; Verma 1999).

Peas are an important model plant for various physiological, biochemical and genetic studies. In addition, they represent the fourth most important legume crop in the world and are therefore a target for crop improvement (Casey and Davies 1993). Here we report the production of transgenic pea plants which produced a high level of the proline. Also the transgenic pea plants were more resistant to salinity compared with non- transgenic plants.

Materials and Methods

Plant material

Mature pea (*Pisum sativum* L. cv. Green Arrow) seeds were sterilised in 70% (v/v) ethanol (1 min) followed by 1% (w/v) sodium hypochorite (20 min) and washed five times with sterile distilled water. The seeds were soaked overnight in an ehrlenmeyer flask containing 100 ml of sterile distilled water, in 4℃. After imbibition, seeds were used for transformation procedures.

Bacterial strain and vector

The full length of *Arabidopsis* P5CS cDNA was synthesized and cloned (by our group) in the binary vector pBI121 (Construct S) (Figure 1A). To verify the intactness of construct S, its mobility in agarose gel was compared with pBI121 (Figure 1B). The mass difference between construct S and pBI121 indicates positioning of exogenous fragment (P5CS). For further certification PCR and dot blot were applied (Figures 1C and 2B). Construct S was transferred into *Agrobacterium tumefaciens* strain LBA4404. Besides this construct (driving by cauliflower mosaic virus 35S promoter and the nopaline synthase nos terminator) has gus reporter gene and kanamycin resistance marker. Growth of the *Agrobacterium tumefaciens* containing pBI-P5CS for inoculation was made by shaking in 10 ml Luria borth (LB) supplemented with 50 mg l^{-1} kanamycin for 24 h at 28℃. From this culture of *Agrobacterium* 100 μl was added to each of the two flasks containing 10 ml LB without antibiotic; *Agrobacterium* was grown at 28℃ until optical densities at 600 nm was reached to 0.5 and 1. These densities corresponded to about 6×10^{8} cell ml^{-1} and 12×10^{8} cell ml^{-1} respectively.

Transformation procedure

Transformation procedure was done as described by Schroeder et al. (1993). Seeds after imbibition were removed from flask and testae excised. The explants were cut from the

