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Short communication

Genetic transformation of the 'W Murcott' tangor: comparison between different techniques

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ABSTRACT

Genetic transformation of citrus has been mainly conducted *via Agrobacterium tumefaciens* using juvenile tissues of the epicotyl segments obtained from the *in vitro* germination of seeds. Transformation of the stem segments obtained from the mature material is an alternative to overcome or reduce the juvenile phase reported in perennial species such as citrus. However, some citrus species, including mandarins, are recalcitrant to these methods or have low genetic transformation efficiency. Thus, the alternative is the use of juvenile explants with high regenerative potential, such as cell suspensions obtained from citrus embryogenic callus or direct incorporation of DNA into protoplasts using polyethylene glycol (PEG). The present study aimed to compare these four genetic modification techniques for 'W Murcott', one of the main fresh market mandarin cultivars in the United States. The transgenic plants were obtained by the different techniques using a vector containing the EGFP gene under the control of the CaMV35S promoter. The genetic transformation efficiency was low, utilizing either juvenile epicotyl or mature stem segments, with an average of 1.23% and 0.33%, respectively. The genetic transformation efficiency using cell suspensions and cell suspension-derived protoplast explants were higher, with an average of 29% and 11%, respectively. Our results reveal the importance of utilizing cell suspension-derived cultures for the efficient transformation of 'W Murcott'.

1. Introduction

Genetic transformation has emerged as a powerful tool for genetic improvement of citrus species hindered by their high level of heterozygosity, nucellar polyembryony, self- and cross-incompatibility and long juvenile period (Talon and Gmitter, 2008). Genetic transformation allows the incorporation of selected traits into an elite genotype without altering the genetic background, making it possible to introduce useful characters in commercial cultivars (Gambino and Gribaudo, 2012; Van Nocker and Gardiner, 2014).

Genetic transformation of citrus has been mainly conducted *via Agrobacterium tumefaciens* (Dutt and Grosser, 2009; Peña et al., 1995) using epicotyl segments obtained from the *in vitro* germination of seeds (Almeida et al., 2003a; Molinari et al., 2004; Peña et al., 2004). Given the polyembryonic nature of most citrus types, it is possible to transform nucellar seedlings to obtain transgenic events while maintaining the genetic characteristics of the mother plant (Peña et al., 2004). However, for some species the adoption of this method poses difficulties due to the low supply of seeds such as in seedless sweet oranges, lemons or satsuma mandarins; there are also difficulties for monoembryonic species like clementine mandarins where only the zygotic embryo develops from the seed, thus carrying a variable combination of the genetic content from both parental lines. In addition, there is a strong impact of the genotype on citrus organogenesis and genetic transformation (Ghorbel et al., 1998; Moore et al., 1992). Among the citrus species, juvenile tissues from mandarins hybrids are more difficult to infect and transform with *A. tumefaciens* (Cervera et al., 1998) resulting in low genetic transformation efficiency (Dutt et al., 2009). Thus, the alternative is the use of unconventional juvenile explants with high regenerative potential, such as the cell suspensions obtained from citrus embryogenic callus (Li et al., 2002; Guo and Grosser, 2005; Dutt and Grosser, 2010). Another alternative, to circumvent responsiveness of genotype to transgene insertion through *A. tumefaciens,* includes the direct incorporation of DNA into protoplasts using electroporation (Niedz et al., 2003) or PEG-mediated DNA incorporation (Fleming et al., 2000; Omar et al., 2007), allowing the introduction of gene

Abbreviations: EGFP, enhanced green fluorescent protein; YEP, yeast extract peptone; PEG, polyethylene glycol; CaMV, cauliflower mosaic virus; NPTII, neomycin phosphotransferase II; NOS, nopaline synthase

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constructs directly into the genome of the plant cell.

Transformation of the juvenile material, such as protoplasts, epicotyl segments, and cell suspension results in the production of juvenile plants that drastically prolong the time required to analyze mature traits, particularly the characteristics related to fruits. *Agrobacterium*-mediated transformation of mature material could ensure earlier fruit production of adult transgenic plants, bypassing the juvenile phase or reducing this period (Almeida et al., 2003b; Cervera et al., 2008; Kobayashi et al., 2003). Despite this important advantage, mature material can show a decline in its response to organogenesis induction in tissue culture, with a progressive loss of transformation competence and regeneration (Von Aderkas and Bonga, 2000), requiring more studies on *in vitro* protocol adjustments.

Mandarins and their hybrids are an important group in world citrus production, and 'W Murcott' (*C. reticulata* Blanco x *C. sinensis* L. Osbeck), a tangor of unknown parentage is one of the main fresh cultivars marketed to consumers in the United States. However, obtaining transgenic plants from mandarins and their hybrids is a challenge especially using conventional *Agrobacterium*-mediated epicotyl transformation techniques (Cervera et al., 1998). Considering this, the present study aimed to compare the four commonly used genetic modification techniques used in citrus for 'W Murcott' transformation: *Agrobacterium*-mediated transformation using cell suspension cultures, seed-derived epicotyl segments, mature stem segments as explants and PEG-mediated transformation using protoplasts as explant.

