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Agrobacterium-Mediated Transformation of Pomegranate (*Punica granatum* L.) 'Yousef Khani' Using the *gus* Reporter Gene

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Abstract
In this study, an efficient *Agrobacterium*-mediated transformation method was developed for pomegranate (*Punica granatum* L.), a difficult-to-transform plant. *In vitro* shoot segments were inoculated with *Agrobacterium tumefaciens* strain *LBA4404* harboring the binary vector *pBI121* carrying the neomycin phosphotransferase (*nptII*) gene as a selectable marker and *β-glucuronidase* (*gus*) gene as a reporter. After 28 d in WPM selection medium containing 50 mg L^{-1} kanamycin, 59 new shoots proliferated. *gus* analysis was performed on these putative transgenic shoots, of which 32 stained positive. Positive staining shoots were cut and cultured in selection medium for 2 subsequent subcultures until final *gus* analysis. After three months of the selection period, 6 putative transgenic shoots were obtained. Presence of the *gus* and *nptII* genes was confirmed by polymerase chain reaction. Southern blot analysis confirmed that T-DNA was stably integrated into the genome of three out of six PCR-positive plants. The transgenic plants were rooted and successfully acclimatized.

Keywords: genetic transformation, polymerase chain reaction, selectable marker, transgenic plants.

Abbreviations: CaMV, cauliflower mosaic virus; **CRD**, completely randomized design; **CTAB**, cetyltrimethylammonium bromide; **DIG**, digoxigenin; ***Gfp***, green fluorescent protein; ***gus***, *β-glucuronidase;* ***nos***, nopaline synthase; ***nptII***, neomycin phosphotransferase.

Introduction

Pomegranate (*Punica granatum* L.) is an economically important fruit tree of the tropical and subtropical regions of the world that is cultivated for its delicious fruits, pharmaceutical properties, and ornamental usage (Naik and Chand, 2011). However, there are several constraints to the productivity of the pomegranate orchards, resulting in serious adverse economic impacts on growers. For example, the carob moth [*Ectomyelois ceratoniae* (Zeller) (*Lepidoptera*:*Pyralidae*)] is a serious problem in pomegranate in many countries, such as Iran, USA, and Turkey, causing estimated losses of 50% of total yield (Carroll *et al*., 2006; Mirkarimi, 2000; Ozturk *et al*., 2005). The development of pomegranate cultivars resistant to pests should have a tremendous impact on crop productivity.

Genetic improvement of pomegranate by conventional breeding is a difficult and time-consuming process due to heterozygosity, the time interval between generations, asexual propagation, and length of field evaluations (Jalikop, 2010). Additionally, conventional breeding has met with limited success due to the lack of some desirable genes in germplasm. An alternative to overcome these limitations is the introduction of new traits by *Agrobacterium*-mediated genetic transformation, which is the most commonly used method for transferring genes into plants cells (Chauhan and Kanwar, 2012). Genetic engineering has

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been employed to develop fruit crops with improved horticultural traits such as disease resistance, pest resistance, herbicide tolerance, cold tolerance, salt tolerance, and improved plant and fruit characters and shelf life (Gomez-Lim and Litz, 2004).

In vitro propagation of pomegranate has been reported by a number of researchers (Naik and Chand, 2011; Chauhan and Kanwar,2012).However,genetic transformation of pomegranate remains inefficient. There are only two reports on genetic transformation of ornamental and wild pomegranates (Kanwar *et al*., 2008; Terakami *et al*., 2007). Terakami *et al*. (2007) established an *Agrobacterium*-mediated transformation system for pomegranate var. 'Nana' (dwarf pomegranate) using strains *LBA4404* and *EHA105*, both harboring binary vector *pBin19-sgfp* carrying the neomycin phosphotransferase II (*nptII*) and green fluorescent protein (*gfp*) genes. Kanwar *et al*. (2008) also transformed wild pomegranate using *Agrobacterium* strain *LBA4404* harboring *pBI121* carrying β-glucuronidase (*gus*) and *nptII* genes. Currently, there is no report describing the genetic transformation of commercial pomegranate cultivars, and there is a strong need for such a protocol.

