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Cultivar-dependent Gene Transfer into Citrus using *Agrobacterium*

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Florida's citrus industry is currently under severe onslaught from two non-indigenous but now endemic pathogens causing the diseases huanglongbing (HLB) and citrus canker. None of the commercial cultivars are resistant to either disease, which result in severe losses to the industry. Genetic transformation of citrus, to incorporate gene(s) for disease resistance not found in citrus, is gaining importance due to the possibility of incorporating resistance to these diseases. In an effort to transform a large number of cultivars via *Agrobacterium*-mediated transformation, epicotyl explants of several citrus scion and rootstock cultivars were evaluated for their ability to incorporate transgenes into their genome. It was determined that genetic transformation efficiency depended on cultivar studied, and the cultivars were either relatively easy to transform, moderately difficult, or recalcitrant to *Agrobacterium* infection. In general, of the cultivars investigated, Carrizo and 'Duncan' had the highest transformation efficiency (over 40%), followed by 'OLL8', a 'Valencia' like somaclone, and 'Hamlin' (20% to 40%). *Poncirus trifoliata,* 'Marsh' and 'Flame' grapefruit, and 'Valencia' sweet orange were moderately difficult (10% to 20%), while 'Mexican Lime', 'Volkamer' lemon, *Citrus macrophylla*, and tangerines were recalcitrant (less than 10%). Our results also suggested that final recovery rate of transgenic plants suitable for transfer to a greenhouse were significantly lower than the total transgenic plants obtained after transformation since the cultivars also varied in their ability to regenerate after in vitro grafting.

Genetic improvement of citrus is a long-term process due to several factors including nucellar polyembryony, pollen and ovule sterility causing incompatibility, long juvenile period, and a high level of heterozygosity (Grosser and Gmitter, 1990). Numerous improvements have been made in the last century for the development of fresh fruit and processed citrus. However, since the mode of inheritance for most traits of agricultural importance is unknown (Pena et al., 2007), strategies to improve scion cultivars require much effort. Even with rootstocks, commercial cultivars do not satisfy all selection criteria for a particular location (Castle, 1987).

Several strategies exist for citrus improvement, including conventional breeding and protoplast fusion to produce allotetraploid somatic hybrids that combine the intact nuclear genome of complementary cultivars, and genetic transformation (Grosser and Gmitter, 1990; Pena et al., 2007). Recently, citrus improvement using genetic transformation is being used frequently as increasing competition in international markets and disease pressure have stimulated worldwide interest in citrus improvement (Grosser et al., 2000). Much progress has been made since the first report of genetic transformation of citrus using epicotyl explants by Moore et al. (1992) and several cultivars have been transformed.

Currently, citrus canker and huanglongbing (HLB) are the two major diseases threatening the Florida citrus industry. Canker results in leaf-spotting and blemishing on the surface of the fruit, often resulting in defoliation, shoot dieback, and fruit drop. HLB affects all cultivated citrus varieties and causes a rapid decline of trees and the production of unmarketable fruit. Both these diseases are caused by non-indigenous bacterial pathogens and cause substantial economic losses. Incorporation of gene(s) via genetic engineering can potentially confer resistance in susceptible cultivars, while maintaining the varietal fidelity. Our research objective was to genetically modify citrus cultivars that can be grown in Florida and evaluate their transformation efficiency in order to optimize conditions for genetic transformation in a wide range of cultivars.

Materials and Methods

Plant materials. Epicotyls segments originating from juvenile nucellar seedlings of several citrus rootstock and scion cultivars were used for genetic transformation experiments (Table 1). The seeds were extracted from fruit, the outer seedcoat removed, surface-sterilized in a 0.6% (v/v) sodium hypochlorite solution for 10 min, and rinsed with sterile deionized water. Seeds were germinated in 15-cm-long glass culture tubes containing 15 mL of solid MS medium [consisting of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 30 g·L^{-1} sugar and 7 g·L^{-1} agar, pH 5.8]. The culture tubes were kept in the dark for 3 weeks at 25 °C, and subsequently moved to the light for an additional week, allowing the etiolated explants to turn green.

Genetic transformation. Light green epicotyl segments were harvested and cut obliquely into 1-inch-long segments to expose the cambial ring. These segments were incubated in liquid CM medium [MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 13.2 μM BAP, 0.5 μM NAA and 4.5 μM 2,4-*D*, 30 g·L^{-1} sucrose, 0.5 g·L^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.8]. Cut explants were incubated in this medium for 3 h before incubation with *Agrobacterium* to determine effect on transformation efficiency. The *Agrobacterium* strain used was EHA105 containing a binary vector with the LIMA gene as

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Table 1. Genetic transformation of different citrus rootstock and scion cultivars.