2. Materials and methods

2.1. DNA construct

The binary vector pC2300-EGFP was used for all transformation experiments (Supplementary Fig. 1). This vector contains the EGFP gene under the control of a CaMV 35S promoter and the *npt*II gene under the control of the NOS promoter.

2.2. Agrobacterium-mediated transformation

The *Agrobacterium tumefaciens* strain EHA105 containing the pC2300-EGFP binary vector was used for all transformation experiments. For epicotyl, mature stem and cell suspension transformation, a single *A. tumefaciens* colony was cultured in liquid YEP medium containing 100 mg L^{-1} kanamycin and 20 mg L^{-1} rifampicin on a shaker (185 rpm) at 26 °C for 2 days. Two milliliters of a vigorously growing *A. tumefaciens* culture, initiated the night before, was seeded into 48 ml YEP medium containing appropriate antibiotics. The cells were cultured for 3 h before being collected by centrifugation at 5000*g* for 6 min at 25 °C and resuspended to an OD_{600} of 0.3 using liquid co-cultivation medium (CM) (Dutt and Grosser, 2009) for all experiments.

2.2.1. Genetic transformation using juvenile epicotyl explants

In vitro nucellar seedlings were obtained as described by Dutt and Grosser (2009). One cm-long epicotyl segments were incubated in the *A. tumefaciens* suspension for 5 min. Subsequently, explants were blotted dry on sterile paper towels, placed on semi-solid co-cultivation medium supplemented with 100 μM acetosyringone and incubated in the dark at 25 °C for 2 days before transfer to regeneration and selection medium. The shoots produced were evaluated for EGFP-specific fluorescence, and the EGFP-positive shoots were grafted onto Carrizo rootstock seedlings followed by PCR confirmation for the presence of transgene, as described by Dutt et al. (2012). The main steps in the transformation process are illustrated in Fig. 1. The regeneration efficiency was calculated as the total number of shoots produced per total number of inoculated explants. The transformation efficiency was calculated as the total number of EGFP-positive shoots per total number of inoculated explants.

2.2.2. Genetic transformation using mature stem segments

Mature trees budded onto Carrizo citrange rootstock and maintained in a low humidity air-conditioned greenhouse were used as sources of explants. All trees were drenched with an Imidacloprid solution (Admire® Pro, Bayer Crop Sciences, USA) 3 months before being pruned to initiate new flushes. Newly formed shoots from the lateral branches were collected and surface sterilized as described by Cervera et al. (2005). One cm-long stem segments were incubated in the *A. tumefaciens* suspension for 10 min. Subsequently, the explants were blotted dry on sterile paper towels, placed on solid co-cultivation medium supplemented with 100 μM acetosyringone and incubated in the dark at 25 °C for 2 days before transfer to regeneration and selection media, essentially as described by Cervera et al. (2005). The subsequent steps are as described for juvenile epicotyl explants and are illustrated in Fig. 1. The transformation efficiency was calculated as described for juvenile epicotyl explants.

2.2.3. Genetic transformation using juvenile cell suspension cultures

Initiation of embryogenic suspension cell masses were as described by Dutt and Grosser (2010). Embryogenic cells were incubated in an *A. tumefaciens* suspension for 20 min. Subsequently, the cells were blotted dry on sterile filter paper disks, plated on solid EME medium supplemented with maltose (EME-M) and 100 μM acetosyringone. The cells were incubated in the dark at 25 °C for 5 days. Subsequently, embryogenic cell cultures were transferred to an EME-M-based selection medium containing appropriate antibiotics and maintained at 28 °C on a standard 16-h light/8-h dark cycle. Developing embryos were observed for EGFP-specific fluorescence. Transgenic embryos were subsequently enlarged on maturation and germination media. All cell culture protocols were essentially as described by Dutt and Grosser (2010). EGFP-positive shoots were transferred for further root development and growth into rooting medium followed by the PCR confirmation for the presence of the transgene. The main steps are illustrated in Fig. 1. The transformation efficiency was calculated as the total number of EGFP-positive shoots per total number of embryos produced (EGFP-negative + positive).

2.3. PEG-mediated transformation using protoplast

Endotoxin-free plasmid DNA containing the pC2300-EGFP construct was utilized for all protoplast transformation experiments. Cell suspension cultures from the same batch as that used for *Agrobacterium*-mediated cell suspension culture transformation experiments were utilized for all protoplast transformation experiments. A PEG-mediated transformation protocol as described by Fleming et al. (2000) was utilized. EGFP-expressing cells were visualized and plantlets obtained through the process of somatic embryogenesis, which was similar to that described for the cell suspension culture-mediated transformation process and described in detail by Dutt et al. (2010). *In vitro* plantlets were tested by PCR, and the positive plants were acclimated in a similar manner to those obtained from the *Agrobacterium*-mediated transformation and subsequently utilized for molecular analyses. The main steps are illustrated in Fig. 1. The transformation efficiency was calculated as described for juvenile cell suspension cultures.

2.4. Molecular analyses and recovery

All transgenic lines were confirmed for gene integration using EGFP gene-specific primers. Copy numbers of thirteen randomly selected lines: four each from transformed juvenile epicotyl explants, juvenile suspension culture explants and protoplast-derived explants, as well as one line from the mature transformation experiment were evaluated by qPCR as detailed earlier (Dutt et al., 2016).