Journal of Plant Physiology and Breeding

2015, 5(1): 55-66 ISSN: 2008-5168

Agrobacterium tumefaciens-Mediated Transformation of Two Economically Important Strawberry Cultivars with *P5CS* Gene

Bahman Bahramnejad[1]*, Sirwan Nasri[1], AliAkbar Mozafari[2] and Adel Siosemardeh[3]

Received: January 21, 2015 Accepted: May 17, 2015
[1]Department of Agricultural Biotechnology, Faculty of Agriculture, University of Kurdistan, Sannandaj, Iran
[2]Department of Horticulture, Faculty of Agriculture, University of Kurdistan, Sannandaj, Iran
[3]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Kurdistan, Sannandaj, Iran
*Corresponding author: E-mail: b.bahramnejad@uok.ac.ir

Abstract
The purpose of this research was to transform two economically important cultivars of strawberry with *P5CS* gene, which encodes Δ1-pyrroline-5-carboxylate synthetase (*P5CS*), the key enzyme in proline biosynthesis. Shoots were obtained on MS basal medium supplemented with 2% glucose and 4 mg/l TDZ for Camarosa and Kurdistan cultivars. For genetic transformation, a binary vector *PBI121* containing *P5CS* gene under control of the 35SCaMV promoter was used. Transformed cells (explants) were regenerated on the selective regeneration medium containing 75 mg/l kanamycin and 500 mg/l cefotaxime after five days of pre-incubation and 72 h of co-cultivation with *Agrobacterium*, while control explants failed to grow in the same medium. The presence of the transgene in the plant genome was confirmed by PCR. The morphology of the transgenic plants was normal as controls. Drought stress was applied using polyethylene glycol (PEG) 6000 at concentrations equal to 0, -5 and -7 Bar, respectively. Proline content was four times higher in the transformed leaves compared to that of the untransformed plants while the proline content in the roots was similar in both transgenic and wild-type plants. Overproduction of *P5CS* also increased chlorophyll content, shoot length, shoot fresh and dry weight in the transgenic plants under drought-stress conditions.

Keywords: *Agrobacterium*; Drought tolerance; Proline; Δ -Pyrroline-5-carboxylate synthetase (P5CS); Strawberry

Introduction

Strawberry is flavorful and nutritious fruit enjoyed by millions of people in all climates with an increasing demand. The berry is recognized for its low-calorie carbohydrate, high fiber contents and a source of natural antioxidants (Wang *et al.* 1996) including carotenoids, vitamins, phenols, flavonoids, dietary glutathione and endogenous metabolites (Larson 1988). About 65% of the Iran's strawberry is produced in the Kurdistan province (Anonymous 2013).

Drought stress is one of the most important environmental factors and its effects on plant productivity are receiving more and more interest based on the effect of climate change on water resources. Strawberry (*Fragaria* x *ananassa* Duch.) with a shallow root system and a large leaf area is very susceptible even to short periods of water deficit. Genotypic differences in drought tolerance have been observed for strawberry cultivars (Klamkowski and Treder 2008).

Many studies have indicated a positive relationship between accumulation of proline and plant drought tolerance. Proline accumulation in higher plants is a characteristic physiological response to osmotic stress and its degradation can provide carbon, nitrogen and energy source after stress (Hare *et al.* 1999). Significant differences among strawberry cultivars have been observed for proline accumulation. When ‘Kurdistan’ and ‘Selva’ cultivars were under severe drought stress (Ghaderi and Siosemardeh 2011), ‘Kurdistan’ had

higher proline content compared to 'Selva', but proline was reduced in both cultivars one day after rewatering. Free proline content in leaves of strawberry cv. Camarosa showed an increase by increasing salinity (Rahimi *et al.* 2011). Proline content also has been considered as an indicator of drought-stress or stress-tolerance (Stanisavljevic´ *et al.* 2009; Neocleous and Vasilakakis 2012).

Different strategies have been used to increase drought tolerance in plants including proline overproduction and accumulation. Increased drought tolerance has already been obtained in some plant species such as Tobacco (Yamchi *et al.* 2007) and wheat (Vendruscolo *et al.* 2007) and increased salt tolerance in rice (Zhu *et al.* 1998), *Carrizo citrange* (Molinari *et al.* 2004) and chickpea (Ghanti *et al.* 2011) by introduction of Δ1-pyrroline-5-carboxylate synthetase gene (*P5CS*) which is involved in proline synthesis. Proline is synthesized in the cytosol, mainly from glutamic acid via two intermediates, glutamic -γ-semialdehyde (GSA) and Δ-pyrroline-5-carboxylate. The first and the final steps of the proline biosynthesis are catalyzed by P5C synthetase (P5CS) and Δ-pyrroline- 5-carboxylate reductase, respectively (Hare *et al.* 1999).

The objective of this study was the *Agrobacterium tumefaciens*-mediated genetic transformation of two economically important strawberry cultivars, Kurdistan and Camarosa, with the *P5CS* gene in order to increase proline biosynthesis in the transgenic plants. Studies concerning organogenesis in leaf explants from a selected genotype were first performed in order to establish a protocol for plant regeneration that was subsequently used in the genetic transformation.

