J. Agr. Sci. Tech. (2016) Vol. 18: 423-435

Agrobacterium Mediated Transformation of Somatic Embryos of Persian Walnut Using *fld* Gene for Osmotic Stress Tolerance

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ABSTRACT

Somatic embryos of Persian walnut were transformed with two strains of *Agrobacterium tumefaciens i.e.* LBA4404 and C58, and two plasmids, namely, pBI121 with *nptII* and *gus* genes for improving the transformation protocol, and p6u-ubi-FVTI plasmid containing the *hpt* and *fld* genes. The transformation frequency was 10%. PCR and RT-PCR analysis proved the presence and expression of the genes. The transgenic and non-transgenic somatic embryos of Persian walnut were exposed to four salinity levels (0, 50, 100, and 200 mM NaCl) and four osmotic stress (0, 1.5, 5, and 10% PEG) levels. After 20 days, the number of survived, secondary and cotyledonary somatic embryos, as well as fresh and dry weights of embryos were evaluated. In addition, the transgenic and non-transgenic regenerated plantlets with 3 leaves and 2.5 cm length were subjected to 200 mM NaCl. In both experiments, the main effects of *fld*-transformation and stress treatments on evaluated parameters were significant. Transgenic somatic embryos showed no significant differences at 0 and 200 mM NaCl and 0 and 1.5% PEG. Significant differences of transgenic vs. non-transgenic somatic embryos were observed at 50 and 100 mM NaCl and 5 and 10% PEG. Non-transgenic plantlets on medium containing 200 mM NaCl showed complete necrosis and died after 10 days, while transgenic lines continued growth until 45 days. Our results clearly showed that expression of *fld* gene increased stress tolerance in *fld* transformant lines of walnut, and also revealed that expression of this specific cyanobacterial protein may provide a powerful tool to improve tolerance to environmental stresses.

Keywords: Flavodoxin, Salinity stress tolerance, Walnut transformation.

INTRODUCTION

Salinity and drought are the major environmental factors limiting plant growth and productivity in arid and semi-arid regions (Lotfi *et al*., 2009). Regarding high sensitivity of walnut to drought and salinity, breeding for salt stress and finding walnut genetic recourses that can tolerate salinity and drought is important. Causing specific and unspecific reactions in plants at the cellular and whole plant levels, these reactions lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and yield (Wang *et al*., 2001). Some key enzymes in plants utilize reduced ferredoxin as an electron donor. These enzymes are

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[DOR: 20.1001.1.16807073.2016.18.2.8.8]

$NADP^+$ reductase ($NADP^+$ to NADPH), nitrite reductase (nitrite to ammonia), glutamate synthase (2-oxoglutarate to glutamate), sulfite reductase (sulfite to sulfide), and thioredoxin reductase (Hase *et al*., 2006).

Genetic engineering is a forceful tool for tolerance to abiotic stress (Hu and Xiong, 2014). Zurbriggen *et al*. (2007) reported that transgenic tobacco expressing the *Flavodoxin* gene showed an increase in drought tolerance. Under stress conditions, especially oxidative stress, the ferredoxin plays an important role in the protection and delivery of reducing equivalents to various metabolic pathways such as antioxidant metabolism, amino acid metabolism, etc. The Ferredoxins (Fd) are present in plants as iron–sulfur proteins in many different electron transfer pathways (Knaff, 2005) and exist in the photosynthesis from photosystem I to $NADP^+$ (Sandman *et al*., 1990).

Flavodoxin is a small soluble electron carrier protein (~19 kD) containing flavin mononucleotide as an electron carrier and flavoprotein that acts as a redox center transferring electrons at low potentials and shuttles electrons among an excess of donors and acceptors (Pueyo *et al*., 1991; Pueyo and Gomez-Moreno, 1991). Flavodoxin does not occur in higher plants, rather, it was found in prokaryotes and some eukaryotic algae (Erdner *et al*., 1999).

Flavodoxin is expressed under stress, replacing ferredoxin in the photosynthetic electron transport (Sandmann *et al*., 1990). In its reduced state, flavodoxin can interact with ROS (e.g. the SuperOxide Radical) and revert to its original redox state in the presence of an appropriate electron source (Mayhew and Ludwig, 1975). In cyanobacteria and enterobacteria, Flavodoxin level increases during exposure of the cells to the redox-cycling herbicide Methyl Viologen (MV) (Zheng *et al*., 1999; Yousef *et al*., 2003; Singh *et al*., 2004), and at other stress conditions such as water-deficit or drought (Tognetti *et al*., 2006). Flavodoxin was used to replace plant ferredoxin in transgenic tobacco plants and showed significant tolerance to several stresses (Tognetti *et al*., 2006).