In many woody plants, the proliferation of escapes and chimeric shoots at high frequencies has been reported (Domínguez *et al*., 2004; Gago *et al*., 2011). For transformation systems generating considerable numbers of escapes and chimeras, the use of marker genes conferring a phenotype allowing visual screening, such as *gus*, could be recommended to recover transformants, since screening reveals transformation more efficiently than lethal selection (Christou and McCabe, 1992; Kim and Minamikawa, 1996). Moreover, by utilizing assays of the *gus* reporter gene, it is possible to rapidly assess the importance of various conditions and thereby select the most effective parameters for achieving *T-DNA* transfer from *Agrobacterium* to pomegranate explants. Therefore, the aim of our research was to optimize the genetic transformation of pomegranate using the *gus* reporter gene. In this paper, we describe for the first time a protocol for *Agrobacterium*-mediated transformation of 'Yousef Khani', a leading Iranian pomegranate cultivar.

Materials and methods

Plant material and culture conditions

Experiments were carried out using *in vitro* shoot segments (Fig. 2A) of *Punica granatum* L. 'Yousef Khani' which were proliferated in WPM medium (Lloyd and McCown, 1980) containing 9.2 μM Kinetin and 0.54 μM NAA, 3% sucrose, 0.6% agar. The pH was set to 5.6-5.8 prior to autoclaving (121°C for 15 min). Cultures were maintained at 25±1°C with white fluorescent light (30-40 μmol m^{-2} s^{-1}) and a 16 h photoperiod.

Sensitivity to kanamycin

To determine the effects of kanamycin that inhibit the shoot proliferation of non-transformed explants, *in vitro* shoot segments were placed on a proliferation medium supplemented with different concentrations of kanamycin (0, 12.5, 25, 50, and 100 mg L^{-1}). Culture conditions were the same as those described earlier. Antibiotics were filter-sterilized and added to the media before use. Survival rates were determined after four weeks (Table 2). The experiment was set up in a completely randomized design (CRD) consisting of 5 replicate jars, each with two *in vitro* shoots. The data were analyzed using SAS Version 9.1. Significant differences were assessed using Duncan's multiple range test at $P< 0.05$. Data expressed as percentages were subjected to arcsine transformation before statistical analysis. Non-transformed data are shown in Table 2. This experiment was repeated three times.

Bacterial strain and plasmid

Agrobacterium tumefaciens strain *LBA4404* harboring the binary vector *pBI121* was used for transformation. The T-DNA region of the plasmid contains the neomycin

phosphotransferase gene (*nptII*) driven by the *nos* promoter and the *β -glucuronidase* (*gus*) reporter gene driven by the cauliflower mosaic virus *35S* promoter (Fig. 1).

Agrobacterium was grown in liquid LB medium (10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract) supplemented with 50 mg L^{-1} kanamycin at 28°C for 24 h on a shaker at 140 rpm. The suspension was centrifuged at 5,000 rpm for 15 min, then the pellet was resuspended in liquid WPM medium supplemented with 9.2 µM Kinetin and 0.54 µM NAA and diluted to an OD600 of 0.3–1. Immediately prior to the infection of explants, the bacterial suspension was supplemented with acetosyringone to achieve a final concentration of 100 µM (Terakami *et al*., 2007).