Materials and Methods

Plant regeneration

Young leaf explants were collected from strawberry (*Fragaria ananassa*) field grown cultivars of 'Kurdistan' and 'Camarosa'. Explants were washed for 30 min in tab water and sterilized by commercial sodium hypochlorite solution (0.5% effective chlorine) for 15 min, then washed three times in the sterile distilled water under aseptic conditions. Leaf segments approximately 0.5 cm square were used as explants. The leaf explants were put in MS medium containing glucose (2%), 4 mg/l TDZ and 0.7% agar and the pH of medium adjusted to 5.8 before autoclaving for 20 min at 121.5°C. The cultures were kept in the dark at the growth room for two weeks and then transferred to fresh medium under white fluorescent tubes providing a photon flux density of approximately 40 μmol m^{-2} s^{-1}, 16-h photoperiod and a temperature of 25 ± 2°C for four weeks. Then, the percentage of explants regenerating shoots were measured.

Transformation of strawberry cultivars

The *Agrobacterium tumefaciens* strain C58 (pGV3101) containing the binary vector pBI121-p5cs was used for genetic transformation. This vector carried the *Nicotiana tabacum P5CS* gene under control of the CaMV35S constitutive promoter, and the neomycin phosphotransferase selectable marker gene (*nptII*) under control of *nos* promoter.

For the preparation of inoculums, a bacterial colony was transferred to LB liquid medium supplemented with kanamycin (50 mg/l) and rifampicin (20 mg/l) and incubated for 16 h on an

orbital shaker (28°C/180 rpm). When the bacterial cultures grew to OD600 =1 , cells were pelleted by centrifugation in 5000 rpm for 10 min at room temperature, and the resulting pellet was resuspended in 25 ml liquid MS medium, and then acetosyringon was added (100µM).

Explants were precultured on MS medium supplemented with glucose (2%), 4 mg/l TDZ, agar (7 g/l), at pH of 5.8 (Figure 1a). Afterwards, explants were inoculated with the bacterial suspension for 20 min and then dried on the sterile filter paper to remove excess of bacteria. The material was incubated (co-cultivated) for three days in darkness at 23°C in MS medium without selection. After co-cultivation, the explants were washed with sterile distillated water for five min and in 800 mg/l cefotaxime solution for 15 min and dried on sterile filter paper. Then explants were transferred to the same medium containing 800 mg/l cefotaxime and incubated for five days. Then explants were transferred to selection medium which was the same medium supplemented with 75 mg/l kanamycin and cefotaxime reduced to 500 mg/l. After 5 weeks, explants were transferred to the second selection medium that was MS medium supplemented with glucose (1%), sucrose (2%), agar (7 g/l), 0.2 mg/l BA, 2 mg/lGA3, 50 mg/l kanamycin and cefotaxime (500 mg/l) and pH 5.8 for 4 weeks.

Plant DNA extraction and PCR analysis

Total gDNA was extracted from the transgenic and wild-type plants using CTAB method with minor modification (Saghai-Maroof *et al.* 1984). Quality and quantity of the extracted DNA were checked by agarose gel and NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The putative transgenic plants were screened for the presence of T-DNA by polymerase chain reaction (PCR) analysis using primers F1 (5'-GGATTGATGTGATATCTCCACTGACG-3)' and R1 (5'-CCTTCAACATCGCTCAGAAGAATCAG-3′). PCR reaction was carried out with 100 ng of genomic DNA, 4 µL mastermix, 3µL H_2O, and 1 µL of each primer. Thirty-five PCR cycles were used for amplification (with denaturation at 94°C for 3 min, an annealing at 50°C for 1 min, an elongation at 72°C of 1 min and further final extension at 72°C for 10 min), after an initial denaturation step at 94°C for 4 min. Amplifying a fragment of 900 bp wild-type strawberry (not infected with *A. tumifaciens*) DNA was used as a negative control and the plasmid was used as a positive control. Amplified DNA fragments were electrophoresed on agarose gel (1.2%) containing ethidium bromide (0.5µg/ml) and visualized and photographed under UV light. In total, 15 lines of PCR positive T0 plants for Kurdistan and 10 for Camarosa were obtained.

Analyses of transgenic plants

Leaf disc senescence assay

The leaves of transgenic and wild-type plants were removed and leaf segments of 1 cm^2 were floated in petri plates having different concentrations (0, 8, 16%) of polyetylenglycol (PEG). The effects of PEG treatment on leaf discs were assessed by observing phenotypic changes and quantified by estimating their chlorophyll

content (Arnon 1949). Chlorophyll estimation was done from the samples after 21 d incubation at 25°C under 16-h photoperiod.

Physiological assessment of transgenic plants

To assess drought tolerance, 40-day-old wild type and five transgenic lines for each cultivar were transferred into a medium containing PEG 6000 at concentrations of 0, 8 and 16%. Several traits including proline content, chlorophyll content, shoot length, fresh weight and dry weight were measured in the transgenic and wild-type plants under stress conditions. Proline content was measured in the leaves by the method as described by Bates *et al.* (1973). Fully developed matured leaf segments were homogenized with 3% sulphosalicylic acid and the homogenates were centrifuged at 3000 *g* for 20 min. The supernatant was treated with acetic acid and acid ninhydrin; after boiling for 1 h, the absorbance at 520 nm was determined using spectrophotometer (Bates *et al.* 1973). Proline content was expressed as μM g^{-1} fresh weight (FW).