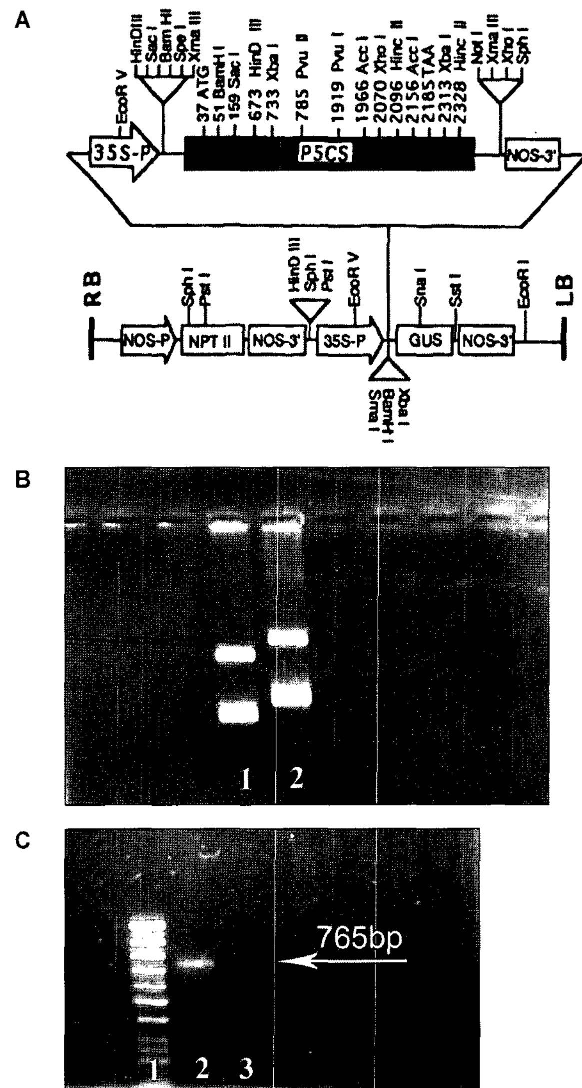

Figure 1. (A) Restriction map of the plasmid pBI-P5CS used for producing transgenic plants. Double strand cDNA of P5CS derived from *Arabidopsis thaliana* was placed between the CaMV35S promoter and gus gene. The resulting construct (construct S) was inserted into the *Bam*H1 site of vector pBI121. (B) Comparative electrophoretic movement of pBI-P5CS (construct S) and pBI-121 without P5CS in a 1% agarose gel stained with ethidium bromide. Lane1; pBI-121 without P5CS. Lane2; pBI-P5CS (construct S). (C) The 765 bp PCR product of *Arabidopsis thaliana* P5CS by applying primers. Lane1; molecular size marker gene ruler 50 bp (Roche). Lane 2; PCR Product of pBI-P5CS (construct S). Lane 3; PCR product of pBI121.

embryonic axis of pea seeds. One cotyledon was removed from each seed and the root end was cut off. The remainder of the axis was sliced longitudinally into three segments, using a scalple blade wetted with suspension of *Agrobacterium tumefaciens*. Inoculation of slices of embryonic axis was done for each concentration in different times: 30s, 60s, 90s and 120s. Wet segments were transferred on B5 medium (Brown and Atanassov, 1985) and cultured under controlled environment (17h light period, 300 μmol quanta m^{-2} s^{-1}, day/night : temperatures of 24/18℃), for three days. Also in this study transgenic pea plants were produced with *Agrobacterium tumefaciens* bearing vector pBI121 alone (without P5CS) as control plants.

Plant regeneration

After 3 days of co-cultivation, explants were washed three times with sterile water containing 500 mg l^{-1} of cefotaxime. The explants were placed on filter paper for drying excess liquid and were transferred to a callus induction medium (Schroeder et al., 1993) which consisted of MS macro and micro (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 2 mg l^{-1} of BAP, 2 mg l^{-1} of NAA, 3% (w/v) sucrose supplemented with 0.75% agar, with 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The pH of the medium was adjusted to 5.8 before autoclaving. The explants were incubated in 17h light period, 300 μmol quanta m^{-2} s^{-1}, day/night : temperatures of 24/18℃ for 12 days. The explants that remained in medium contain kanamycin were counted.

Then explants were transferred to shoot induction medium (Schroeder et al 1993) contained MS macro and micro, B5 vitamins, BAP (4.5 mg l^{-1}) and NAA (0.02 mg l^{-1}). Medium was changed at every 20 days. Any shoots produced there after were excised from the explants and transferred to MS medium supplemented with B5 vitamins, 3% sucrose, 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime and BAP (1 mg l^{-1}), to enhance shoot elongation. Elongated shoots were removed from the explants and transferred to B5/2 rooting medium, with half- strength B5 salts and vitamins, 3% sucrose and NAA (0.185 mg l^{-1}) (Polowick et al., 2000).

DNA isolation

Total DNA was isolated from leaves of non-transgenic and transgenic plants as described by Dellaporta et al. (1983).

Histochemical assay for gus

The leaves of transgenic pea plants were histochemically assayed for gus gene expression as described by Jefferson (1987), that used X-gluc (5- bromo-4- chloro-3- indolyl-ß-D-glucuronic acid) as a substrate for the ß-glucuronidase enzyme gene and the indigo blue color of the cells and tissues were observed.

Polymeras chain reaction (PCR) analysis

To determine the presence of the transferred P5CS gene in transgenic pea plants, PCR was performed on total genomic DNA extracted from the non-transgenic and transgenic plants with following primers:

5'- GGATTGATGTGATATCTCCACTGACG-3' (CaMV 35S promoter specific sense primer) and 5'-CCTTCAACATCGC TCAGAAGAATCAG-3' (P5CS gene specific antisense primer).