High concentrations of salt cause ion imbalance and hyperosmotic stress in plants. Therefore, salinity has two main effects: the osmotic secondary stress and the toxic effect because of ion accumulation. In order to separately analyze the first effect of this type of stress, PolyEthylene Glycol (PEG) is used. PEG is a non-penetrating and non-ionic osmotic stress-inducing compound. It is an extremely hydrophilic organic polymer that is able to remove much of the free water within a solution. It does not cause any ionic stress and is mostly being used to stimulate osmotic stress particularly at *in-vitro* conditions (Verslues *et al*., 2006). Many research works have studied the effects of NaCl and PEG on growth and ion absorption, such as those in bean, maize, and sorghum (Kawasaki *et al*., 1983), orange (Zecri and Parsons, 1990), bean and cowpea (Vasquez Tello *et al*., 1990).

Walnuts (*Juglans* spp.) are among deciduous trees found primarily in the temperate areas and commercially cultivated in the United States, western South America, Asia, and central and southern Europe. We, therefore, made use of already described *flavodoxin* gene to achieve two major goals: firstly, to establish a rapid and efficient transformation system for somatic embryos, and secondly, to evaluate the effect of the over expression of cyanobacterial *flavodoxin* gene on a crop plant such as Persian walnut in salinity and osmotic conditions.

MATERIALS AND METHODS

Vector Construction and Transformation Confirmation

Binary vectors, pBI121 containing the *nptII* (neomycin phosphotransferase) plus *gus* (uid A) genes and p6u-ubi-FVTI containing *fld* (Cyanobacterial Flavodoxin) and *hpt* (Hygromycin resistance) genes as selectable marker were used in this study

(Figure 1). Both vectors were transferred into the Agrobacteria strains (C58 and LBA4404) by triparental mating. The protocol was improved by Agrobacteria strain C58, which harbours PBI121 with *nptII* and *gus* genes.

Plant Material

A repetitively embryogenic culture line initiated from immature cotyledons of a Persian walnut (*Juglans regia* L.) apomict genotype named G79 as explained by Vahdati *et al.* (2006) was used for this study. This line had been maintained by secondary embryogenesis with sequential subculture proliferation at one-week intervals on a hormone-free DKW (Driver and Kuniyuki, 1984) medium in dark at 25°C. Individual, white, normally developed embryos, with 2-12 mm length were used for this study.

Culture Media and Growth of Bacteria

The *Agrobacterium tumefaciens* strains were grown at 27-29°C in Luria-Bertani (LB) containing 10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 10 g L^{-1} NaCl, and 15 g L^{-1} Bacto-agar for solid medium. The pH was adjusted at 7.0 using 1N NaOH. Liquid Media were supplemented with Kanamycin (50 mg L^{-1}) for pBI121, Streptomycin (200 mg L^{-1}) for p6u-ubi-FVTI. Thereafter, bacterium cultures were placed on an orbital shaker at 27°C with 200 rpm, for 24 hours (Rodriguez and Tait, 1983). The cultures were grown to an absorbance at 600 nm of 0.5 This *Agrobacterium* suspension was used for infection.

Agrobacterium cells were precipitated by centrifugation at 4,500 rpm for 10 minutes and re-suspended in DKW to a density of approximately ~5×10^{8} cells mL^{-1} (Widdel, 2007) hormone-free medium containing 100 µM Acetosyringone (Leple *et al.*, 1992; Han *et al.*, 1996; McGranahan *et al.*, 1987) and hormone-free medium without Acetosyringone. In all selective media, antibiotics were filter-sterilized and added to the autoclaved media. These re-suspended cells were used for inoculation.

Inoculation and Co-cultivation with *Agrobacterium*

For inoculation, 10-15 somatic embryos were divided into sterile Petri dishes with aliquots from inoculum cultures at room temperature (23±2°C). After 20-30 minutes, the embryos were blotted lightly dry on sterile filter paper and plated on solid basal DKW medium (Driver and Kuniyuki, 1984; McGranahan *et al.*, 1987) in the dark for co-cultivation (Vahdati *et al.*, 2002). Then,