Chlorophyll content was quantified using acetone and by the method described by Arnon (1949). The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min. The absorbance of the supernatant was recorded at 646 and 663 nm wavelength using spectrophotometer and chlorophyll content was calculated. Data on shoot length, fresh weight and dry weight was scored after a period of one month for controls and transgenic plants grown under PEG stress conditions for both cultivars, Camarosa and Kurdistan.

Statistical analysis

The significance of PEG treatment effects and the effects of the transgene were determined using analysis of variance with three replicates. Differences among treatment means was analyzed using the LSD (least significant difference) procedure at 5% probability level.

Results

Plant regeneration

Callus formation occurred for all leaf explants. Callus induction began after 15 d on MS medium containing 2% glucose and 4 mg/l TDZ (Figure 1 a, b, c). Direct shoot regeneration was obtained 30 days after culture on the same medium (Figure 1 d, e, f). After five weeks, 30 and 70% of the explants were regenerated for Camarosa and Kurdistan, respectively.

Plant genetic transformation

Leaf disc of two cultivars Camarosa and Kurdistan were transformed using *A. tumefaciens* strain C58 (pGV3101) containing the binary vector pBI121-p5cs with *P5CS* gene. The leaf disks with 48 h co-cultivation failed to grow after five weeks on co-cultivation medium, while leaf disks with 72 h co-cultivation were regenerated efficiently. After inoculation, co-culture and cultivation on selection medium, some calli were formed on leaf explants and the shoots regenerated (Figure 1a, 1b and 1c). After pre-selection, the explants were transferred to fresh selective regeneration media for five weeks with 75 mg/l kanamycin for the selection of transformed cells and to inhibit further agro-bacterial growth (Figure 1). In the control explants (non-inoculated), calli bleached and died

following the culture on medium supplemented with 75 mg/l kanamycin. Adventitious buds sprouted from green calli after 42 d. In the non-inoculated explants, 70 and 30% callus induction occurred for Camarosa and Kurdistan on kanamycin free medium, respectively. Transformation efficiency was evaluated as the number of PCR positive plants per total number of inoculated explants. On average, contamination rates were 20% and 17% for Camarosa and Kurdistan, respectively.

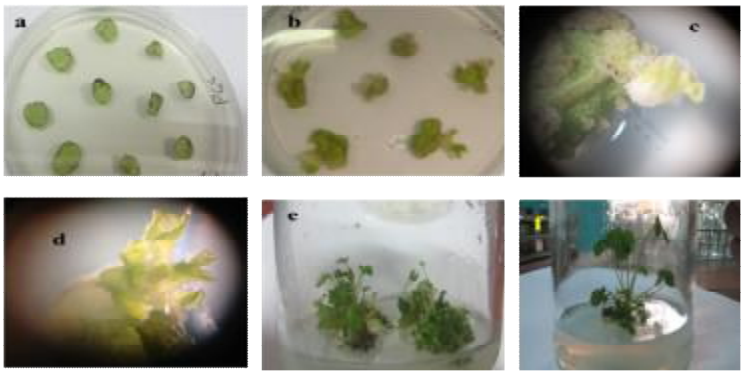

Fig.1 *In vitro* plant regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation of Comarosa variety of strawberry using leaf disc method. (a) Explants on regeneration medium after co-culture.(b, c) Early callus development in leaf disc region 15 days after co-culture in the regeneration medium in darkness. (d) Early developed shoot indirectly, in explants, 30 days after co-culture in the regeneration medium (selection medium) in darkness. (e) Differentiation of shoot after co-culture in the second selection medium in darkness. (f) Transgenic plantlets.

PCR analysis

The confirmation of genetic transformation was done by PCR to confirm the insertion of the *P5CS* gene. To confirm transformation event at the molecular level, total DNA was extracted and analyzed from the leaves of both non-transformed and transgenic plants (Figure 2). Putative transgenic plants were screened by PCR using 35S and *P5CS* gene-specific primers to detect the presence of the transgene in the transgenic plants. PCR analysis showed the amplification of the predicted 900 bp fragment in kanamycin-resistant transformed plants for both cultivars (Figure 2). No amplification product was detected in the negative control, while positive plasmid control produced the same bands as transgenic plants.

Leaf disk and plants senescence assay

Leaf disks and plantlets obtained from primary transgenic plants and wild-type plants were cultured on MS medium containing 0, 8 and 16% PEG stress. Leaf disks and plantlets from the transgenic plants remained green in the presence of 16% PEG, whereas leaf disks and plantlets from the wild-type were highly susceptible to PEG and showed chlorophyll bleaching symptoms (Figure 3).

