Full Length Research Paper

Genetic transformation of olive somatic embryos through *Agrobacterium tumefaciens* and regeneration of transgenic plants

Mahboobeh Jafarzadeh-Bajestani [1#], Maryam Khodai-Kalaki [1#], Nasrin Motamed[1]*, Omidreza Noorayin[2]

[1]The University College of science, Faculty of Biology, University of Tehran, Tehran, Iran.
[2]Department of Biochemistry, National Center of Genetic Engineering and Biotechnology, Tehran, Iran.

Transformed olive plants were regenerated from inoculated somatic embryos with *Agrobacterium tumefacience* strain GV3101, which carries the plasmid pBI-*P5CS* containing *Arabidopsis thaliana P5CS* cDNA, kanamycin marker (*nptII*) gene and *uidA* reporter gene. Initially, repetitively embryogenic cultures were established from radicles and cotyledonary segments of mature olive zygotic embryos. Single somatic embryos at cotyledon stage were used for transformation. Through repetitive somatic embryogenesis, non-chimer secondary embryos were selected and propagated on kanamycin containing medium. Resistant embryos were converted to plantlets by subjecting them to desiccation. Transformation and *P5CS* gene expression was confirmed by β-glucuronidase (GUS) assay polymerase chain reaction (PCR) and reverse transcription (RT)-PCR analysis.

Key words: *Olea europaea*, somatic embryogenesis, transformation, β-glucronidase, *P5CS* gene.

INTRODUCTION

Olive is a long-lived evergreen tree with high economic importance due to nutritional value of its oil. Olive breeding through conventional methods has not been very successful. Gene transfer techniques offer a highly desirable approach for genetic improvement in respect to traditional methods (Rugini and Gutiérrez Pesce, 2006). Using somatic embryos for genetic transformation is an important tool for the development of superior genotypes. This has been the method of choice for genetic manipulation of some woody, including walnut (McGranahan et al., 1990, 1988), avocado (Cruz-Hernández et al., 1998),

rose (Li et al., 2002) and olive (Rugini et al., 1999). The regeneration of transgenic plants from somatic embryos is a viable alternative to the leaf disk transformation method in plants that are recalcitrant to adventitious bud formation but undergo secondary somatic embryo-genesis. Another advantage of this system is to eliminate chimer transformants by multiplying embryos through secondary or repetitive somatic embryogenesis (Dandeker and MacGranahan, 1989; McGranhan et al., 1990).

P5CS gene codes for Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme that catalyzes two steps of proline biosynthesis in plants (Zhang et al., 1995; Peng et al., 1996). Proline as an osmolyte seems to have diverse roles under osmotic stress conditions, such as stabilization of proteins, membranes and subcellular structures as well as protecting cellular functions by scavenging reactive oxygen (Kishor et al., 2005). It acts as an osmoprotectant, increasing the ability of plant cells to retain water without disturbing normal cellular functions (Kishor et al., 1995, 2005).

Using genetic engineering in order to increase levels of compatible solutes such as proline appears to be a promising approach in an effort to increase the ability of plants to tolerate environmental stress (Zhu et al., 1998).

---

*Corresponding author. E-mail: motamed2@khayam.ut.ac.ir. Tel: (+9821) 61112472. Fax: (+9821) 66405141.

#These authors equally contributed to this article.

