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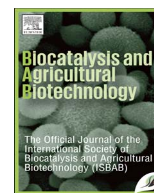

Transformation of pomegranate (*Punica granatum* l.) a difficult-to-transform tree

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ABSTRACT

In this study, an *Agrobacterium*-mediated transformation method was developed for pomegranate, a difficult-to-transform plant. We first optimized callus induction and shoot regeneration efficiency. Induction of calluses was best achieved from the intermodal stem sections on WPM medium supplemented with 12 µM BA and 8 µM NAA. The highest frequency of shoot regeneration (69.33%) and number of shoots (7.16) per piece of callus were obtained when calluses were incubated on WPM medium supplemented with 12 µM BA and 2 µM NAA. Callus pieces were inoculated with *Agrobacterium tumefaciens* strain *EHA105* harboring the binary vector *pBin19* carrying the neomycin phosphotransferase (*nptII*) gene as a selectable marker and green fluorescent protein (*GFP*) gene as a reporter. After 4 weeks in WPM selection medium (the best callus induction medium supplemented with 50 mg L^{-1} kanamycin), putative transgenic calluses were obtained. From such calluses transformed shoots began to appear approximately 3 week after the kanamycin resistance transgenic callus pieces had been placed on the best shoot regeneration medium containing 50 mg L^{-1} kanamycin. *GFP* fluorescence was observed in putatively transformed calluses and shoots. Molecular analysis confirmed the integration of the *nptII* transgene in transformed shoots. The transgenic shoots were rooted and successfully acclimatized.

1. Introduction

Pomegranate (*Punica granatum* L.) is a significant fruit tree of the tropical and subtropical areas of the world that is grown for its enjoyable fruits, medicinal properties and ornamental horticulture. However, there are some restrictions to the productivity of the pomegranate orchards, resulting in serious adverse economic impacts on growers. For instance, the carob moth [*Ectomyelois ceratoniae* (Zeller) (Lepidoptera:Pyralidae)] is a serious problem in pomegranate in many countries such as Iran, the USA and Turkey, causing estimated losses of 50% of the total yield (Carroll et al., 2006; Mirkarimi, 2000; Ozturk et al., 2005). In addition, pathogens such as grey mould rot (*Botrytis cinerea*), heart rot (*Alternaria alternate*) and bacterial blight (*Xanthomonas axanopodis*) are the important pomegranate diseases adversely affecting crop production in all main growing areas (Munhuweyi et al., 2016). The development of pomegranate cultivars with resistance to these pests and diseases should have great impact on crop efficiency.

Genetic improvement of pomegranate by conventional breeding is a laborious and time consuming method because of heterozygosity, time interval between generations, vegetative propagation, and duration of field evaluations. In addition, traditional breeding has met with inadequate success due to the lack of desirable genes such as genes conferring resistance to disease and pest in the germplasm (Gupta and Pandey, 2014). A substitute to dominate these restrictions is the introduction of new characteristics by *Agrobacterium*-mediated genetic transformation, an established technique for transferring genes into plants cells (Chauhan and Kanwar, 2012). Genetic engineering has been employed to develop fruit crops with better horticultural characteristics such as pest resistance, disease resistance, cold tolerance, herbicide tolerance, salt tolerance, and made better plant and fruit characters and shelf life without affecting their desirable genetic and phenotypic background (Gomez-Lim and Litz, 2004; de Campos et al., 2011; Ziemienowicz, 2014).

In vitro propagation of pomegranate has been reported by a number of researchers (Valizadeh Kaji et al., 2013; Chauhan and Kanwar, 2012; Naik and Chand, 2011); However, genetic transformation of pomegranate remains inefficient and there is a strong need for an efficient transformation protocol. We previously reported the development of a transformation protocol for pomegranate cultivar,

Abbreviations: GUS, β-glucuronidase; *GFP*, green fluorescence protein; NAA, naphthaleneacetic acid; BA, benzyladenine; WPM, woody plant medium; nptII, phosphotransferase II gene; NOS, nopaline synthase promotor and terminator; CTAB, Cetyltrimethylammonium bromide.

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'Yousef Khani' using intermodal stem sections as the explant and using *Agrobacterium* strain *LBA4404* harboring *pBI121* carrying β-glucuronidase (GUS) and *nptII* genes (ValizadehKaji et al., 2014). However, the limitation of previous protocol is low transformation efficiency and the proliferation of escapes and chimeric shoots at high frequencies.

The effective introduction of new favourable genetic features generally needs the development of an effective and dependable transformation procedure. The use of visible markers, which provides straight observation of transformation occurrences, leads to a more accurate and easier assessment of different treatments and methods. They can enhance transformation performance by decreasing the time and quantity of material to be applied and screened (Baranski et al., 2006) permitting the most effective, dependable and reproducible transformation procedure to be recognized. Markers such as β-glucuronidase (GUS), luciferase (LUC) or b-galactosidase (LacZ) have been very successful instruments as reporters for gene transfer and gene expression in transgenic plants. Nevertheless, these need either ruinous tests of the examined sample or the adding of exogenous materials for their manifestation. These markers generally do not present the feasibility of defining the precise transgenic status of plants. In contrast, green fluorescence protein (*GFP*) marker, in principle, permits observing in situ transgene expression from primary stages of the transformation of growth and development of living transgenic plants. In addition, *GFP* appearance does not need the adding of any exogenous materials. Therefore, plants can carry on their growth and development, and can be examined at every growth step (Heim et al., 1995; Chiu et al., 1996). This indicates the validation for using *GFP* as a visible indicator throughout genetic transformation and regeneration of transgenic plants.