Proline accumulation

A significant difference ($P<0.01$) for the proline content was seen between the control and transformed plants under different PEG concentrations (Figure 4 a, b). Overall, in the 0, 8

and 16% PEG stresses, the average proline content in the leaves of Kurdistan variety increased by 165.2%, 191.1% and 111.1%, compared with the non-transgenic plants, respectively. The average proline content in leaves of Camarosa increased by 133.4%, 137.5% and 140.3% compared with the wild-type plants under 0, 8 and 16% PEG stresses, respectively. There was no obvious difference in the proline content between the Kurdistan and Camarosa

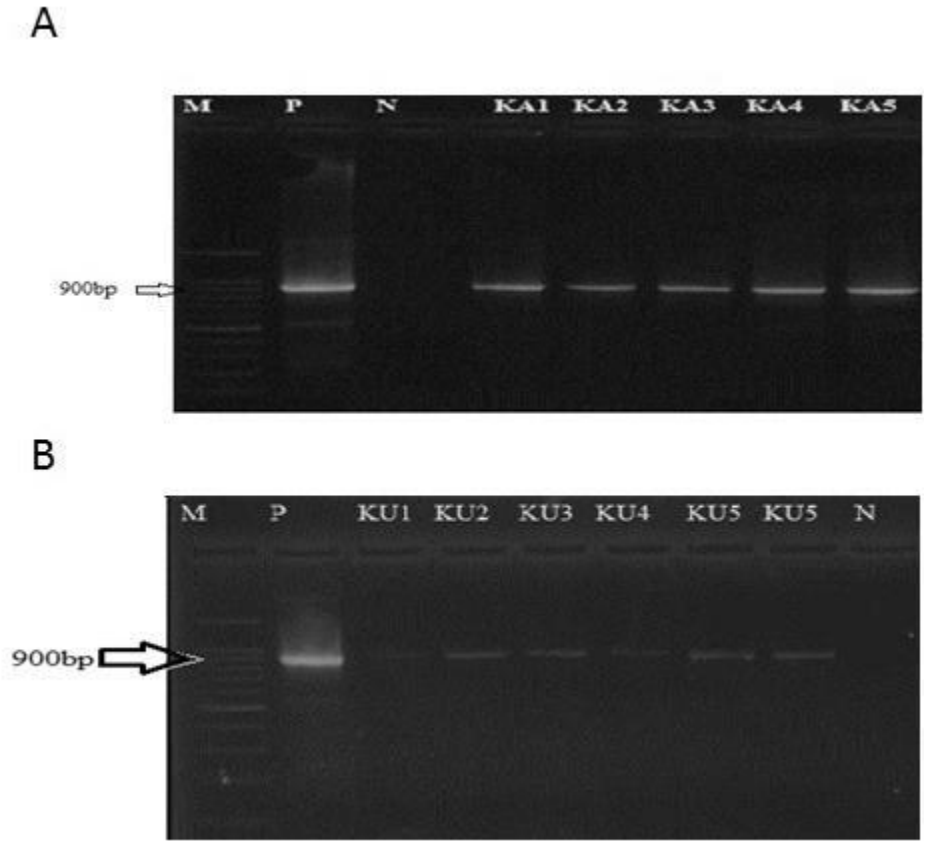

Figure 2. PCR analysis of transgenic plants of p5cs gene (900bp) in the genome of selected transformed strawberry cultivars Camarosa (A) and Kurdistan (B); KA: Camarosa transgenic shoots; KU: PCR of Kurdistan transgenic shoots; N: negative control (Non-transgenic strawberry plants); P: positive control DNA from plasmid; M: molecular marker (100 bp DNA ladder).

.

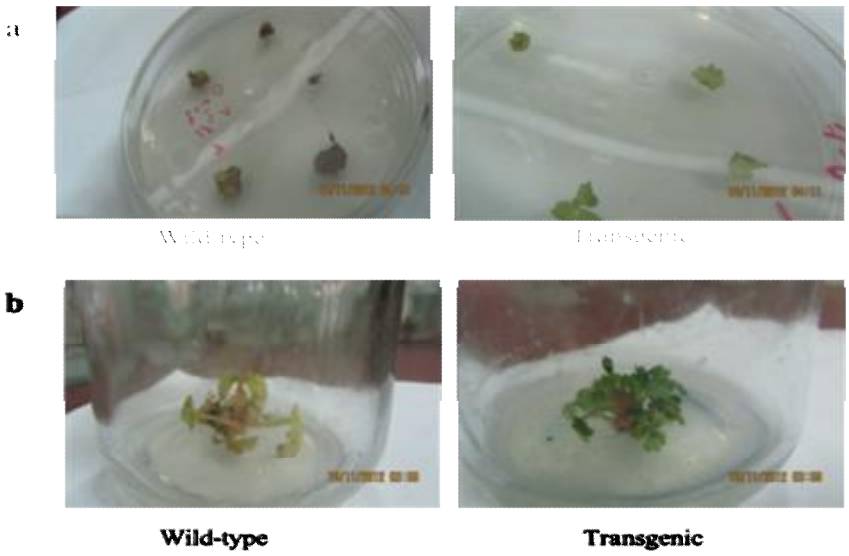

Figure 3. Effect of drought stress on leaf and plantlets of Kurdistan variety; (a) transgenic plants were analyzed for tolerance to PEG stress by leaf senescence assay. Leaves from transgenic and wild-type plants were treated with 16% PEG (-7 bar) for 21 days. Leaf from transgenic plants remained green, whereas leaf from the wild-type plants were highly susceptible to PEG and showed chlorophyll bleaching symptoms. (b) Transgenic and wild-type plants were treated with 16% PEG (-7 bar) for 21 days. The transgenic plants survived and continued to grow, while wild-type plants failed to withstand drought conditions.

under normal and stressed conditions. However, significant differences in the proline content (P<0.0001) were seen between different levels of PEG under normal and PEG-stress conditions.