Abbreviations: GUS, β-Glucuronidase; **IBA,** indole-3-butyric acid; **2ip,** N^6 (2-isopentenyl) adenine; **NAA,** naphtaleneacetic acid; **BA,** 6-benzyladenine; **Km,** kanamycin; **OMc,** olive medium in which macroelements were replaced with the ones proposed by Bourgin and Nitch; **IBA,** indole-3-butyric; **CTAB,** cetyl trimethylammonium bromide; **DEPC,** diethylpyrocarbonate.
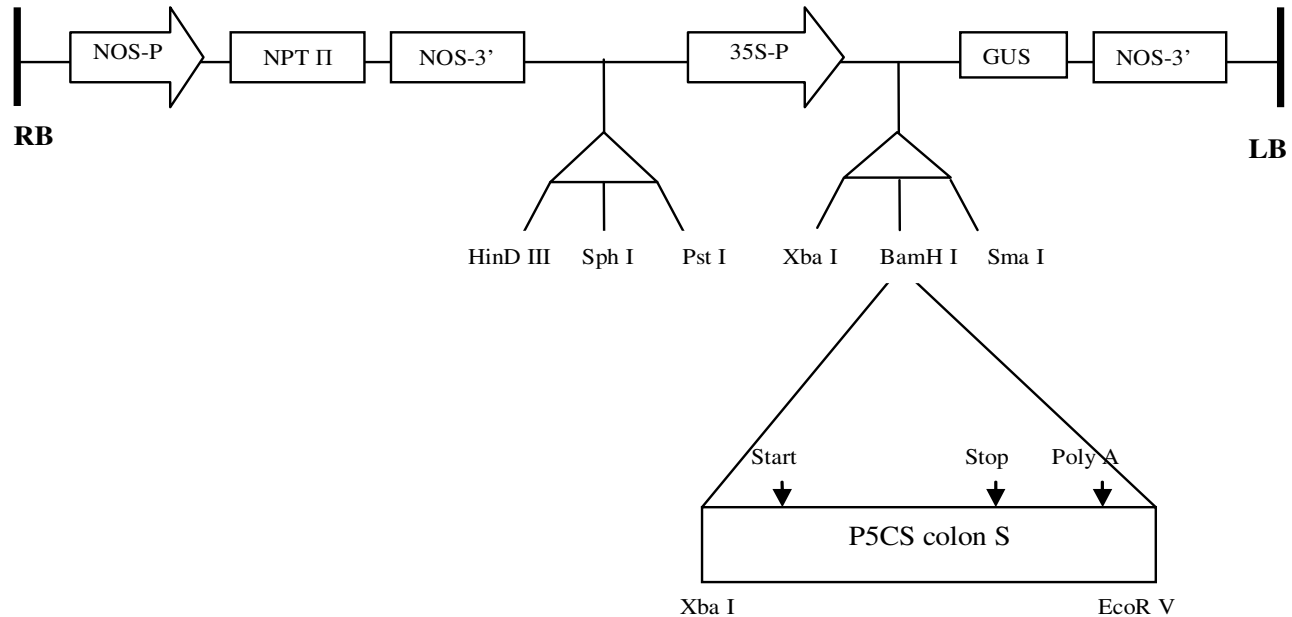
Figure 1. Schematic diagram of pBI121-*P5CS.*

Overexpression of *P5CS* in olive plant could lead to a better understanding of stress mechanisms in perennial plants as well as stress tolerance and improved olive growth under osmotic conditions. In this study, our aim is to develop an efficient method for olive genetic trans-formation as well as the insertion of *P5CS* gene to olive genome

MATERIALS AND METHODS

Plant material

Mature seeds of *Olea europaea* cv. Zard were used in this study. This cultivar is endemic in Iran and produces about 55% oil in dry weight. Seeds were peeled by removing woody pericarp. They were surface sterilized with 70% ethanol for 1 min followed by 50% commercial bleach (active sodium hypochlorite) plus few drops of tween 20% for 20 min. The seeds were rinsed three times with sterile distilled water and kept in Petri dishes containing 10 ml sterile distilled water for 48 to72 h in darkness at 4°C.

The embryos were excised from the endosperm, then radicle and proximal cotyledon segments of zygotic embryos were separated as explants. Explants were placed on olive medium (OMc) medium (modified OM medium with BN macroelements, Canas and Benbadis 1988) supplemented with 1 gL^{-1} casein hydrolysate, 30 gL^{-1} sucrose and either growth regulators of 24 µM IBA /2.5 µM 2ip (Orinos and Mitrakos, 1991; Mitrakos et al., 1992) or 24 µM NAA /2.5 µM BA. The cultures were kept at 25 ± 1°C in darkness. After 3 weeks, calli were transferred to OMc medium without any plant growth regulators and maintained for two months in darkness at 25 ± 1°C to develop embryos. Somatic embryo lines were established by subculturing embryos on solid basal OMc medium without growth regulators in the dark at 25 ± 1°C. Secondary embryos were transferred to fresh medium every 3 to 4 week to multiply the lines. These somatic embryo lines were used as a source for transformation experiments. The pH of all media were adjusted to

5.8 before adding 6 gL^{-1} Difco Bacto agar and autoclaved at 121°C for 20 min.