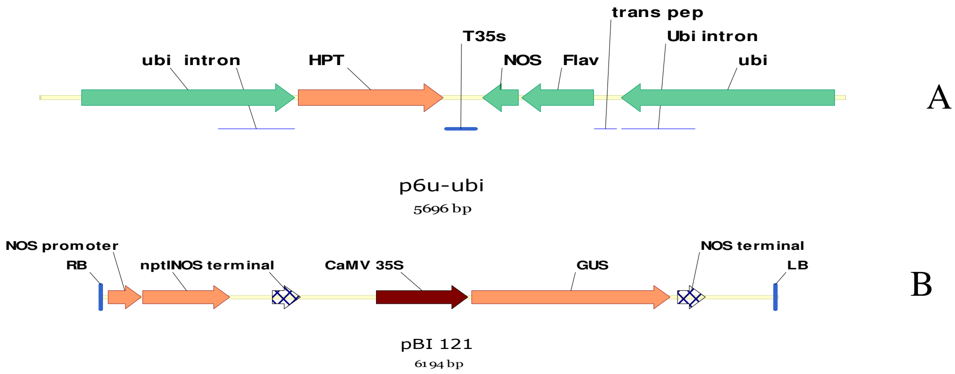

Figure 1. *Chimeric* gene map of the recombinant binary vectors. (A) p6u-ubi carrying the *fld* and *hpt* genes driven by Ubi promoter. (B) pBI121 carrying the *gus* and *nptII* genes driven by CaM 35S promoter.

embryos were transferred after 48 hour to DKW basal medium supplemented with 75 and 120 mg L^{-1} Hygromycin for p6u-ubi-FVTI vector and 50 mg L^{-1} Kanamycin for pBI121. Cefotaxime 250 mg L^{-1} was used to control the *Agrobacterium*. Cultures were maintained in dark at room temperature with sequential subcultures at 1 week intervals on the same medium for 1-2 months.

Somatic embryo germination and plantlet regeneration

Antibiotic resistant embryos were transferred to maturation medium described by Vahdati *et al*. (2008). Walnut tissue culture procedure explained by McGranahan *et al*. (1987) and Vahdati *et al*. (2006) was used for germination and regeneration of somatic embryos.

Embryos Proliferation and Genetic Selection

Secondary embryos (E_1 generation) were derived from original inoculated embryos. These were maintained as proliferation sub-clones on the selective medium for 2 months. E_{1-4} lines that proliferated well and did not turn brown were considered to be resistant to Kanamycin and Hygromycin as potential transformants. The non-inoculated secondary embryos were used as controls.

GUS (uidA) Expression Assays

Somatic embryos E_1 and E_2 generations inoculated with C58 strain containing pBI121 were screened for GUS activity by histochemical assay according to Jefferson *et al*. (1987). The staining solution contained 1mM X-Gluc, diluted with 10 mM EDTA, 0.1M sodium phosphate buffer pH 7.1, 0.1% Triton X-100 and 0.5 mM potassium ferricyanide. Staining was carried out at 37°C for 18 to 20 hours. After staining, clearing was done in 70% ethanol. The transformed embryos showed blue colour (indigo precipitate) and non-transformed ones were considered as control.

Plant DNA Isolation and Polymerase Chain Reaction (PCR) Analysis

DNA was isolated from 1.0-5.0 mg of the somatic embryos and their derivatives leaves using the method of Murray and Thompson (1980). PCR was performed using *hpt* gene specific primers (hpt-5: 5´ AGA ATC TCG TGC TTT CAG CTT CGA 3´, hpt-3: 5´ TCA AGA CCA ATG CGG AGC ATA TAC 3´) and *fld* specific primers (fld-5: 5´GCG ATC GTC TGT TAA GTC 3´, fld-3: 5´CTA CGG TAC TCA AAC TGG 3´) for amplification of 650 and 495 bp fragments, respectively. PCR conditions were as follow: 94°C for 5 minutes and then 35 cycles of 94°C for 1 minute, 58°C for 1 minute (for *fld)* and 57°C for 1 minute (for *hpt*), 72°C for 1 minute and 72°C for 5 minutes. The PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining.

Isolation and Analysis of RNA

Total RNA was isolated following procedure of Chang *et al*. (1993). For synthesizing cDNA, 1 µg total RNA from each sample was used in a 10 µL RT reaction using oligo dT as a primer with the following program: 72°C for 10 minutes; 42°C for 60 minutes and 72°C for 5 minutes.

After the completion of cDNA synthesis, two microliters was used for amplification of *fld* in a 20 µL PCR reaction. The same primers described above were used for PCR amplification of *fld* cDNA from the transgenic lines. The PCR reactions were preheated to 94°C for 5 minutes, followed by 30 cycles with denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, elongation at 72°C for 1 minute.

Abiotic Stress Tolerance Assay

Evaluation of Salt Stress Tolerance

Transgenic and non-transgenic (control) somatic embryos of Persian walnut at globular stage were exposed to different concentrations (0, 50, 100, 150, 200 mM) of NaCl in DKW medium under in vitro conditions. For each replicate, 10 embryos were cultured in a 10 cm Petri-dish containing 25 mL of DKW. Cultures were grown at 25±2°Ċ in the dark. After 20 days of culture, various growth parameters including number of survived embryos, number of secondary embryos, number of cotyledonary embryos as well as fresh and dry weight of embryos were evaluated.