Cultivar	Ease of culture in vitro[z]	Response to in vitro grafting[y]
Rootstock		
Carrizo	+++[x]	+++
Poncirus	+++	+++
Volkamer lemon	+	+++
C. macrophylla	+	+++
Grapefruit		
Duncan	+++	++
Marsh	++	++
Flame	++	++
Sweet orange		
OLL#8	+++	+++
Hamlin	+++	+++
Valencia	+	+
Lime/lemon		
Mexican lime	++	+++
Lemon	+	+
Tangerine/tangelo		
Murcott	+	---[w]
Dancy	+	---
Orlando	+	---

[z]Ease of culture in vitro is defined by the ability of the explants to readily produce shoots after co-cultivation with *Agrobacterium* in a selection medium.

[y]Response to in vitro grafting is defined as the ability of the transgenic shoots to survive the process of in vitro grafting.

[x]+++, <80% success; ++, 40+ to 80% success; +, >40% success.

[w]No data.

described by Dutt et al. (2008). A single *Agrobacterium* colony derived from *Agrobacterium* stock 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 d (Dutt et al., 2007). Two milliliters of a vigorously growing culture 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 re-suspended in liquid CM medium. The optical density (OD_{600}) of the *Agrobacterium* suspension was adjusted to 0.3 before incubation with explants. The explants were blotted dry and placed horizontally on solid CM medium supplemented with 100 μM Acetosyringone for a co-cultivation period of 2 or 3 d depending on the cultivar before being transferred into a shoot regeneration medium. This medium contained the antibiotics, kanamycin (70 mg·L^{-1}), and timentin (400 mg·L^{-1}) for selection of transgenic shoots and to eliminate the *Agrobacterium*, respectively. The transformation efficiency of putatively transgenic shoots was evaluated as the number of green fluorescent protein (GFP) positive plants per total number of inoculated explants (Cervera et al., 1998).

Selection of regenerant plants. GFP-specific fluorescence was evaluated using a Zeiss SV11 epi-fluorescence stereomicroscope equipped with a light source consisting of a 100-W mercury bulb and a FITC/GFP filter set with a 480-nm excitation filter and a 515-nm longpass emission filter producing a blue light (Chroma Technology Corp., Brattleboro, VT). GFP expressing transgenic shoots were transferred onto RMG medium (MS salts and vitamins supplemented with 1 μM BAP and 2.89 μM GA_3, 0.5 g·L^{-1} MES, 25 g·L^{-1} sucrose, pH 5.8. Eight g·L^{-1} agar was added before autoclaving, while the GA_3 was added as a filter sterilized solution following autoclaving) for shoot elongation.

In vitro micrografting. Elongated transgenic shoots were micro grafted in vitro onto rootstock seedlings of Carrizo citrange or C35 citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] as described by Dutt et al. (2008) to expedite the recovery of transgenic plants. After a month of growth in vitro, the grafted shoots were potted into a peat based commercial potting medium (Metromix 500, Sun Gro Horticulture, Bellevue, WA) and acclimated to greenhouse conditions.

Molecular analysis of transformants. Polymerase chain reaction (PCR) was carried out in a thermal cycler (MJ Research, Waltham, MA) using GoTaq® Green Master PCR Mix (Promega Corp., Fitchburg, WI). LIMA gene-specific oligonucleotide primers: LI-51, 5´GGGATCCCCGGGTACCACCA 3´ and LI-32, 5´CTATAAGAAGCATGCTCTAG 3´ were used to confirm the presence of specific transgenes in citrus plants. Amplified DNA fragments were electrophoresed on a 1% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA) and visualized under UV light. All images were recorded and digitized. A Southern blot analysis was performed to confirm stable integration of the transgene and determine copy number in selected transgenic plants (Dutt and Grosser, 2009).

Ex vitro micrografting. PCR positive plants were grown in a greenhouse under 40% shade cloth and approximately 31/21 °C day/night temperatures. A rapid ex vitro micrografting technique as described by Skaria (2000) was modified wherein tender shoots from young transgenic plants were excised, cut into pieces containing a single internode along with 1 cm of attached stem and prepared for micrografting onto 6 mo-old vigorously growing Carrizo rootstock. The rootstock was decapitated and a cut made in the center of the stem through the pith. A tapering cut was made on the transgenic stem, exposing the pith and a wedge graft union was made between the rootstock and transgenic scion. To stabilize the graft union, a thin strip of Nescofilm® was used to wrap around the wedge. A 200-μL pipette tip was placed on top of the graft to maintain a high level of humidity around the bud. The grafted plant was placed under low light and room temperature conditions for 2 weeks following grafting. The plants were subsequently transferred to 80% shade and high humidity levels (80% to 90%) maintained in a greenhouse. After 3 weeks, plants were transferred into another greenhouse that was shaded with a 40% shade cloth. The pipette tip was then removed and all side branches as well as rootstock leaves were removed to allow the bud to grow.