Chlorophylls content

A significant difference (P < 0.01) in chlorophyll content was seen between the control and transformed plants under different PEG concentration (Figure 4 c, d). In the Kurdistan variety, chlorophyll content was significantly higher in the transgenic plants as compared to the wild-type plants at 0, 8 and 16% PEG stress. However, for Camarosa average chlorophyll content was significantly higher in the transgenic plant only at 8 and 16% PEG stress. There was significant difference in the chlorophyll content between different levels of PEG in the transgenic plants for both cultivars.

Biomass of transgenic and wild-type plants

To determine the effect of proline accumulation on plant growth and development, we measured shoot length, shoot fresh and dry weight in both the control and transgenic plants under normal and PEG stressed conditions. Wild type and transgenic plants in the Kurdistan cultivar showed significant difference (P<0.05) for all characters when grown under PEG conditions (Figure 5a, 5c, 5e). Similarly Camarosa control and transgenic plants showed significant difference (P<0.001) for these three characters when grown under PEG (Figure 5b, 5d, 5f). Compared to the control plants, fresh biomass of shoots in the Kurdistan transgenic plants were 0.6%, 8.5% and 16% longer and 0, 14.5 and 47.5% greater (Figure 5 c, d). In Camarosa compared to the control plants, shoots on transgenic plants were 8%, 13% and

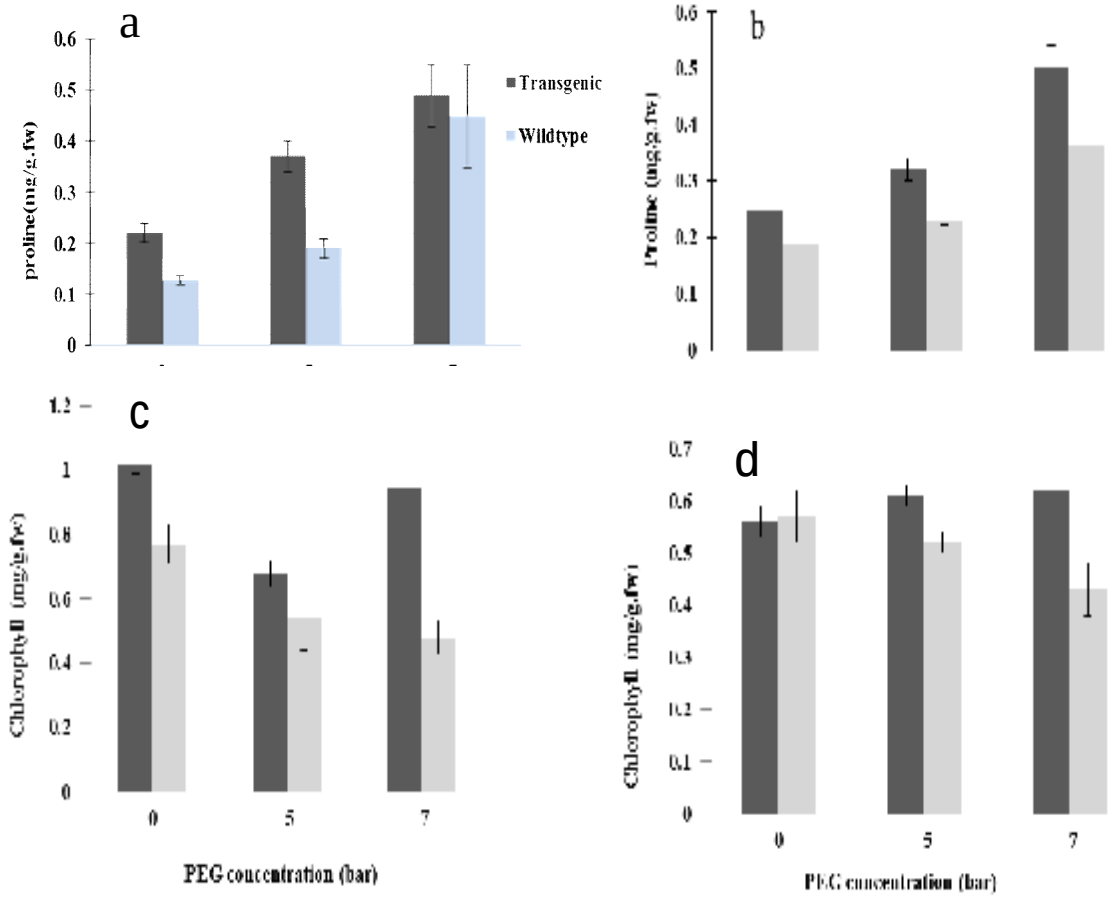

Figure 4. Proline and chlorophyll content of the leaves in wild-type and transgenic plants exposed to various concentrations of polyetylnglycol (PEG). Proline content of Comarosa (a) and Kurdistan (b) varieties; Chlorophyll content in Comarosa (c) and Kurdistan (d). The bars represent mean ± standard errors.

34% longer and 32%, 25% and 97% greater for the fresh biomass. However, both fresh and dry weight were significantly different ($P<0.05$) in different PEG conditions for the Camarosa variety (Figure 5 e**,** f).