Evaluation of Osmotic Stress Tolerance

Transgenic and non-transgenic somatic embryos at globular stage were exposed to four osmotic treatments (0, 1.5, 5 and 10% PEG 6000) in DKW medium to determine osmotic stress tolerance. Culture conditions and measured characteristics were similar to the previous experiment.

Salinity Tolerance Bioassay of Transgenic Plants Using NaCl

Non- transgenic control and *fld* transgenic *in-vitro* plantlets with 3 leaves and 2.5 cm length were selected for salinity stress bioassay. Stress treatments were applied by culturing plantlets on DKW basal medium supplemented with 200 mM NaCl. Growth of the plantlets was monitored for 45 days.

Statistical Analysis

Data were analyzed Using SAS software (version 9.1, SAS Institute, Inc.. Cary, NC, USA). Means were compared by Duncan's Multiple Range Test (DMRT). *P* values less than 0.05 were considered significant.

RESULTS

Recovery of Transgenic Plants

Kanamycin and hygromycin resistant cotyledonary embryos continued growth after co-cultivated with *Agrobacterium* in culture medium, while the untransformed explants stopped their growth after 2-3 weeks and died after one month on selection medium. The initial formation of cotyledonary embryos was observed after 2-3 weeks on the maturation media. The transformed explants were placed and maintained on the maturation selection medium for shooting. The shoots grown from somatic embryos were transferred to DKW proliferation medium. The rate of transformation was 10%. Twelve putative transgenic plants with well- developed leaves and root systems were transferred to soil under greenhouse conditions.

GUS Expression Assay

Histochemical GUS activity was measured in embryos transformed with C58 strain containing pBI121 for improving the protocol. Putatively-transformed somatic embryos were selected on the basis of growth on selection medium and the test for GUS expression.

All putative transgenic embryos showed positive results after the GUS assay. Untransformed embryos were negative for the *GUS* gene (Figure 2).

PCR and RT-PCR Analysis

The amplification of DNA from walnut somatic embryos displayed DNA fragment band of 495 bp for *fld* gene (Figure 3) and 650 bp for the *hpt* gene (Figure 4). This amplification could not be attributed to the

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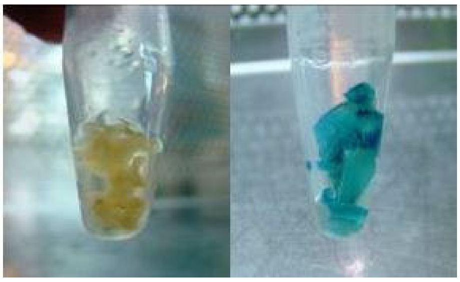

Figure 2. Gus staining phenotype in walnut somatic embryos transformed with pBI121 harbouring *gus* gene (right) and untransformed embryos (left) after 24 hours in X-Gluc solution.

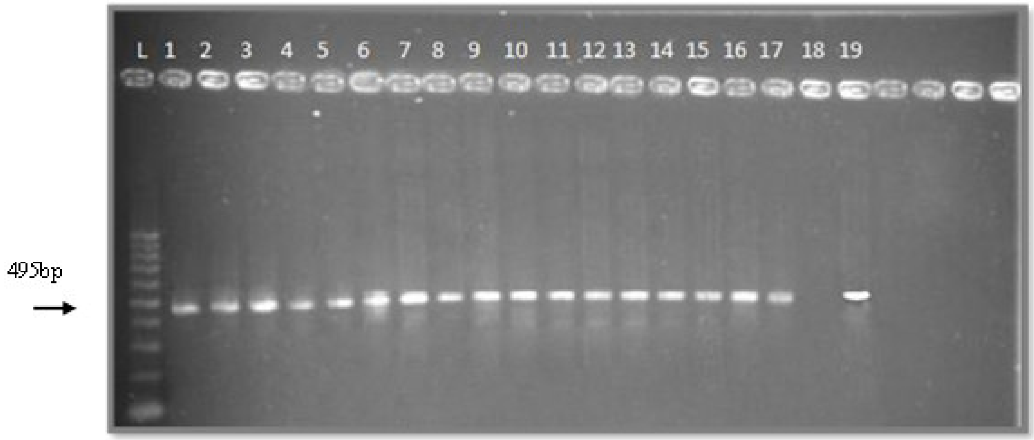

Figure 3. PCR product of hygromycin resistant SEs amplified by *fld* specific primers (495bp). [L: 100 bp DNA ladder (Gene Ruler); Lanes 1 to 17: DNA from hygromycin resistant SEs; Lane 18: DNA from untransformed control, Lane 19: DNA from plasmid].