Agrobacterium strain

Agrobacterium tumefaciens strain pGV3101 harboring the binary vector pBI121-*P5CS* was utilized, containing its T-DNA Km resistance marker gene (*nptII*) under the control of NOS promoter, *Arabidopsis thaliana P5CS* cDNA and β-glucuronidase (GUS) reporter gene *(uidA)*, both under the control of the cauliflower mosaic virus 35S promoter which is a constitutive promoter (Figure 1) . The bacteria were grown overnight at 28°C in 15 ml LB liquid medium supplemented with 15 µg/ml km in an orbital shaker at 150 rpm.

Agrobacterium-mediated transformation

Single somatic embryos (0.5 to 0.7 cm in size) in the cotyledonary stage were inoculated with bacterial suspension at OD = 0.4 for 20 min, blotted on dry sterile filter paper. Embryos were co-cultivated with *Agrobacterium* for 48 h in OMc medium at 25°C in the dark. The embryos were then transferred to OMc medium supplemented with 25 mgl^{-1} Km and 350 mgl^{-1} cefotaxime. Embryos were regularly subcultured every 3 to 4 weeks to the same medium for 4 months. During this period, resistant embryos produced secondary embryos on their base and some of these secondary embryos were chosen for GUS activity as the representatives of the line.

GUS histochemical analysis

β-Glucuronidase enzyme activity was detected in transformed embryos chosen from earlier mentioned resistant lines using x-glu (5-bromo-4-chloro-3-indolyl-D-glucuronic acid) at pH 7, after overnight incubation at 37°C as described by Jefferson et al. (1987).

Germination of somatic embryos

After four months selection, kanamycin (Km) resistant embryos were propagated through repetitive somatic embryogenesis. Well-grown embryos irrespective of their morphology were chosen randomly. They were put in sealed empty Petri dishes for desiccation and kept in darkness for 3 days at 25°C. For germination, desiccated embryos were transferred to 80 × 15 mm jars containing 20 ml ½MS medium (All vitamins, micro and macro elements were half strength). They were kept in a growth chamber at 25 ± 1°C with 16/8 h photoperiod under white fluorescent lamp which gave photon flux density of about 40 µmol m^{-2} s^{-1} .

DNA isolation and PCR

Plant genomic DNA was extracted from leaves of control and putative transformed olive plantlets according to the cetyl trimethylammonium bromide (CTAB) method (Murray and Tompson, 1980). PCR was carried out using specific primers previously designed by Yamchi et al. (2006). The forward primer was located in the 35S promoter (R2Fw: 5'-GGATTGATGTGATATCTCCACTGACG-3') and the reverse primer in the *P5CS* gene (available at NCBI data basis with the acc no. D32138) (R6Rev: 5'-CCTTCAACATCGCTCAGAAGAATCAG-3'), which was permitted to amplify a fragment with an expected size of 765 bp. The PCR reaction were carried out in 25 µM volume with the following condition: Predenaturation at 95°C for 10 min, 35 cycle of denaturation at 94°C for 1 min, 47.5°C annealing for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified products were visualized in a 1% agarose gel stained with ethidium bromide using ETP as a running buffer (Sambrook et al., 1989).

RNA extraction and reverse transcription (RT)-PCR analysis of *P5CS* expression

Total RNA was extracted from leaf tissue of transgenic and control plants using RNX plus extraction kit (Fermentas), treated with RNase-free DNaseI and suspended in 30 µl of diethylpyrocarbonate (DEPC) treated water. For RT-PCR, cDNA synthesis was performed according to Fermentas protocol using M-MuL reverse transcriptase and ribonuclease enzyme. According to this protocol, 5 µl of total RNA from each sample were used in a 20 µl RT reaction using the reverse primer mentioned earlier with the following program: 70°C for 10 min, 37°C for 5 min, 42°C for 2 h and 70°C for 10 min. After completion of cDNA synthesis, 1 µl of the RT reaction was used for amplification of *P5CS* in a 25 µl PCR reaction. Twenty eight PCR cycles were performed with the same program as for DNA amplification using the following primers: 5'-GGA TTG ATG TG A TAT CTC CAC TGA CG-3' and 5'-CCT TCA ACA TCG CTC AGA AGA ATC AG-3' and Fermentas Taq polymerase. Aliquots of each reaction were run on 1% agarose/TBE 0.5× gel and visualized with ethidium bromide staining.