Discussion

In this study, *Agrobacterium*-mediated transformation of two economically important strawberry cultivars, Kurdistan and Camarosa, was reported with *P5CS* gene. P5CS is a rate-limiting enzyme in the proline synthesis via the glutamate pathway. In most plant species such as *Vigna aconitifolia* (Karthikeyan *et al.* 2011), *Opuntia streptacantha* (Silva-Ortega *et al.* 2008) and *Oryza sativa* (Kumar *et al.* 2011), increasing of *P5CS* expression resulted in more proline accumulation. Similarly our results showed that over expression of *P5CS* in both strawberry cultivars significantly increased proline content. Amount of proline and soluble carbohydrates increased under severe drought stress in Kurdistan (Ghaderi and Siosemardeh 2011). Neocleous and Vasilakakis (2010) and Stanisavljevic´ *et al.*

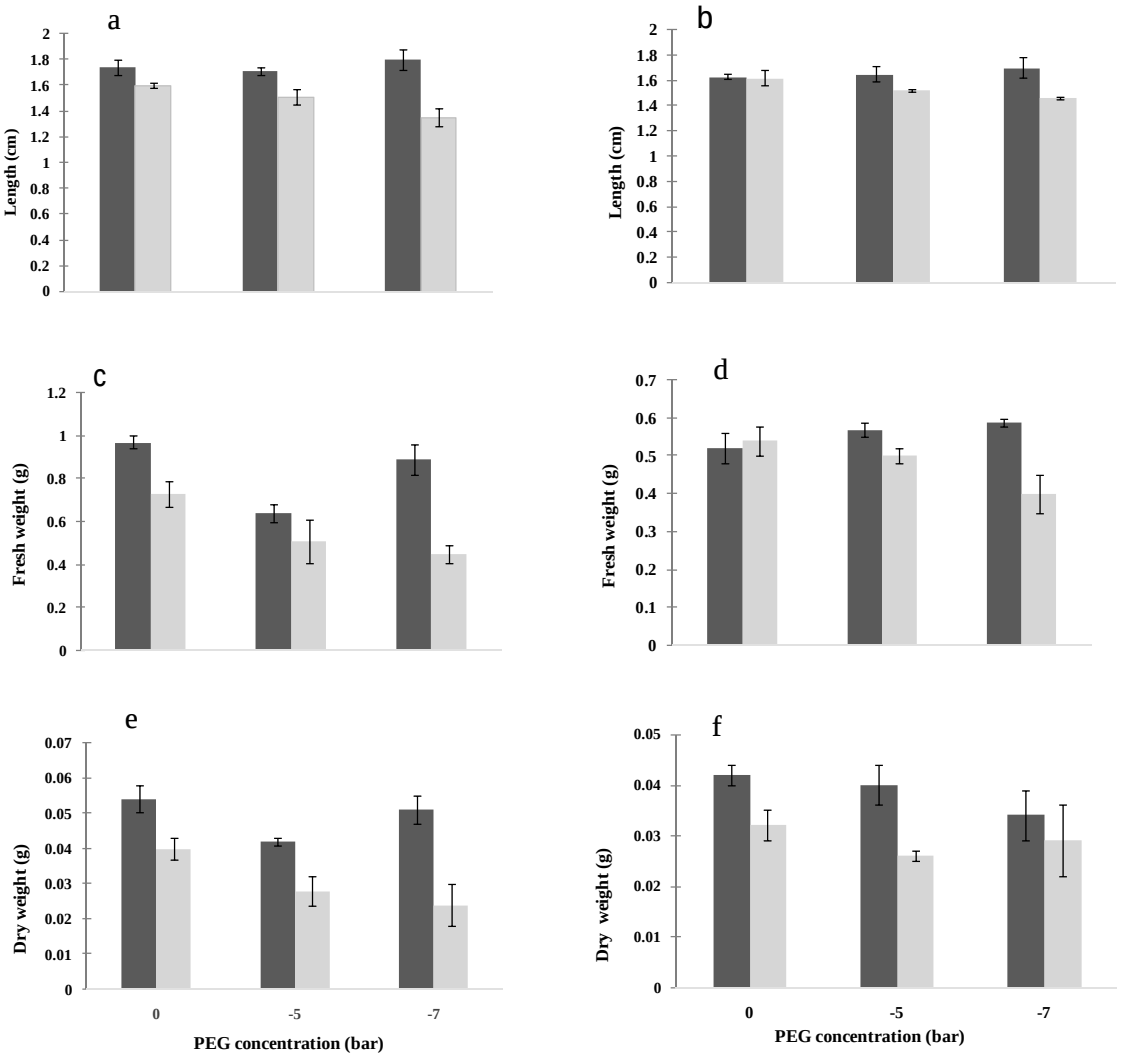

Fig. 5 Shoot length, fresh weight and dry weight in Comarosa and Kurdistan wild-type and transgenic lines under drought stress with polyetylnglycol (PEG). Shoot length in Comarosa (a) and Kurdistan (b); fresh weight in Comarosa (c) and Kurdistan (d); dry weight in Comarosa (e) and Kurdistan (f). The bars represent mean ± standard errors.