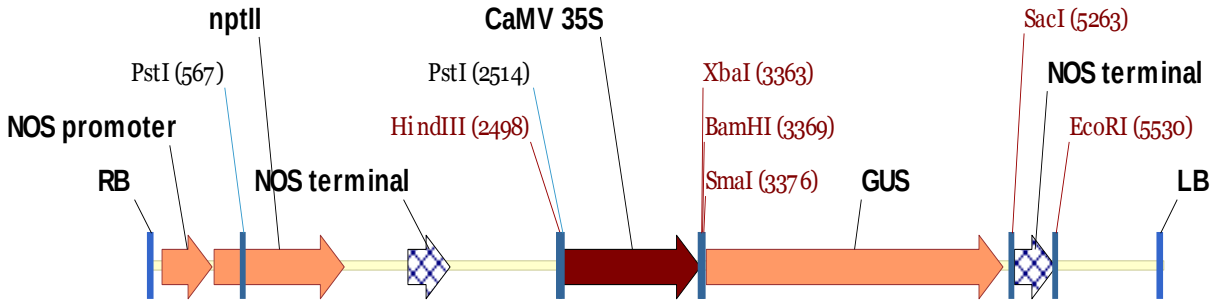

Fig. 1. Structure of T-DNA region of plasmid pBI121. *nos* promoter = nopaline synthase gene promoter; *nptII* = coding region of neomycin phosphotransferase gene, conferring kanamycin resistance; *nos* Terminal = transcriptional terminator from the nopaline synthase gene; CaMV promoter = cauliflower mosaic virus 35S promoter; *gus* = coding region of the *β-glucuronidase* reporter gene

Co-cultivation, selection, and proliferation of transgenic plants

In vitro shoot segments (1 cm in length) were immersed in 20-30 ml of the *Agrobacterium* suspension for 10 min and slowly shaken. In order to improve the transformation efficiency, several wounds were meticulously made throughout the explants using a needle prior to their inoculation in the *Agrobacterium* suspension. Explants were dried on sterilized paper and placed in the solid co-cultivation WPM medium with the same supplements as used in the liquid medium. After co-cultivation for 3 d in darkness at 25°C, the *in vitro* shoot segments were washed three times with sterile distilled water and then transferred onto the WPM selection medium containing 9.2 µM Kinetin and 0.54 µM NAA, 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime at 25°C for 28 days. This experiment was repeated three times.

The *in vitro* shoots were maintained under these conditions for 2 subsequent subcultures (3 months in total). Proliferating shoots from axillary buds (Fig. 2B) were selected and transferred to proliferation WPM medium supplemented with 9.2 µM Kinetin and 0.54 µM NAA. Putative transgenic shoots were identified by kanamycin-resistance and homogeneous expression of reporter genes in leaves as determined by histochemical *gus* staining (Jefferson *et al*., 1987). Transformed shoots were transferred to half-strength WPM medium containing 5.4 µM NAA and 50 mg L^{-1} kanamycin for rooting. One month later, well-rooted plants were removed from the culture medium. The roots were washed gently with tap water to remove agar, and then plants were transferred to small plastic pots containing autoclaved cocopeat-perlite mixture (1:1). The pots were covered with polyethylene bags to maintain high humidity and kept at 25±1°C in artificial light (50 µmol m^{-2} s^{-1}) provided by white fluorescent tubes for 3-4 weeks. For hardening of the plants, polyethylene bags were opened gradually, from a few minutes a day until normal conditions.

Fig. 2. Proliferation of transgenic pomegranate cultivar 'Yousef Khani' (A) *In vitro* shoot segments forming shoots after infection with LBA4404 on selection WPM medium after 12 days; (B) Shoot elongation after 30 days; (C) A transgenic shoot rooted on WPM medium containing kanamycin; (D) A fully developed transgenic plantlet before transplantation into the greenhouse; (E) Three transgenic plantlets 5 months after transfer to the greenhouse

Histochemical gus assay

Five leaf sections per each kanamycin resistant shoot were placed in X-gluc solution (Jefferson *et al*., 1987) and incubated for 24 h at 37°C. The explants having no *Agrobacterium* infection were used as control for *gus* histochemical assay. After X-gluc treatment, the leaf sections were rinsed with 70% ethanol for 12 h to remove chlorophyll. After degreening, explants were observed under a microscope and photographed. Plantlets having partially stained leaves or positive and negative leaf sections in the same shoot were considered to be chimeric ones.

PCR analysis

Total DNA was isolated from leaves and shoots of putative transgenic and non-transgenic plants using the cetyltrimethylammonium bromide (CTAB) method as described by Doyle and Doyle, (1987). The presence of *nptII*, *gus*, and *virC* genes was checked by PCR using specific primers presented in Table 1. PCR amplification for the *gus* gene was performed in a mixture of total volume 25 µl that contained 20 ng of genomic DNA or 5 ng of plasmid DNA, 0.25 µM of each primer, 1 mM dNTPs, 2.5 µl 10 X PCR Buffer, 1.5 mM of MgCl2, and 1 unit of Taq DNA Polymerase. The PCR conditions for amplification of the *gus* gene fragment were 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min with a drop to 4°C.