RT-PCR

For RT-PCR, RNA was prepared from the leaves of transgenic and non-transgenic plants. The first strand cDNA synthesis was performed as follows: a sterile tube that contained 12 μl total RNA and 1 μl primer (5'- GCAAGAC TAAGTGGTAAAGTGGATCT-3') was incubated at 70℃ for 5 minutes and chilled on ice, then 4 μl 5X reaction buffer, 2 μl dNTP (10 mM) and 0.5 μl ribonuclease inhibitor were added and incubated at 37℃ for 5 minutes; 1 μl Reverse Transcriptase was added to reaction mixture and incubated at 42℃ for 90 min. The reaction was stopped by heating at 70℃ for 10 minutes and then chilled on ice. The first strand cDNA was then employed as a template DNA in PCR for the amplification of the P5CS cDNA. The following primers were used:

Forward primer : 5'- CCAAGGGCAAGTAAGATACTGAACAT-3'
Reverse primer : 5'- GCAAGACTAAGTGGTAAAGTGGATCT-3'

The RT-PCR product was separated on a 0.8% agarose gel.

Dot blot hybridization

pBI P5CS was digested with *Eco*RV/ *X*ba1, the resulting insert was subjected to gel purification and DIG DNA labeling (Roche Applied Science Gmbh, Germany). Genomic DNA was extracted from both non-transgenic and transgenic plants. A volume containing 10 μg of extracted plant DNA was denatured by NaOH (5 M) and NaCl (1 M) for 30 minutes, at room temperature. Following denaturation, added 5 μl SSC 20X and rapidly transfer on ice. Then blotted onto nylon membrane (Roche), baked at 120℃ for 30 min, membrane was hybridized overnight with 10 pmol/ml DIG-labeled probe in the hybridization solution [5XSSC (saline sodium citrate buffer: 20X corresponding to 3M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent, Roche]. Following washes at room temperature

and at 65℃ bound DIG-labled probes were detected using DIG luminescent detection kit (Roche) according to the manufacturer's instructions.

Southern Blot Hybridization analysis

Genomic DNA from transgenic and non-transgenic pea plants was isolated using the protocol of Dellaporta et al. (1983). For southern blots, 20 μg of genomic DNA was digested with *Sac*I and *Bcl*I restriction enzymes, electrophoresed in 0.8% (w/v) agarose gel, transferred onto Hybond-N^+ membranes. The P5CS gene-specific probe was generated using *Xba*1 and *Eco*RV digest of pBI121-P5CS to isolate a 2500 bp fragment. The restriction fragment was purified using the High Pure PCR Product Purification Kit (Roche Co.). Hybridization was done as described Sambrook et al. (1989).

Bioassay for tolerance

Enhanced tolerance to salt stress of transgenic plants in comparison with non- transgenic plants was assayed by treating plants with nutrient solution containing 0 (control), 100, 150 and 200 mM of NaCl and plants were grown under controlled environment, 17 h light periods, 300 μmol quanta $m^{-2}s^{-1}$, day/night: temperatures of 24/18℃. The plants leaves were harvested after 20 days and analysed for proline content and photosynthetic rate.

Proline content of leaves

200 mg of fresh leaves of mature plants were powdered in liquid nitrogen. 10 ml of 3% sulfosalicilic acid was added to each sample and centrifuged at 1300 rpm for 10 minutes. 2 ml of the supernatants from each sample, 2 ml of acidified ninhydrin solution (1.25 g ninhydrin in 30 ml acetic acid at boiling temperature to which 20 ml of ortho phosphoric acid was added) and 2 ml of 100% acetic acid were mixed and boiled for one hour. To stop further reaction, samples were immediately transferred into ice-water and left to cool down for at least 20 minutes. To each sample, 4 ml of toluene was added at room temperature, mixed well, and the absorbance at 520 nm was measured (Bates et al., 1973). To assay the proline content, a standard curve was prepared by measuring the absorbance at 520 nm of the specified concentrations of prepared proline.

Leaf gas exchange

Photosynthetic rate was determined in transgenic and non-transgenic plants under salt stress applying an infrared gas (CO_2) analyser (*225 MKS, Analytical Development Co., U.K.*) as described by Khavari-Nejad (1980 and 1986).

Statistical analyses

To study the effects of salinity, completely randomized design with four replications was conducted. The statistical analysis was performed using SAS software.