Determination of free proline content

The proline content was determined in leaf samples derived from transgenic and control plants under normal conditions according to Mc Mannus et al. (2000) by measuring the quantity of the colored product of proline reaction with ninhydric acid. The absorbance was read at 518 nm using a Shimadzu model UV160 spectro-photometer. The proline concentration was determined from a standard curve and calculated on fresh weight basis. As the proline content varies from leaf to leaf and also with the age of the plants, leaves with the same age and size were selected.

Statistics

The data presented in this paper is the mean of three independent experiments. All data were analyzed for significance (P = 0.05) by analysis of variance (ANOVA) with mean separation by Duncan´s test using statistical software SPSS (13.0).

RESULTS AND DISCUSSION

Genetic transformation and regeneration

By using both naphtaleneacetic acid/6-benzyladenine (NAA/BA) and indole-3-butyric (IBA)/2ip treatment, calli from segments of mature zygotic embryos showed similar morphogenic response. In calli derived from radicle and proximal cotyledon segments, rhyzogenesis was obser-ved alongside embryogenesis. The embryos were propa-gated by secondary embryogenesis on a medium lacking growth regulators.

The Km sensitivity of somatic embryos was previously tested (data not shown). A concentration of 25 mgl^{-1} Km completely inhibited somatic embryogenesis. It was also lethal to embryos and turned them to brown color. Therefore, this concentration was used for selection of transformants. Inoculated embryos (E0) firstly showed poor survival at selection medium but after a month, secondary somatic embryos (E1) appeared more on radicle than cotyledon regions. E1 secondary embryos were transferred to fresh medium containing same con-centration of antibiotics and propagated for 3 to 4 months. E0 embryos were omitted from the experiment because of the possibility of being chimer. Some somatic embryos did not survive or failed to propagate new embryos. These embryos were omitted from the experi-ment. Table 2 indicates the transformation results.

The resistant embryos were chosen for GUS assay. The expression of *uid A* gene was very low in the first somatic lines showing a pale blue color. Following repetitive somatic embryogenesis, most of the somatic embryos from resistant clones showed deep blue colors indicating that they were not chimer (Figure 2).

For regeneration, well-grown embryos at cotyledonary stage were transferred to ½ Murashige and Skoog (MS) medium after desiccation. A control group of both embryos were directly transferred to ½MS medium without being desiccated. Only few mature embryos (5.7%) regenerated to plantlets. Germination percentages increased to about 50% when embryos were pretreated by desiccation (Figure 3 and Table 1).

Regenerated plants from Km-resistant were used to verify the presence of *P5CS* gene by PCR. Transformed olive plants (lane 4) showed amplified products of nearly 765 bp from *Arabidopsis P5CS* gene, while non trans-genic control plants (lane 3) showed no band (Figure 4). As shown in Figure 4, the negative control (water instead of DNA) in lane 2 and pBI121 in lane 5 did not show any band. To elucidate *P5CS* expression, RT-PCR was carried out. As shown in Figure 5, a 700 bp amplification

Table 1. The effect of desiccation on regeneration of non-transformed embryos. Regeneration percentages followed by same letter are not significantly different at the 5% probability level.

Pretreatment	Regeneration medium	Embryos tested	regeneration%
Control	1/2MS	52	5.7[a]
Desiccation	1/2MS	62	50[b]
Partial desiccation	1/2MS	43	48/8[b]

Table 2. Summery of transformation experiment. Number of E_1 embryos is higher due to repetitive somatic embryogenesis of E_0 lines.