For *nptII* and *virC* genes, the PCR reactions and programme conditions followed Vidal et al., (2010). PCR products were separated in 1% (w/v) agarose gels in 1x TAE buffer at 80 V. After electrophoresis, gels were stained with ethidium bromide and visualised under UV light.

Southern blot hybridization

For Southern blot analysis, genomic DNA (10-20 µg) extracted from leaves of PCR-positive plants was digested overnight with BamHI, which cuts a single site within the T-DNA (Fig. 1).

Table 1. Sequence and fragment length of primers used in PCR assay of transgenic plants of pomegranate 'Yousef Khani'

Target gene	Primer sequence 5'→3' { Forward / Reverse	Fragment length (bp)
gus	ACCTCGCATTACCCTTACGCTGAA AATCGCCGCTTTGGACATACC	450
nptII	GTCATCTCACCTTGCTCCTGCC AAGAAGGCGATAGAAGGCGA	472
virC	ATGATTTGTAGCGGACT AGCTCAACCTGCTTC	730

Digested DNA fragments were separated on a 1% (w/v) agarose gel and subsequently transferred to a positively charged nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany) by capillary blotting. The *gus* probe (450 bp) was generated from plasmid *pBI121* and labelled with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany). Prehybridization, hybridization, washing of blots, and detection were performed according to the instruction manual of the DIG labelling and detection system (Roche Diagnostics, Mannheim, Germany). Hybridizing bands were visualized with anti-DIG antibody-alkaline phosphatase and CDP-Star (Roche) on X-ray films. Genomic DNA extracted from a non-transformed plant and plasmid DNA served as negative and positive controls, respectively.

Results

Sensitivity to kanamycin

In order to determine the appropriate concentration of selection agent to effectively screen transformed shoots, *in vitro* shoot segments were cultured on WPM medium supplemented with different concentrations of kanamycin. The addition of increasing concentrations of kanamycin significantly decreased survival rates ($P < 0.05$) (Table 2). After 4 weeks of culture, 100% survival rates were attained in explants cultured on medium lacking kanamycin. On medium containing kanamycin, maximum shoot induction (63%) was obtained at 12.5 mg L^{-1}. At 25 mg L^{-1}, 70% of explants bleached and died. Further increase in the level of kanamycin to 50 and 100 mg L^{-1} totally inhibited shoot production (Table 2). To minimize escape and prevent necrosis, 50 mg L^{-1} kanamycin was chosen as the selection antibiotic in the transformation experiments.

Table 2. Sensitivity of *in vitro* shoot segments of pomegranate 'Yousef Khani' to kanamycin

Concentration of kanamycin (mg L^{-1})	Number of explants cultured	Number of explants with shoots	Survival rates (%)*
0	30	30	100 ± 0.0 a
12.5	30	19	63 ± 1.5 b
25	30	9	30 ± 2.5 c
50	30	0	0 ± 0.0 d
100	30	0	0 ± 0.0 d

* Values represent the mean ± SD.
Means with different letters are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

Regenerating and analysis of transgenic plant expressing gus

Pomegranate *in vitro* shoots segments (Fig. 2A) were transformed using *Agrobacterium* strain *LBA4404* harboring the binary vector *pBI121-gus*. After 28 d in the selection media, 59 new shoots proliferated (from the total 180 initial explants). The average

number of shoots produced per explant was 0.33. *gus* analysis was performed on all 59 putative transgenic shoots, of which 32 stained positive (Table 3). Positive staining shoots were cut and cultured in selection medium for 2 subsequent subcultures until final *gus* analysis. After three months of the selection period, 11 chimeric and 6 full transgenic shoots (i.e., 6.1% and 3.3% of initial explants, respectively) were obtained. *gus* activity was clearly observed in leaves of all 6 transformed shoots (Fig. 3A). In contrast, no *gus* activity was detected in untransformed explants(Fig. 3B). Transformed shoots were rooted and acclimatized to normal conditions (Figs. 2C, D, E).