Results

Grain legumes have generally been difficult to regenerate and transform. To harness the potential of biotechnology, efficient and reliable transformation systems are necessary (Grant et al., 1995). Of the major grain legume crops-soybean, chickpeas, peas, cowpea, peanut, common bean, faba bean and lentils-confirmed transgenic plants have been produced in all except faba bean, lentils and cowpea (reviewed by Christou 1994). There are some of reports for *Agrobacterium* mediated transformation of peas (Grant et al. 1995; Puonti-Kaerlas et al. 1990; Schroeder et al. 1993).

In this report, we used from embryonic axis of mature pea seeds as explant. Transformation of explants was done via *Agrobacterium tumefaciens*. Also effect of different concentraions of *Agrobacterium* and times of inoculation for obtaining the best result were investigated. Of the two used concentrations of *Agrobacterium* and the four time periods, the best results for inoculation of explants, were obtained when they were immersed for 90s in a concentration of 6×

Table 1. The living explants percent in medium consist of kanamycin (50 mg l^{-1}) obtained after inoculation of explants in 4 times and two concentrations of *Agrobacterium tumefaciens.*

Concentration of *Agrobacterium tumefaciens* (Cells, ml^{-1})	Time of inoculation (s)			
	30	60	90	120
6×10^8	71.0 ± 2.9^b	63.6 ± 5.7^b	91.0 ± 3.9^a	50.0 ± 6.1^{cd}
12×10^8	24.0 ± 2.4^e	46.4 ± 4.3^d	45.6 ± 6.3^d	51.3 ± 3.2^{cd}

Values are means ± SE (n=10)
Numbers followed by the same letter are not significantly different (P>0.05).

10^8 cell ml^{-1} (OD = 0.5) of bacterial suspension. After transfering explants to selective medium that contained kanamycin (50 mg l^{-1}), the number of explants remained were counted (Table 1).

Putative pea transformants were screened by using PCR. Total DNA was extracted from the non-transgenic and transgenic pea plants. The forward and reverse primers landed to the nucleotide sequences of the CaMV 35S promoter and P5CS cDNA respectively. Of the 25 putative transgenic pea plants, 5 had a 765 bp size band in the pea genome (In Figure 2A. result of a sample was shown). The result from PCR showed that 20% of the plantlets were transformed.

To investigate whether the P5CS gene is expressed in the transgenic pea plants, RT-PCR was performed. Total RNA, isolated from 15 transgenic and non-transgenic plants. RT-PCR analysis showed a 640 bp size band in 4 transgenic plants, but no band was detected in the non-transgenic plants (In Figure 2C result of a sample was shown). Further confirmation for presence and integration of P5CS with genomic DNA was done by applying dot blots. The probe for identifying of P5CS was made by applying *Eco*RV/*Xba*1 (Figure 2B). Southern-blot analysis was used to investigate transmission of the P5CS gene in the transgenic plants (Figure 2D).

To verify whether in transgenic pea plants were modified amino acid content, the proline content of leaves was measured in non- transgenic and transgenic plants under non-slaine and saline conditions (Figures 3A & 3B). In transgenic plants proline content was significantly increased in respect of non-transgenic plants. In salinity treatments, in all of NaCl concentraions proline content of transgenic plants was higher than that of in non-transgenic plants.

Because among the damages caused by saline stress in plants, the reduction of the photosynthetic processes is one of the most important (Delfine et al., 1999), we measured photosynthetic rate in non-transgenic plants and transgenic plants in presence of salinity. Salinity treatment significantly decreased photosynthetic rate in non-transgenic plants in respect of transgenic plants (Table 2).