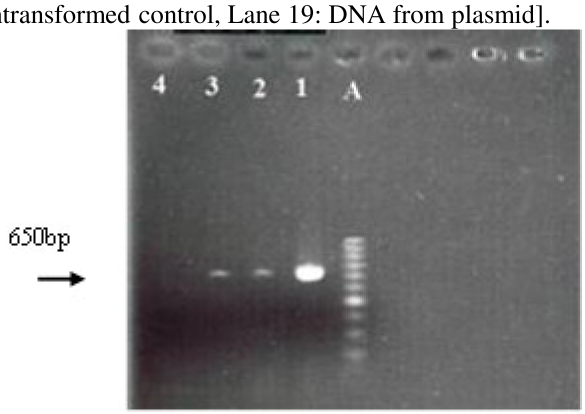

Figure 4. The PCR amplified from differently transformed walnut somatic embryos with the specific primer for *hpt* gene. [From right to left: Line A: Molecular size marker (Lambda DNA/EcoRI+HindIII); Line 1: Positive control (Plasmid); Line 2: Transformed embryos; Line 3: Transformed leaves, Line 4: Negative control].

presence of contaminating bacterial DNA, because the somatic embryos had been cultured on selection medium for more than two months and then cultured on cefotaxime-free medium, and if there was contamination, the bacteria would have grown. A band of the same size was observed in the PCR amplification of plasmid (positive control), while no amplification was observed in the negative control (non-inoculated walnut somatic embryo).

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RT-PCR was performed to detect the expression of putative transcription of *fld* gene in transgenic plants. The results showed that 495 bp fragments of the *fld* were amplified in transgenic plants (Figure 5).

Analysis of Transgenic Walnut Somatic Embryos for Tolerance to Salinity Stress

The growth of transgenic somatic embryos was improved compared to non-transformed line (Figure 6). When Somatic Embryos (SEs) were cultured on DKW medium supplemented with different concentrations of NaCl, the number of survived, secondary, and cotyledonary somatic embryos, as well as fresh and dry weights of embryos of non-transformed SEs, decreased drastically with increasing concentrations of NaCl. The evaluated parameters of the transgenic SEs also decreased, but to a lesser extent in comparison with the salt-stressed non-transformed SEs. Means comparison using Duncan's Multiple Range Test revealed that transgenic somatic embryos showed no significant differences in measured parameters at 0 mM NaCl after 20 days as compared to non-transgenic SEs (Figure 7). Significant differences of measured parameters between transgenic and non-transgenic SEs were observed at 50 and 100 mM NaCl (Figure 7). At the concentration of 50 mM NaCl, reduction in growth parameters was higher in the case of non-transformed versus transgenic SEs. In particular, significant differences were observed for the number of secondary embryos and fresh and dry weights, but not

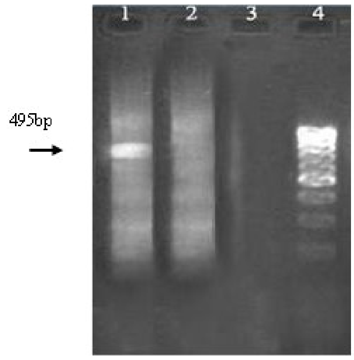

Figure 5. Analysis of *fld* gene expression. RT-PCR was done on the RNA extracted from walnut leaves. (Line 1: Transgenic plant that amplify a *fld* fragment; Line 2: Untransformed plant; Line 3: Negative control, Line 4: Molecular size marker).

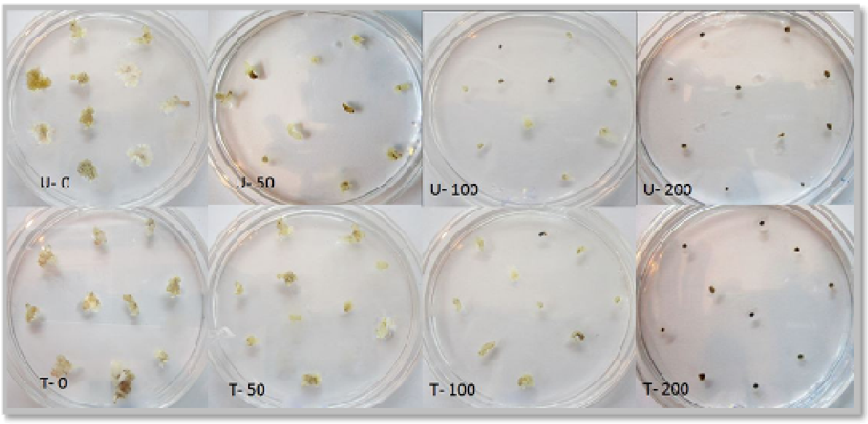

Figure 6. Growth of transgenic (T) and non-transgenic (U) SEs of Persian walnut on DKW containing different concentration of NaCl.