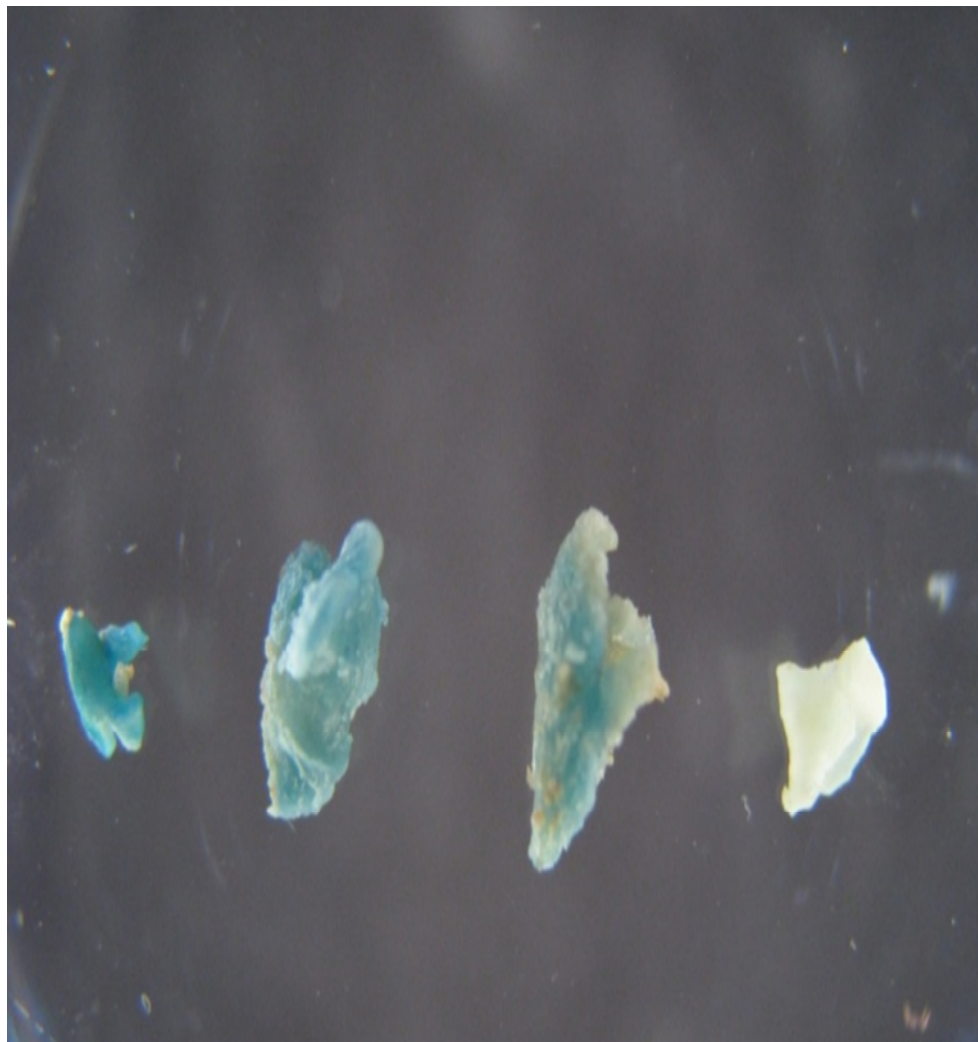| Transformation experiment	Number
Somatic embryos co-cultivated with *Agrobacterium*	2160
Resistant E_0 somatic embryos	227
GUS+ resistant E_1 somatic embryos	384
Regenerated somatic embryos in selection medium by desiccation treatment	185

Figure 2. X-gluc assay of transformed embryos.

Figure 3. Regenerated transgenic olive plants.

product of *P5CS* could be observed in case of transgenic plant but not for the control non transgenic plant which indicates successful transformation of somatic embryos.

Free proline level

Proline concentration was determined in transgenic and control plants in normal condition without the presence of any antibiotics. The values were listed in Table 3. The proline level produced by the constitutive expression of the *P5CS* transgene reached 1960.12 µg/g FW compared with control plants (330.28 µg/g FW).

There are a few reports about gene transfer into olive; most of them have involved *Agrobacterium*-mediated transformation ((Rugini and Gutiérrez Pesce, 2006). The only DNA delivery system has been used in olive up to now is microprojectile bombardment (Lambardi et al., 1998). *Agrobacterium*-mediated system was used to transform the cvs Dolce Agogia and Moraiolo with the GUS gene (Mencuccini et al., 1999). No transformed shoots have been recovered; only 7% of shoot regenerated from cv Dolce Agogia and 4% from Moraiolo. Rugini et al. (2000) transferred *rol A, B* and *C* gene and osmotin gene to Moraiolo and Canino cultivars using embryogenic masses with attached embryos at different stages. In our study, we used well-grown independent cotyledonary embryos for *Agrobacterium* inoculation to increase the rate of transformation.