Discussion

Enviromental stresses, such as drought, increased soil salinity, and extreme temperature, are major factors that

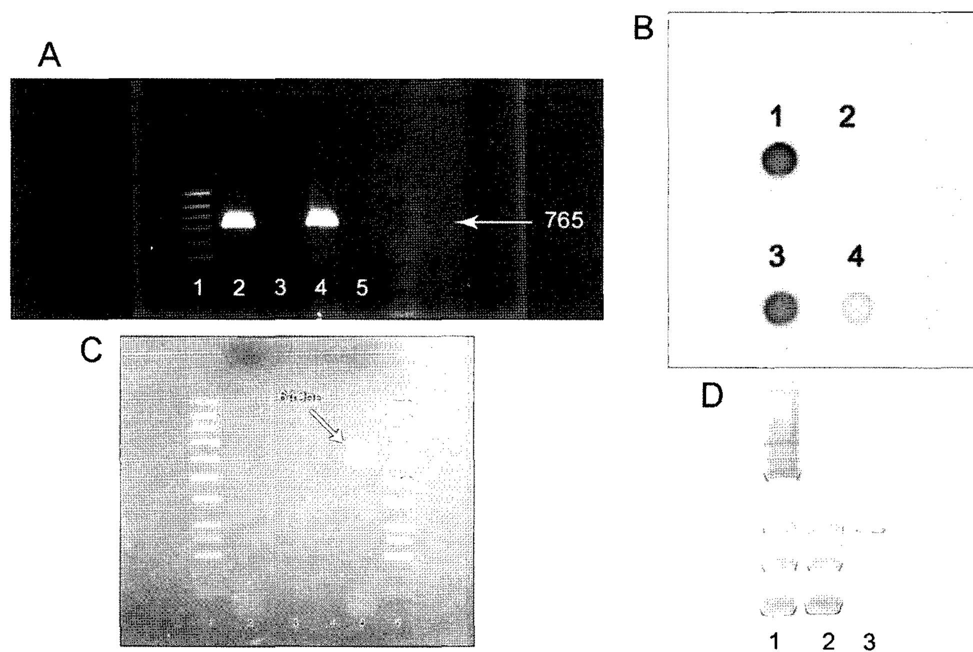

Figure 2. (A) Determination of transformation by PCR analysis. Lane1; Molecular size marker gene ruler 50 bp. Lane2; PCR product of transgenic pea plants. Lane 3; PCR product of non-transgenic pea plants. Lane 4; PCR product of Plasmid pBI-P5CS. Lane 5; PCR product of control plants (transformants produced with vector pBI without P5CS). (B) Dot-blot hybridization non-transgenic and transgenic pea plants.(1) pBI-P5CS (Construct S). (2) pBI121. (3) transgenic pea plants.(4) non-transgenic pea plants. (C) RT-PCR analysis showing the expression of mRNA, corresponding to P5CS in leaves of transgenic pea plants. Lanes 1 and 6 ; Molecular size marker gene ruler 50 bp. Lane 2; H_2O. Lane 3; RT-PCR product derived from transcripts of P5CS control plants . Lane 4; RT-PCR product derived from transcripts of P5CS in non-transgenic pea plants. Lane 5; RT-PCR product dervied from transcripts of P5CS in transgenic pea plants. (D) Southern blot analysis of DNA from transgenic and non-transgenic pea plants. Lane1; transgenic pea plants. Lane 2; non-transgenic pea plants. Lane 3; vector pBI-P5CS.

Table 2. The effects of NaCl on CO_2 exchange (μmol CO_2 m^{-2} s^{-1}) in non-transgenic, control and transgenic plants.

NaCl (mM)	Non-transgenic plants	Control plants	Transgenic plants
0	5.2 ± 0.71^{a}	5.1 ± 0^{a}	4.5 ± 0.36^{ab}
100	3.5 ± 0.2^{c}	3.8 ± 0.16^{bc}	4 ± 0.15^{bc}
150	1.1 ± 0.1^{e}	0.8 ± 0.01^{e}	3.5 ± 0.02^{c}
200	0	0	2 ± 0.06^{d}

Values are means ± SE (n=4)
Numbers followed by the same letter are not significantly different (P>0.05).