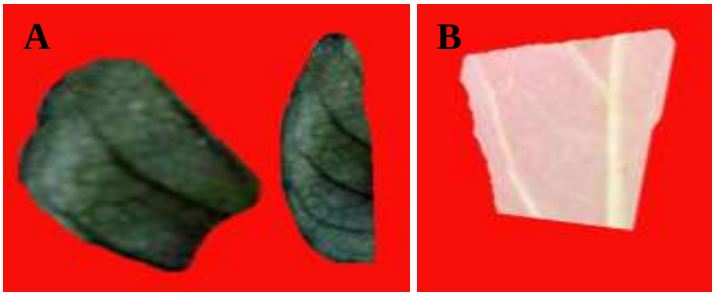

Fig. 3. Detection of *gus* activity in leaf sections of transgenic plants of pomegranate 'Yousef Khani' (A) compared with untransformed control; (B) after 24 h of staining with X-Gluc

Table 3. Number and frequency of *gus* positive and negative proliferated shoots obtained from *in vitro* shoots segments of pomegranate 'Yousef Khani' under selection at 50 mg L^{-1} kanamycin after 28 d

Experiment number	Number of explants	*gus* positive*	*gus* negative**
1	60	8 (13%)	11 (18%)
2	60	11 (18%)	8 (13%)
3	60	13 (21%)	8 (13%)

*, **Values in parentheses indicate the percentage of *gus* positive and negative shoots of total number of explants studied.

PCR analysis

To confirm the presence of *gus* and *nptII* genes in 6 *gus* positive plants, PCR analyses were conducted on these putative transformants along with one non-transgenic plant (negative control) and the plasmid DNA (positive control). DNA fragments corresponding to the *gus* gene (450 bp) and *nptII* gene (472 bp) were amplified for all 6 *gus* positive plants (Figs. 4A and B, lanes 1–6) as well as for the plasmid DNA, whereas the corresponding band was not detected in the untransformed control, indicating that the T-DNA of the binary plasmid vector was present in the genome of the transgenic plants.

The absence of *Agrobacterium* contamination in transformed shoots was confirmed by the absence of the 730 bp band corresponding to the *virC* gene (Fig. 4C). This indicates that kanamycin resistant shoots do not contain any residual *Agrobacterium*.

Southern blot analysis

Six putative transgenic plants (PCR-positive) were analyzed by Southern blot hybridization. Since the genomic DNA was digested with BamH1, with only one restriction site within the T-DNA region (Fig. 1), one band would be expected for each T-DNA integration. Variable hybridization patterns were observed in three out of the six plants tested, indicating the expected random integration of the transgene into the 'Yousef Khani' genome. Examination of BamH1 digests (Fig. 5) revealed that one insert (lane 4) or two inserts (lanes 3, 5) were present in the analyzed transgenic plants. No hybridization signals could be detected for the untransformed control.