In this study, we showed that olive somatic embryos are susceptible to *Agrobacterium*. Olive transformation by using somatic embryos has a very high efficiency because according to Lambardi et al. (1999) these somatic embryos differentiate from epidermal cells with mainly unicellular origin which are accessible for *Agrobacterium* and they are easier to be transformed. According to McGranhan et al. (1988), if competent cells for both transformation and regeneration are chosen, non-chimer plantlets will regenerate in a shorter period.

Somatic embryos co-cultured with *Agrobacterium* could result in chimeric secondary embryos. With each subsequent generation of secondary embryos, the frequency of completely transformed embryos increased (McGranahan et al., 1988). The relatively low frequency of embryo transformation is compensated for abundant proliferation in secondary somatic embryogenesis (Ninkovic et al., 1995).

Generally, plant transformation can be carried out using tissues at various stages of differentiation. In preliminary experiments, in addition to secondary somatic embryos, zygotic radicle and proximal cotyledon segments were directly used as explants for transformation (data not shown). These explants induced no callus and subsequently, no somatic embryogenesis, probably due to inhibitory effect of cefotaxime and Km on callus induction. The explants should have lost their ability to induce callus under a long period of selection pressure, but somatic dicotyledon embryos derived from embryogenic calli of olive (*O. europaea L.*) were successfully transformed by *A. tumefaciens* using a plasmid containing *A. thaliana P5CS* gene.

In olive, secondary embryogenic process is not yet completely controlled and the possibility of somatic embryos to develop into complete plants is still low (Benelli et al., 2001; Lambardi et al., 2002). In our experiment, somatic embryos showed a very poor conversion rate to plantlets (5.7%); however, the germination frequency was significantly improved by undergoing
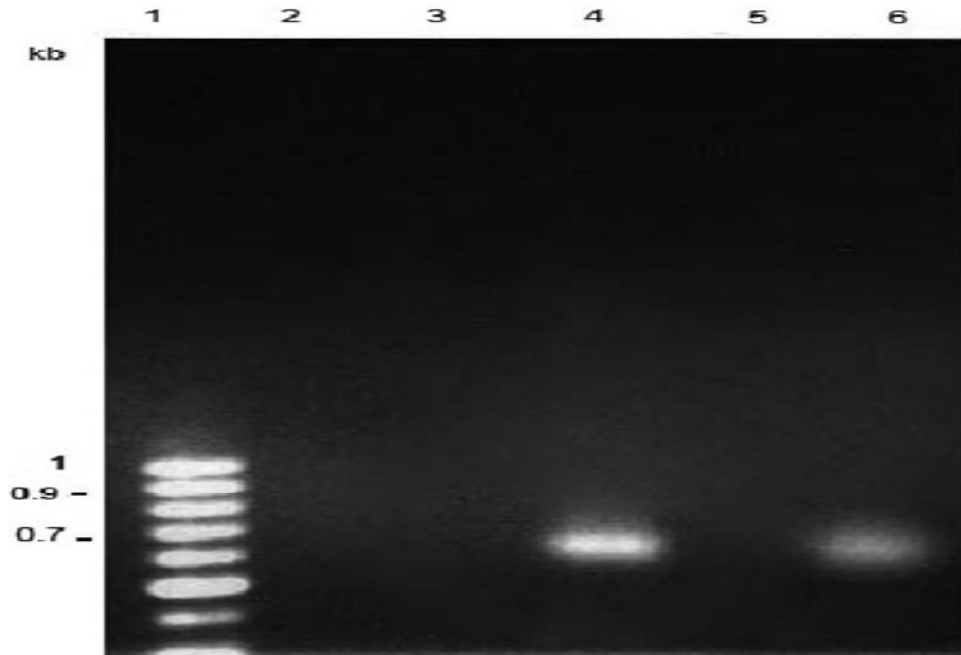
Figure 4. PCR detection of *P5CS* gene in transformed olive plant. Lane 1: 1 kb DNA ladder, lane 2: negative control, lane 3: non-transformed plant, lane 4: transformed plant, lane 5: pBI121, lane 6: pBI121-*P5CS.*
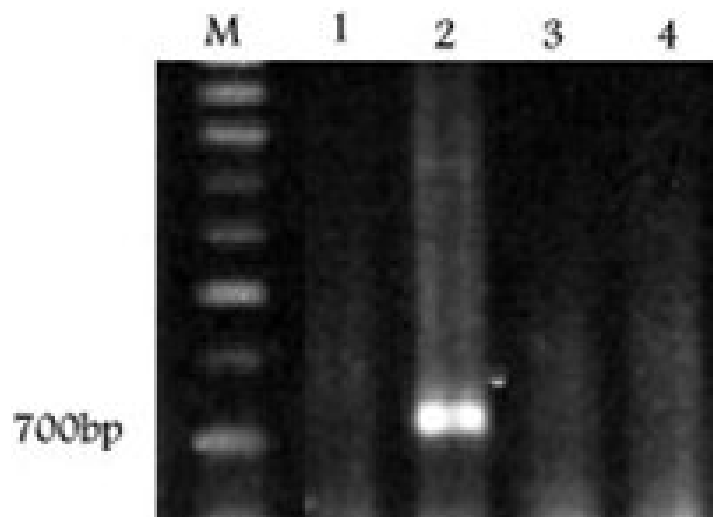