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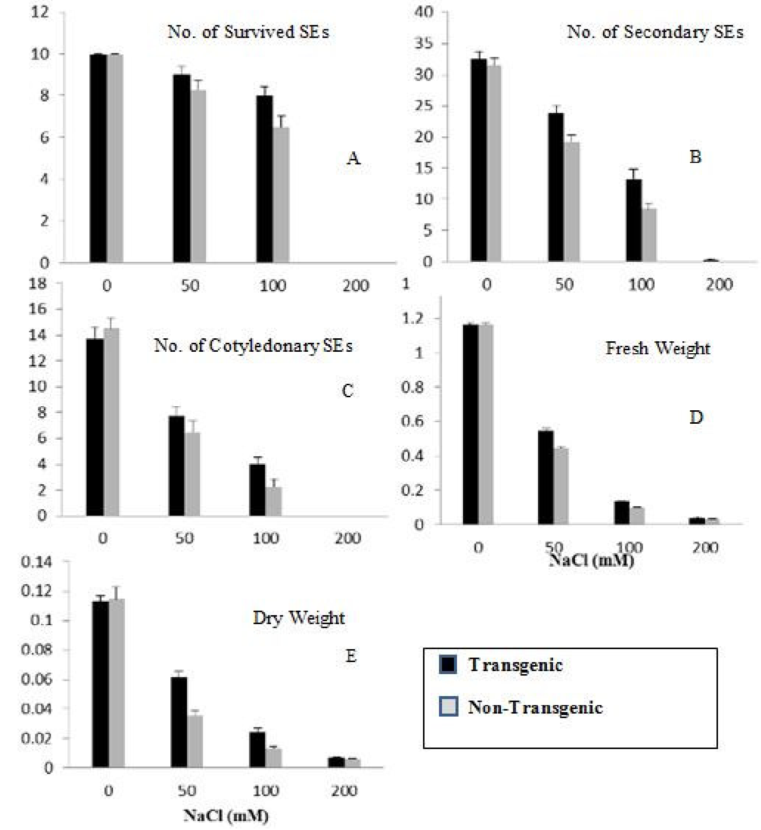

Figure 7. Effect of different concentrations of NaCl on the number of survived embryos (A); the number of secondary embryos (B); the number of cotyledonary embryos (C); fresh weight (D), and dry weight (E) of transgenic and non-transgenic somatic embryos of Persian walnut.

for the number of survived and cotyledonary embryos (Figure 7).

With increasing the concentrations of NaCl to 100 mM NaCl, differences between transgenic and non-transgenic SEs were more noticeable. Significant differences between transgenic and non-transgenic SEs in this salt treatment was observed for the number of survived and cotyledonary embryos as well as fresh weight of embryos (Figure 7). No growth occurred at 200 mM NaCl in both transgenic and non-transgenic SEs. However, transgenic SEs survived for 16 days in 200 mM NaCl, while non-transformed SEs showed complete necrosis and died after 12 days. Generally, the number of cotyledonary embryos was not affected by transformation with *fld* gene.

Analysis of Ttransgenic Walnut SEs for Tolerance to Osmotic Stress

As expected, PEG had an adverse effect on all of the growth parameters tested in the non-transgenic SEs. Similarly, the transgenic somatic embryos were also negatively affected in their growth; but, the reductions in many of the growth parameters were less severe compared to those observed in the untransformed controls. There were no significant differences between transgenic and non-transformed SEs in 0 and 1.5% of PEG in DKW medium for all evaluated parameters (Figure 8). It is interesting to remark that at 1.5% PEG, the number of cotyledonary embryos was significantly increased in both