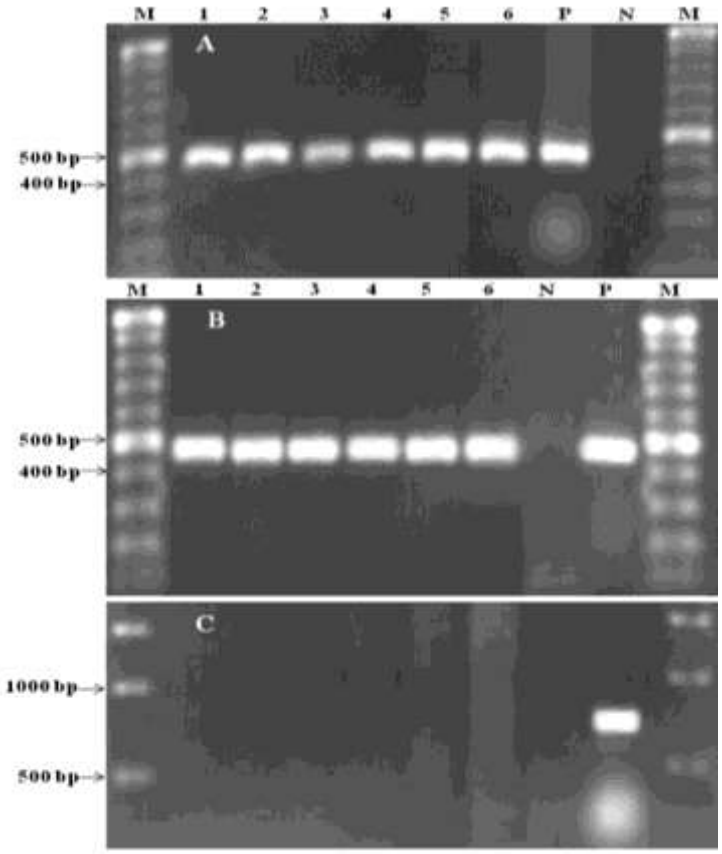

Fig. 4. A PCR analysis for detecting *gus* (A), *nptII* (B) and *virC* (C) genes in transgenic plants of pomegranate 'Yousef Khani'. The sizes of the amplified fragments for the *gus*, the *nptII*, and the *virC* genes are 472, 450, and 730 bp, respectively. Lane M is a ladder marker (100 bp); lanes 1–6 are transformed plants; lane N is an untransformed plant; and lane P is a positive control (plasmid DNA pBI121)

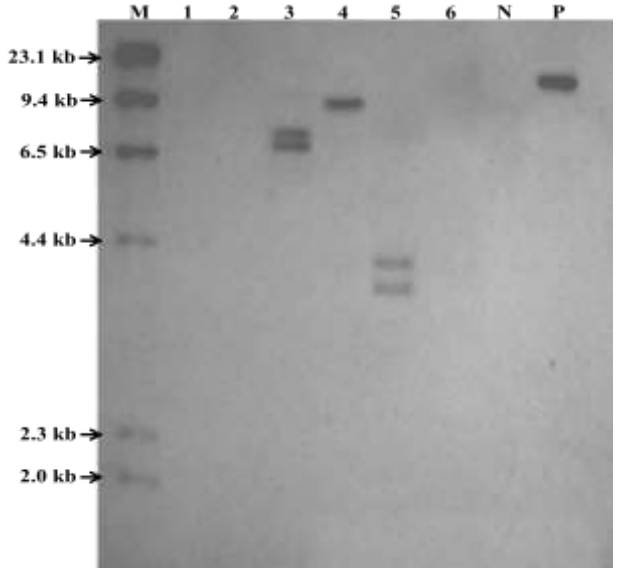

Fig.5.Southern blot analysis of 6 PCR positive plants of pomegranate 'Yousef Khani'. Genomic DNA was digested with BamH1 and hybridized with a digoxigenin (DIG)-labelled fragment containing the *gus* gene as probe. Lane M, Lambda HindIII Dig-labelled molecular marker; lanes 3, 4, 5, transgenic plants; lanes 1, 2, 6 chimerical plants; Lane N, non-transformed; lane P, positive control (plasmid DNA)

Discussion

The *Agrobacterium tumefaciens* strain *LBA4404* was found to be effective for the transformation of pomegranate 'Yousef Khani'. Several studies have reported that *LBA4404* is more effective than other strains for transformation (Chen *et al*., 2002; Tohidfar *et al*., 2005). This strain has also been used for many plant transformations, because the elimination of *LBA4404* from plant tissue is relatively easy in low concentrations of antibiotics (Maheswaran *et al*., 1992).

A number of different explants have been previously used in the development of *Agrobacterium*-mediated transformation protocols for fruit trees (Archilletti *et al*., 1995; Corredoira *et al*., 2004; Domínguez *et al*., 2004; Polin *et al*., 2006; Gago *et al*., 2011). In this work, we used pomegranate *in vitro* shoot segments readily obtainable from *in vitro* culture as transformation targets, and we propagated transgenic shoots via axillary bud proliferation. Previously, Terakami *et al.* (2007) reported the development of a transformation protocol for dwarf pomegranate (*Punica granatum* L. var. nana). In their study, they obtained transgenic plants using adventitious shoots as the explant. After repeated attempts to reproduce their results, we were not successful in generating adventitious shoots.