(2009) suggested that proline content in strawberry may be an indicator of drought-stress or stress-tolerance. Leaf disk assay and plant biomass measurement under PEG condition in the transgenic and wild-type plants showed that higher accumulation of proline in the strawberry transgenic plants may increase plant tolerance.

Plant genetic engineering strategies for abiotic stress tolerance depend upon the expression of genes that are involved in signaling and regulatory pathways or genes that encode proteins conferring stress tolerance or enzymes present in pathways leading to the synthesis of structural metabolites and osmolytes (Apse and Blumwald, 2002; Seki *et al.* 2003; Shinozaki *et al.* 2003; Wang *et al.* 2003). One such gene encoding osmotin protein that is induced in response to salinity and drought was identified in

1980s (Singh *et al.* 1987; LaRosa *et al.* 1992). Since then, numerous studies have been carried out to determine the physiological role of osmotin in abiotic and biotic stress tolerance (Jami *et al.* 2007; Kupchak *et al.* 2008; Lee *et al.* 2010). In the previous reports, an increased level of free proline was found to correlate with improved osmotolerance (Sing *et al.* 1987). While salinity and drought stresses cause detrimental changes in cellular components, a wide range of metabolites including amino acids like proline can prevent these detrimental changes (Vinocur and Altman 2005).

Chlorophyll content is one of the parameters that may be decreased during drought stress. Székely *et al.* (2008) showed that the *P5CS1* Arabidopsis mutants are also compromised in their capacity to withstand salt stress. The mutants showed impaired root elongation, enhanced chlorosis, and ultimate seedling lethality in the presence of NaCl. They also indicated that reduced proline accumulation in the *p5cs1* mutants leads to salt hypersensitivity, not only because of impaired osmotic adjustment but also due to enhanced accumulation of reactive oxygen species (ROS). Enhancement of chlorosis, chlorophyll degradation and lipid peroxidation in the salt-stressed *p5cs1* mutants are thus indicative of ROS damage (Székely *et al*. 2008). In our experiments chlorophyll content was significantly higher under severe drought stress for both transgenic cultivars compared to the untransformed plants. This may indicate the protection of chlorophyll in transgenic plants under PEG stress and shows that the transgenic strawberry plants with P5CS gene were able to tolerate drought stress. Transgenic chickpea over expressed P5CS showed more chlorophyll stability and electrolyte leakage and proline accumulation and finally salt stress tolerance compared to the wild-type chickpea plants (Ghanti *et al*. 2011).

Shoot length, fresh weigh and dry weight was significantly higher in the transgenic plants as compared to the wild-type plants during the stress period at 16% PEG concentration. Transgenic sugarcane overexpressed P5CS also showed significantly higher biomass compared to the control plants after 12 days of water withhold (Molinari *et al.* 2007). Husaini and Abdin (2008) reported that overexoression of tobacco osmotin gene leads to salts stress tolerance of strawberry. In their study, shoot length and root length of transgenic plants were greater than that of wild-type plants even after two weeks of exposure to salt stress. The higher biomass observed in the transgenic strawberry plants may indicate the proline contribution to the enhancement of tolerance to drought stress and proline accumulation in those plants might have played a role in plant growth other than cytoplasmic osmotic adjustment.

In conclusion, the results obtained in this study showed that overexpression of P5CS resulted in more proline accumulation under drought stress condition and stress-induced proline production confers drought stress tolerance in *P5CS*-transformed strawberry cultivars. Results also showed that proline in addition to an osmotic adjustment mediator, is linked to stress tolerance through the protection of the photosynthetic apparatus and producing higher biomass. However, for a decisive conclusion T1 plants or later generations should be evaluated.

Acknowledgment

This work was supported by the Research Center for Strawberry Breeding and Development, University of Kurdistan, Iran. We are thankful to Dr. Ahad Yamchi for kindly providing the vector needed for this research program.

References

Anonymous, 2013. Statistics Yearbook. Organization of Agriculture-Jahad, Kurdistan, Iran (In Persian).

Apse MP and Blumwald E, 2002. Engineering salt tolerance in plants. Current Opinion in Biotechnology 13: 146-150.

Arnon DI, 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology 24: 1-15.

Bates LS, Waldren RP and Teare ID, 1973. Rapid determination of free proline for water-stress studies. Plant and Soil 39: 205-207.

Dhindsa RS, PlumB-Dhindsa P and Thorpe TA, 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany 32: 93–101.

Ghaderi N and Siosemardeh A, 2011. Response to drought stress of two strawberry cultivars (cv. Kurdistan and Selva). Horticulture, Environment and Biotechnology 52: 6-12.

Ghanti SKK, Sujata KG, Vijay Kumar BM, Karba NN, Reddy KJ, Rao MS and Kavi Kishor PB, 2011. Heterologous expression of *P5CS* gene in chickpea enhances salt tolerance without affecting yield. Biologia Plantarum 55: 634–640.

Hare PD, Cress WA and Van Staden J, 1999. Proline synthesis, degradation; a model system for elucidating stress-related signal transduction. Journal of Experimental Botany 50: 413-434.

Husaini A and Abdin MZ, 2008. Overexoression of tobacco osmotin gene leads to salts stress tolerance in strawberry (Fragaria x ananassa Duch.) plants. Indian Journal of Biotechnology 7: 465-471.