Figure 5. Expression analysis of *P5CS* transgenic plants, M: 100bp clone sizer (Norgen) DNA ladder, lane1: negative control, lane2: transgenic plant, lane 3: non-transgenic plant, lane 4: further negative control where RNA instead of cDNA was used for checking the possible DNA contamination with the extracted RNA.

Table 3. Proline content (µg/g FW) of transgenic and non-transgenic plants. Values are the mean ± SD (n=3).

Plant	Proline content (µg/g FW)
Transgenic	1960.12 ± 1.7[a]
Non-transgenic	330.28 ± 0.5[b]

desiccation treatment (50%). We expected these transgenic plants to accumulate high levels of proline which can lead to osmotolerance and better survival in stress conditions. The gene construction used in this study contains a constitutive promoter. Our results indicated that, *P5CS* gene expression driven by this promoter led in 5.9-fold higher free proline when compared with non-transformed plants. The proline assay indicated that, the endogenous proline production is most likely the result of an increase in biosynthesis. There is a direct correlation between over production and accumulation of proline. This has been made in variety of transgenics that were engineered for overproduction of proline (Kishor et al., 1995; Gubis et al., 2007; Zhu et al., 1998; Su and Wu, 2004; Vendruscolo et al., 2007; Sawahel et al., 2002; Han and Hwang, 2003).

Next step in our project is to investigate the efficiency of this transformation on abiotic stress tolerance and the performance of transgenic plants under osmotic stress.

REFERENCES

Benelli C, Fabbri A, Grassi S, Lambardi M, Rugini E (2001). Histology of somatic embryogenesis in mature tissue of olive. J. Horticult. Sci. Biotechnol. 76: 112-119.

Canas LA, Benbadis A (1988). *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea L.*). Plant Sci. 54: 65-74.

Cruz-Hernandez A, Witjaksono Litz RE, Gomez-Lim AM (1998). *Agrobacterium tumefaciens*-mediated transformation of embryogenic avocado cultures and regeneration of somatic embryos. Plant Cell Rep. 17: 497-503.

Dandeker AM, MacGranahan GH (1989). *Agrobacterium*-mediated transformation of somatic embryos as a method for the production of transgenic plants. J. Tissue Cult. Methods, 12: 145-149.

Gubis J, Vankova R, Cervena V, Dragunova M, Hudcovicova M, Lichtnerova H, Dokupil T, Jurekova Z (2007). Transformed tobacco plants with increased tolerance to drought. South Afr. J. Bot. 73: 505-511.

Han KH, Hwang CH (2003). Salt tolerance enhanced by transformation of a *P5CS* gene in carrot. J. Plant Biotechnol. 5: 149-153.

Jefferson RA (1987). Assaying chimiric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.

Kishor PBK, Hong Z, Miao G, Hu CA, Verma PS (1995). Over expression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers osmtolerance in transgenic plants. Plant Physiol. 108: 1387-1394.

Kishor PBK, Sangam S, Amrutha RN, Laximi P, Naidu K, Rao KR, Rao S, Reddy KJ, Theriappon P, Sreenivasula N (2005). Regulation of proline biosynthesis, degredation, uptake and transport in higher plants: its implication in plant growth and abiotic stress tolerance. Curr. Sci. 88: 427-438.