The transformation efficiency (1.6%) obtained in this study is within the range obtained for other tree species. Low transformation frequencies have been obtained in the genetic engineering of fruit trees. For instance, transformation rates of 3% were reported in explants derived from *Citrus clementina* (Cervera *et al.,* 2008), 1.2% in *Prunus serotina* (Liu and Pijut, 2010), and less than 1% in *Prunus dulcis* (Miguel and Oliveira, 1999).

Kanamycin is widely used to select *nptII*-transformed cells. Various concentrations of kanamycin have been reported to inhibit organogenesis; almond (Miguel and Oliveira, 1999), apple cv. Royal Gala (Yao *et al.,* 1995), apple rootstock M26 (Norelli and Aldwinckle, 1993), grape (Scorza *et al.*, 1996), and citrus (Yao *et al.,* 1996) require 5–10, 50, 5, 40, and 100 mg L^{-1}, respectively, in order to prevent proliferation. In this work, a proliferation medium containing 50 mg L^{-1} kanamycin was found to be most suitable for selecting transformed cells (Table 2). Similar results were previously reported for dwarf pomegranate (Terakami *et al.,* 2007). However, using this concentration, the frequencies of escapes (16%) and chimeric shoots (6.1%) were still high. In most transformation systems, proliferation of escapes and chimeric shoots is a major problem (James *et al.,* 1990a; Domínguez *et al.*, 2004; Gago *et al*., 2011). In citrus, the proliferation of escapes and chimeric shoots at high frequencies (60% - >90%) has been reported (Costa *et al.*, 2002; Yu *et al.,* 2002). Raising the concentration of the selective agent is the most obvious strategy to overcome generation of escapes (Niu *et al.*, 2000; Park *et al.*, 1998), but it may inhibit the proliferation of transformed as well as untransformed cells (Harjeet *et al.*, 1997).

To investigate *gus* activity, leaves of plantlets were analysed for *gus* expression with the histochemical *gus* assay from four weeks to three months after infection, when most shoots were already formed. In transgenic pomegranate shoots, histological detection of *gus* activity revealed an intense activity in leaves (Fig. 3A). However, the destructive characteristic of the *gus* assay allows analysis of only a small part of the regenerating shoots. Moreover, the possibility of transgene silencing or very low expression cannot be evaluated with techniques based on expression assays. Therefore, some of the *gus* negative shoots and all 6 *gus* positive shoots were analysed by PCR. The results of PCR analysis showed that all 6 *gus* positive shoots amplified fragments for both *gus* and *nptII* genes (Figs. 4A and B). PCR analysis of regenerated shoots is proposed as a method that permits the identification of transgenic plants (De Vetten *et al*., 2003). Our data strengthen the opinion that selection by

PCR could permit the recovery of an achievable number of transgenic plants. The integration of the transgene into the plant genome and its copy number was well verified by Southern blot analysis. Unexpectedly, three of the putative PCR-positive plants did not show any hybridization signal (Fig. 5, lanes 1, 2, 6). The most conceivable explanation is that these plants might be chimeric. Several studies have reported that a positive Southern blot cannot be obtained with DNA from chimeric shoots (Mathews *et al*., 1998; Domínguez *et al.*, 2004; Petri *et al*., 2008).

All transformed shoots had the ability to root on half-strength WPM medium containing 50 mg L^{-1} kanamycin (Fig. 2C), while control shoots did not root. This confirms the observation of James *et al.*, (1990b) that the ability of *nptII*-transformed shoots of apple to form roots on medium containing kanamycin is a strong indication for the transgenic nature of these shoots.

Conclusions

On the basis of the above-mentioned results, a reliable transformation protocol for pomegranate has been established using *Agrobacterium tumefaciens* as a vector. The *in vitro* shoots proliferation system proved to be an excellent vehicle for the production of transgenic pomegranate plants over relatively short periods. The protocol used here is considered to be an important step towards the development of transgenic pomegranate cultivars with agronomically important genes.

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