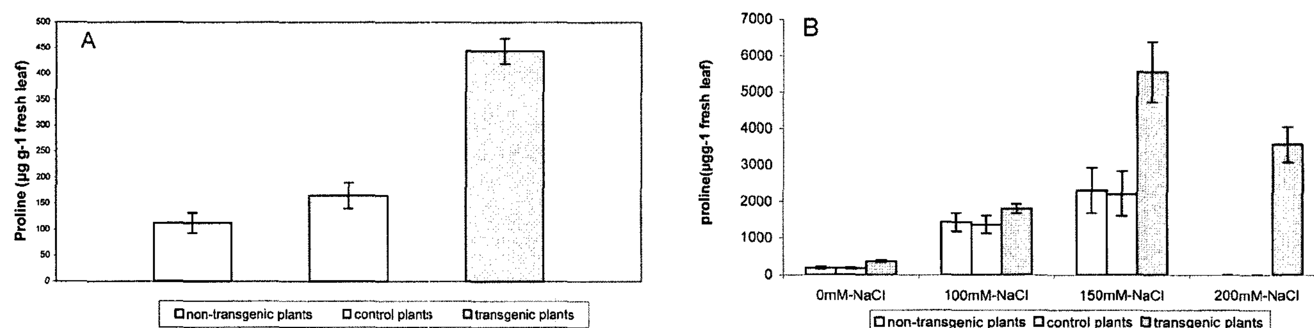

Figure 3. (A) Proline content in leaves of non-transgenic, control and transgenic pea plants before salt-stress conditions. (B) Proline content in leaves of non-transgenic, control and transgenic plants after salt-stress. Control plants were transformed with *Agrobacterium tumefaciens* bearing vector pBI121 without P5CS. Values are mean from four replications. Vertical bars indicate ± SE.

limit plant growth and productivity. Recent progress in plant genetic transformation and the availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (Tarczynski et al. 1993; Pilon-Smits et al. 1995; Xu et al. 1996). Proline is known to play an important role as an osmoprotectant in plants subjected to hyperosmotic stresses such as drought and soil salinity (Delauney and Verma 1993). Kishor overexpressed in tobacco the mothbeam [delta]-pyrroline-5-carboxylate synthase, a bifunctional enzyme able to catalyze the conversion of glutamate to [delta]-pyrroline-5-carboxylate, which is then reduced to proline (Kishor et al. 1995).

In this study we are reporting production of transgenic pea plants that were salt-tolerant by the overexpressing of P5CS gene. The effect of cocentration of *Agrobacterium tumefaciens* and time period for inoculation stage explants were investigated. The number of explants remained in 90s inoculation and 6×10^{8} cell ml^{-1} *Agrobacterium tumefaciens* were significantly more than that of other treatments. There are some reports for transformation of pea (Kathen and Jacobsen 1990; Nauerby et al. 1991; Puonti-Kaerlas et al., 1990; Lulsdorf et al. 1991; Schroeder et al. 1993; Grant et al. 1995), however, time of inoculation and concentration of *Agrobacterium tumefaciens* used in each of them were different. Our results indicate that two factors time and concentration of bacteria for inoculation of explants are important.

As a result of water stress and salinity, proline accumulation was observed in many organisms, inculding bacteria, fungi, algae, invertebrates and plants (for review see Csonka and Hanson 1991; Delauney and Verma 1993; Hanson and Hitz 1982; Yoshiba et al. 1995), so we estimated proline content in leaves of transgenic and non-transgenic plants grown in the saline and unsaline environment. In the non-transgenic and transgenic plants with increasing of concentrations of NaCl proline content was significantly increased, however, in all of treatments of NaCl, proline content of transgenic plants was 2.5 times of that of non-transgenic plants (Figures 3A & 3B).

With increasing concentration of NaCl in culture medium photosynthetic rate in non-transgenic in comparison with transgenic plants was significantly decreased. Besides in 200 mM NaCl, non-transgenic plants died but transgenic plants remained and indicated high efficiency photosynthesis.

Our results indicated that the P5CS gene can be used to improve the salt tolerance of plants through gene transfer, because P5CS increases the production of proline. Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Pollard and Wyn Jons 1979; Paleg et al. 1981, 1984; Nash et al. 1982 ; Brady et al. 1984; Gibson et al. 1984; Rudolph et al. 1986; Santarius 1992; Santoro et al.

1992). Proline may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995) and as a hydroxyl radical scavenger (Smirnoff and Cumbes 1989).

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