Lambardi M, Amorosi S, Caricato G, Benelli C, Branca C, Rugini E(1998). Microprojectile-DNA delivery in somatic embryos of olive *(Oleaeuropaea* L.). Acta Hortic. 474: 505-509.

Lambardi M, Caccavale A, Rugini E, Caricato G (1999). Histological observation on somatic embryos of olive (*Olea europaea L.*). Acta Horticulture, 474: 67-70.

Lambardi M, Benelli C, De Carlo A, Fabbri A, Grassi S, Lynch PT (2002). Medium and long-term in vitro conservation of olive germplasm. Acta Horticulturae, 586: 109-112.

Li X, Krasnyanski SF, Korban S (2002). Optimization of the uidA gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens.* Plant Physiol. Biochem. 4: 453-459.

McGranahan GH, Leslie CA, Uratsu SL, Martin LA, Dandekar AM (1988). *Agrobacterium*-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Bio/Technol. 6: 800-804.

McGranahan GH, Leslie CA, Uratsu SL, Dandekar AM (1990). Improved efficiency of the walnut somatic embryo gene transfer system. Plant Cell Rep. 8: 512-516.

Mc Mannus MT, Bielesk RL, Caradus JR, Barker DJ (2000). Pinitol accumulation in mature leaves of white clover in response to water deficit. Environ. Exp. Bot. 43: 11-18.

Mencuccini M, Micheli M, Angiolillo A, Baldon L, Frugis G, Mariotti D(1999). Agrobacterium-mediated DNA transfer in olive callus *(Olea europaea L.*). Adv. Hort. Sci. 13: 25-28.

Mitrakos K, Alexaki A, Papadimitriou P (1992). Dependence of olive morphogenesis on callus origin and age. J. Plant Physiol. 139: 269-273.

Murray MG, Thomson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. 8: 4321-4325.

Ninkovic S, Miljus-djukis J, Neskovic M (1995). Genetic transformation of alfalfa somatic embryos and their clonal propagation through repetitive somatic embryogenesis. Plant Cell, Tissue Organ Cult. 42: 255-260.

Orinos TH, Mitrakos K (1991). Rhizogenesis and somatic embryogenesis in calli from wild olive (*Olea europaea var.sylvestris*) mature zygotic embryos. Plant Cell, Tissue Organ Cult. 27: 183-187.

Peng Z, Lu Q, Verma DPS (1996). Reciproca regulation of 1-pyrroline-5-carboxylate synthetase and prolinee dehydrogenase genes controls prolinee levels during and after osmotic stress in plants. Mol. Gen. Genet. 253: 334-341.

Rugini E, Gutiérrez-Pesce P, Spampinato PL, Ciarmiello A, D'ambrosio C (1999). New perspective for Biotechnologies in olive breeding: morphogenesis, *in vitro* selection & gene transformation. Acta. Hortic. 474: 107-110.

Rugini E, Rita B, Muleo R (2000). Olive Transformation. In: Jain JM, Minocha SC (ed.). Molecular Biology of Woody Plants, Kluwer Academic Publishers, the Netherlands, pp. 245-279.

Rugini E, Gutiérrez-Pesce P (2006). Genetic improvement of olive. Pomologia Croatica.12: 43-74.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning, a laboratory manual. Cold spring Harbor lab. pp. 301-405.

Sawahel WA, Hassan AH (2002). Generation of transgenic wheat plants producing high levels of osmoprotectant proline. Biotechnol. Lett. 24: 721-725.

Su J, Wu R (2004). Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. Plant Sci. 166: 941-948.

Zhang CS, Lu Q, Verma DPS (1995). Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. J. Biol. Chem. 270: 20491-20496.

Zhu B, Su J, Chang M, Verma, DPS, Fan YL, Wu R (1998). Overexpression of a Δ1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water-and salt-stress in transgenic rice. Plant Sci. 139:41-48.

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

Yamchi A, Rastgar Jazii F, Mousavi A, Karkhane A (2006). Proline Accumulation in Transgenic Tobacco as a Result of Expression ofArabidopsis Δ-1-Pyrroline-5-carboxylate synthetase (P5CS) During OsmoticStress. J. Plant Biochem. Biotechnol. 16: 09-15.