Jami SK, Swathi Anuradha T, Guruprasad L and Kirti PB, 2007. Molecular, biochemical and structural characterization of osmotin-like protein from black nightshade (*Solanum nigrum*). Journal of Plant Physiology 164: 238–252.

Karthikeyan A, Pandian SK and Ramesh M, 2011. Transgenic indica rice cv. ADT 43 expressing a Δ -1-pyrroline-5-carboxylate synthetase (P5CS) gene from *Vigna aconitifolia* demonstrates salt tolerance. Plant Cell, Tissue and Organ Culture 107: 383–395.

Klamkowski K and Treder W, 2008. Response to drought stress of three strawberry cultivars grown under greenhouse conditions. Journal of Fruit and Ornamental Plant Research 16: 79-188.

Kumar V, Shriram V, KaviKishor PB, Jawali N and Shitole MG, 2010. Enhanced proline accumulation and salt stress tolerance of transgenic *Indica* rice by over-expressing *P5CSF129A* gene. Plant Biotechnology Reports 4: 37–48.

Kupchak BR, Villa NY, Kulemina LV and Lyons TJ, 2008. Dissecting the regulation of yeast genes by the osmotin receptor. Biochemical and Biophysical Research Communications 374: 210–213.

LaRosa PC, Chen Z, Nelson DE, Singh NK, Hasegawa PM and Bressan RA, 1992. Osmotin gene expression is posttranscriptionally regulated. Plant Physiology 100: 409–415.

Larson RA, 1988. The antioxidants of higher plants. Phytochemistry 27: 969-978.

Lee B, Damsz B, Woloshuk CP, Bressan RA and Narasimhan ML, 2010. Identification of *Fusarium oxysporum* genes that control cell wall properties using the plant defense protein, osmotin. Eukaryot Cell. *doi*:10.1128/EC.00316-09

Molinari HBC, Marur CJ, Daros E, Campos MKF, Carvalho JFRP, Bespalhok JCF, Pereira LFP and Vieira LGE, 2007. Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiologia Plantarum 130:218–229.

Molinari HBC, Marur CJ, Filho JCB, Kobayashi AK, Pileggi M, LeiteJunior RP, Pereira LFP and Esteves Vieira LG, 2004. Osmotic adjustment in transgenic citrus rootstock *Carrizo citrange* (*Citrus sinensis* Osb. x *Poncirus trifoliate* L. Raf.) overproducing proline. Plant Science 167: 1375-1381.

Neocleous D and Vasilakakis M, 2012. Effects of cultivars and substrates on soilless strawberry production in Cyprus. Acta Horticulturae 926: 435-440.

Rahimi A, Biglarifard A, Mirdehghan H and Borghei SF, 2011. Influence of NaCl salinity on growth analysis of strawberry cv. Camarosa. Journal of Stress Physiology and Biochemistry 7: 145-156.

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW, 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proceedings of the National Academy of Sciences of the USA 81: 8014-8018.

Seki M, Kamei A, Yamaguchi-Shinozaki K and Shinozaki K, 2003. Molecular responses to drought, salinity and frost: common and different paths for plant protection. Current Opinion in Biotechnology 14: 194–199.

Shinozaki K, Yamaguchi-Shinozaki K and Seki M, 2003. Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology 6: 410–417.

Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Agüero JA, Aguado-Santacruz GA and Jiménez-Bremont JF, 2008. Salt stress increases the expression of *p5cs* gene and induces proline accumulation in cactus pear. Plant Physiology and Biochemistry 46: 82-92.

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE and Bressan RA, 1987. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiology 85: 529–536.

Stanisavljevic A, Lisjak M, Vinkovic T, Spoljarevic M, Karakas M, Teklic T and Cosic T, 2009. Proline in strawberry leaf as an indicator of salt stress. In Marić S and Lončarić Z (Eds.). Proceedings of the 44th Croatian & 4th International Symposium on Agriculture, Faculty of Agriculture, Osijek, Croatia, 94–98.

Szekely G, Abrahám E, Cseplo A, Rigo G, Zsigmond L, Csiszar J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, Koncz C and Szabados L, 2008. Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. The Plant Journal 53: 11-28.

Vendruscolo ECG, Schuster I, Pileggi M, Scapim CA, Molinari HBC, Marur CJ and Vieira LGE, 2007. Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. Journal of Plant Physiology 164: 1367–1376.

Vinocur B and Altman A, 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion in Biotechnology 16: 123–132.

Wang H, Cao G and Prior RL, 1996. Total antioxidant capacity of fruits. Journal of Agriculture and Food Chemistry 44: 701-705.

Wang W, Vinocur B and Altman A, 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218: 1–14.

Yamchi A, RastgarJazii F, Mousavi A, Karkhane A and Renu A, 2007. Proline accumulation in transgenic tobacco as a result of expression of *Arabidopsis* Δ1-pyrroline-5-carboxylate synthetase (P5CS) during osmotic stress. Journal of Plant Biochemistry and Biotechnology 16: 9–15.

Zhu B, Su J, Chang M, Verma DPS, Fan YL and Wu R, 1998. Overexpression of a **Δ** 1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt stress in transgenic rice. Plant Science 139: 41-48.