Therefore, the objective of this work was to develop an effective reproducible procedure for the transformation of pomegranate using the *GFP* reporter gene. Here we report for the first time a reliable protocol to produce transgenic pomegranate plants from calluses of the cv. 'Robab', an Iranian leading pomegranate cultivar.

2. Materials and methods

2.1. Callus initiation

To induce callus formation, in vitro shoot and leaf segments of *Punica granatum* L. cv. 'Robab' which were proliferated in WPM (Woody plant medium) medium (Lloyd and McCown, 1980) were excised and placed on WPM medium supplemented with varying concentrations of naphthaleneacetic acid (NAA) (4–16 µM) in combination with benzyladenine (BA) (4–16 µM). Sucrose was added at 30.0 g l^{-1} and myo-inositol at 0.1 g l^{-1}. The pH of the medium was set to 5.6–5.8 prior to autoclaving (121 °C for 15 min) and the medium was solidified with 6 g l^{-1} agar. Each Petri dish contained 20 mL of media and 5 explants. The cultures were maintained at 25 ± 1 °C with white fluorescent light (30–40 µmol m^{-2} s^{-1}) and a 16 h photoperiod. The experiment was conducted in three replicates, each consisting of at least 5 explants. Frequency of callus induction was estimated after 4 weeks of culture as the percentage of explants producing callus (Fig. 2).

2.2. Shoot regeneration and root induction

To determine the ability of the initiated callus to induce shoots, small pieces of callus, 0.5–1 cm^2 were excised from intermodal stem sections and transferred to WPM medium supplemented with various combinations of BA (2–16 µM) and NAA (2–4 µM). The culture conditions were the same as those described earlier. The experiment was conducted in three replicates, each consisting of at least 5 explants. The percentage of calluses producing shoots and mean number of shoots per callus piece were determined after 30 days as shown in Fig. 3.

To induce roots, regenerated shoots (2–4 cm length) were separated from the callus and transferred to half-strength WPM medium containing 5.4 µM NAA as previously described by Valizadeh Kaji et al. (2013).

2.3. Sensitivity to kanamycin

To determine the concentration of kanamycin that inhibits callus growth, four-week-old callus pieces of 'Robab' were cultured on the best callus induction medium (WPM medium containing 12 µM BA and 8 µM NAA) supplemented with different concentrations of kanamycin (0, 12.5, 25, 50 and 100 mg L^{-1}). The experiment was conducted in two replicates with at least 10 explants per replicate.

2.4. Bacterial strain and plasmid

Agrobacterium tumefaciens strain *EHA105* harboring the binary vector *pBin19-GFP*, kindly provided by Dr. N. Alburquerque (Mejora de Plantas, CEBAS-CSIC, Murcia, Spain), was used for transformation. The T-DNA region of the plasmid contains the neomycin phosphotransferase II gene (*nptII*) under control of nopaline synthase promotor and terminator (NOS) and the *GFP* gene under control of the 35 S promotor and the NOS terminator.

Agrobacterium was grown in liquid LB medium supplemented with 50 mg L^{-1} kanamycin at 27 °C for 24 h on a shaker at 140 rpm. The suspension was centrifuged at 3000 rpm for 15 min, then the pellet was resuspended in WPM liquid medium and diluted to an OD600 of 0.5. Immediately prior to the infection of explants, the bacterial suspension was supplemented with acetosyringone to achieve a final concentration of 100 µM.

2.5. Plant transformation

Four-week-old callus pieces obtained on WPM callus induction medium were immersed in the *Agrobacterium* suspension for 10 min with slow shaking. The infected callus pieces were dried on sterilized paper to remove excess bacteria and co-cultivated on the best callus induction medium. After co-cultivation for 3 days in darkness at 25 °C, the infected callus pieces were washed three times with sterile distilled water and then transferred onto the WPM selection medium (the best callus induction medium supplemented with 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime) for 4 weeks under the same culture conditions described above. After 4 weeks, newly emerging kanamycin resistant calluses were separated from the infected callus pieces and subcultured onto the best callus induction medium supplemented with 50 mg l^{-1} kanamycin. The kanamycin resistant calluses were transferred to fresh selection medium every 4 weeks. The transformation experiment was repeated twice with 100 callus pieces per experiment. The transformation efficiency was calculated as the number of kanamycin resistant shoots (obtained from kanamycin resistance callus pieces) per total number of infected callus pieces.

To induce roots, the putative transgenic shoots regenerated from kanamycin resistant calluses were excised and transferred onto half-strength WPM medium containing 5.4 µM NAA and 50 mg L^{-1} kanamycin (ValizadehKaji et al., 2014). 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 acclimatized.

2.6. Microscopic detection of GFP expression

The *GFP* transient expression in the callus pieces and regenerated

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