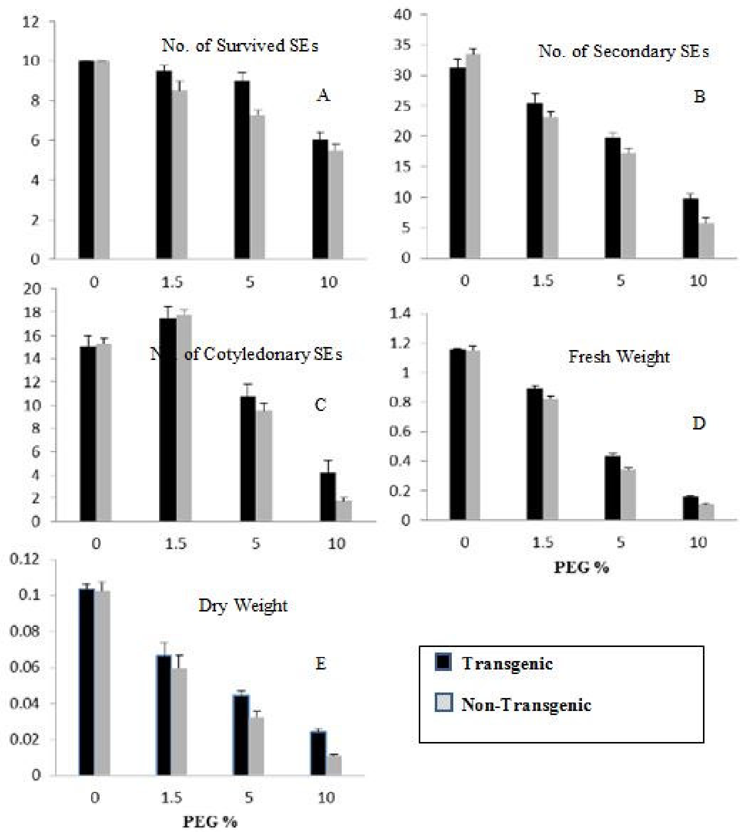

Figure 8. Effect of different concentration of PEG on the number of survived embryos (A), the number of secondary embryos (B), the number of cotyledonary embryos (C), fresh weight (D) and dry weight (E) of transgenic and non-transgenic somatic embryos of Persian walnut.

transgenic and non-transgenic SEs (Figure 8-c). With increasing concentrations of PEG in the culture medium to 5 and 10%, significant differences between transgenic and non-transgenic SEs were observed for most of the evaluated parameters.

Analysis of Transgenic Plantlets for Tolerance to Salinity Stress

The results showed that transgenic plantlets did grow clearly in a better shape at 200 mM NaCl compared with the non-transgenic controls. The control turned brown and died after 10 days (Figure 9-a), while transgenic lines not only did not show any browning symptoms but also they continued their growth up to 45 days on 200 mM NaCl.

DISCUSSION

One part of the Fd set delivers electrons to Fd-NADP$^+$ reductase to produce NADPH, but a considerable part of Fd is used as electron donor to other enzymes such as nitrite reductase, sulfite reductase, Glu-oxoglutarate reductase, and the Fd-Trx Reductase (FTR) (Knaff, 2005). Considering that some metabolic pathways such as carbon fixation, nitrogen and sulfur assimilation, and amino acid synthesis are Fd-dependent, stable supply of Fd or Fd-like protein such as fld is vital to plants especially under stress conditions (Holtgrefe *et al.* 2003). Ferredoxin levels decrease in plants and other organisms exposed to stress

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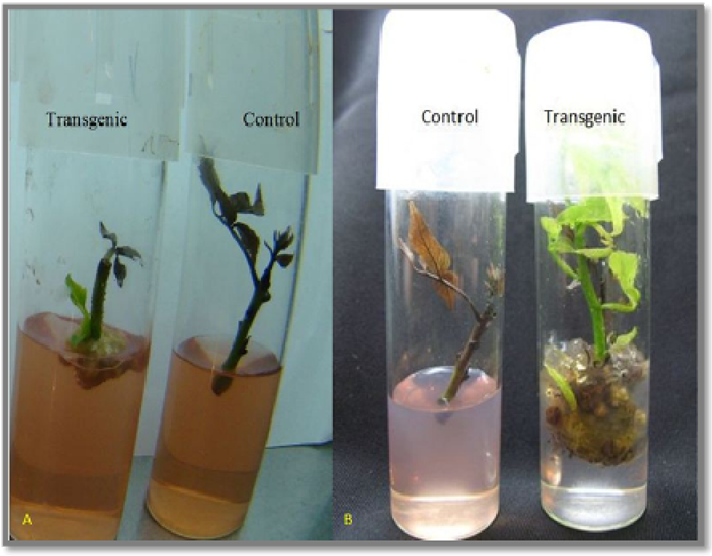

Figure 9. Differences in growth of the transgenic and non-transgenic walnut (control) that were cultured in DKW containing 200 mM NaCl after 10 days (A) and 45 days (B).

(Zurbriggen *et al*., 2008). It was shown repeatedly that under various environmental sources of stress, flavodoxin can replace ferredoxin in plants (Tognetti *et al*., 2006; Zurbriggen *et al*., 2008), enterobacteria (Zheng *et al*., 1999) and cyanobacteria (Yousef *et al*., 2003). Under iron starvation stress condition, replacement of Fd with *fld*, allowed for growth and reproduction of stressed transgenic tobacco plants where this last bacterial protein was overexpressed. The results indicated that the compensatory roles of *fld* were still effective in higher plants in spite of the evolutionary divergence (Tognetti *et al*., 2006).

Absence of differences between transgenic and non-transformed SEs under non-stressed conditions revealed that *fld* expression had no important role in plants grown under normal condition, indicating that under autotrophic growth, Fd is the normal electron carrier (Zurbriggen *et al*., 2007). In stressed plants, the amount of Fd decreases to a limiting level showing that the expression of this iron-sulfur dependent protein is crucial (Tognetti *et al*., 2007). The improved growth of transgenic SEs in terms of phenotypic parameters in NaCl and PEG mediated stresses could be attributed to the expression of the *fld* gene in somatic embryos of walnut. Our observations showed better growth performance of the transgenic SEs on medium supplemented with 50 and 100 mM NaCl and 5 and 10% PEG indicating that *fld* protein can partially replace the function of decaying ferredoxin under salt and osmotic conditions.

No growth occurred at 200 mM NaCl in both transgenic and non-transgenic SEs, demonstrating that the expression of this flavoprotein could not induce resistance to high salinity stress, but partially increased the salinity tolerance threshold. Our findings suggest that fld introduction into the walnut genome imparts abiotic stress tolerance. This is in agreement with the results previously reported on tobacco plants (Tognetti *et al*., 2006; Blanco *et al*., 2011). Taken together, our results showed that an increase in PEG concentration enhanced the number of cotyledonary embryos, which was in accordance with previous reports (Attree *et al*., 1991; Krajnakova *et al*., 2009). Compared with salt stress, the decrease in growth of transgenic and non-transgenic SEs under osmotic stress caused by PEG was relatively low. This difference could be due to the ionic toxicity effect in

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case of NaCl treatments, while PEG only causes osmotic stress and does not provoke any ionic toxic effect on SEs (Munns and Tester, 2008). In addition, we showed here that over-expression of *fld* gene in transgenic lines of Persian walnut could partially decrease some of the adverse effects of salinity and osmotic stresses in terms of phenotypic evaluation and growth parameters. Transgenic plantlets survived and were able to produce new shoots in stress medium, which is in agreement with previous reports in analogous transgenic tobacco (Tognetti *et al*., 2006). All the findings reported here clearly show that the expression of specific cyanobacterial proteins is a powerful tool to enhance the tolerance capacity of crop plants against stressful conditions.

ACKNOWLEDGEMENTS

Thanks to the Iran National Science Foundation (INSF) and the University of Tehran for financial support. Dr. Iunot Gotea and Dr. Pejman Azadi are also acknowledged for their kind collaboration.

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تراریزش جنینهای سوماتیک گردوی ایرانی با استفاده از ژن *fld* به منظور تحمل به تنشهای اسمزی

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چکیده

جنینهای گردوی ایرانی با دو استرین اگروباکتریوم، LBA4404 and C58 و دو پلاسمید: pBI121 دارای ژنهای *nptII* و *gus* برای بهینه سازی پروتوکل تراریزش، وp6u-ubi-FVTI دارای ژنهای *hpt* و *fld* تراریزش شدند. تکرار پذیری و فراوانی تراریزش ۱۰٪ بود. تجزیه PCRوRT-PCR حضور و بیان ژنها را ثابت کرد. جنینهای سوماتیک تراریخت و غیرتراریخت در چهار غلظت شوری (صفر، ۵۰، ۱۰۰ و ۲۰۰ میلی مولار NaCl) و چهار سطح تنش اسمزی (صفر، ۱/۵، ۵ و ۱۰ درصد PEG) قرار گرفتند. پس از ۲۰ روز، تعداد جنین‌های زنده، جنین‌های ثانویه و لپه ای شکل و وزن تر و خشک جنین‌ها ارزیابی شدند. علاوه بر این، گیاهان باززایی شده تراریخت و غیر تراریخت داری ۳ برگ و ۲/۵ سانتی متر طول در برابر ۲۰۰ میلی مولار NaCl قرار گرفتند. در هر دو آزمایش، اثرات اصلی تراریزش با *fld* و تیمارهای تنش روی صفات اندازه‌گیری شده معنی‌دار بود. جنینهای سوماتیک تراریخت اختلاف معنی‌داری در سطوح صفر و ۲۰۰ میلی مولار NaCl و صفر و ۱/۵٪ PEG نشان ندادند. اختلاف معنی‌داری بین جنین‌های سوماتیک تراریخت و غیرتراریخت در غلظتهای ۵۰ و ۱۰۰ میلی مولار NaCl و ۵ و ۱۰ درصد PEG مشاهده شد. گیاهان غیرتراریخت روی محیط دارای ۲۰۰ میلی مولار NaCl به طور کامل نکروزه شدند و پس از ۱۰ روز مردند. در حالی که لاینهای تراریخت به رشد خود تا ۴۵ روز ادامه دادند. نتایج نشان داد که بیان ژن *fld* میزان تحمل لاینهای تراریخت را افزایش می‌دهد و بیان این پروتئین سیانوباکتری خاص سبب افزایش تحمل به شرایط نامساعد محیطی می‌شود.

[DOR: 20.1001.1.16807073.2016.18.2.8.8]