Data analysis. Each experiment was repeated at least twice and treatments were replicated three times. A replicate consisted of 200 cut explants, each approximately 1 inch in length. Data were analyzed to calculate standard error or the mean percentage of transgenic plants using MS Excel.

Results and Discussion

In the present study, we discuss our efforts to transform various citrus rootstock and scion cultivars that will potentially provide material that may be resistant to citrus canker and/or citrus greening. In an attempt to incorporate transgene(s) of interest into as many citrus cultivars as possible, we conducted a series of experiments to evaluate the ability of different citrus scion and rootstock cultivars to incorporate transgenes into its genome following *Agrobacterium* mediated transformation. The general process of transformation to obtain a transgenic plant is outlined in Fig 1. The ability of *Agrobacterium* to incorporate

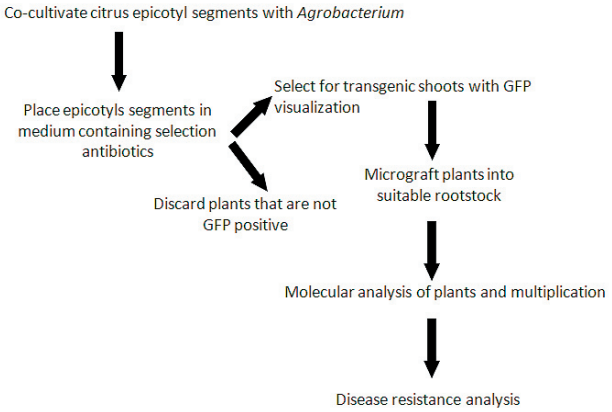

Fig 1. Steps in transgenic plant production.

transgenes into the genome of a particular cultivar depended on the cultivar and there was no co-relation between the different citrus groups (Fig. 2). Putative transgenic lines were observed to be either GFP positive, GFP negative or chimeric, where shoots had segments expressing GFP and other parts that did not. Only transgenic lines constitutively expressing GFP in all parts of the shoot were observed to be suitable for subsequent in vitro grafting. The soil bacterium *Agrobacterium tumefaciens* is able to transfer a particular DNA segment (T-DNA) contained in its tumor-inducing (Ti) plasmid into the nucleus of infected cells. This DNA segment is subsequently integrated into the plant's chromosome (Nester et al., 1984; Binns and Thomashaw, 1988). *Agrobacterium*-mediated transformation also has the unique advantage in that it is possible to get stable transgene integration without rearrangement of either host or transgene DNA and the integration of one or few gene copies into the plant's genome minimizes transgene silencing. This results in incorporation of a gene of interest without otherwise modifying the genotypic or phenotypic makeup.

Of the rootstock cultivars evaluated (Fig. 1A), Carrizo had the highest transformation efficiency. This cultivar was easy to establish in vitro and also had a good response to in vitro grafting (Table 1) wherein small transgenic explants (<5 mm) were wedge grafted into young non-transgenic Carrizo or C-35 rootstocks to expedite the growth process. Several researchers have also produced transgenic Carrizo plants. Cervera et al. (1998) improved the transformation efficiency in Carrizo over results reported by Pena et al. (1995a) by using tomato cell suspension medium (TCSM) feeder plates for co-cultivation and obtained a transformation efficiency of 41.3%. Our results, however, indicated an improvement in the methods previously described for Carrizo. Of the other rootstock cultivars evaluated, *Poncirus trifoliata* had 20% transformation efficiency whereas Volkamer Lemon and *Citrus macrophylla* were both very recalcitrant to *Agrobacterium* mediated transformation with less than 10% transformation efficiency. Difficulties in regeneration of transgenic plants have also been reported by Palacios-Torres et al. (2005) who obtained a few GUS+ shoots following transformation of *C. macrophylla*. However, none of those plants were PCR+. Both 'Volkamer' lemon and *Citrus macrophylla* produced thin epicotyl segments in vitro and which did not survive well after *Agrobacterium* infection. This could have played a role in the inability of explants to successfully regenerate shoots following transformation. However, following regeneration of a transgenic

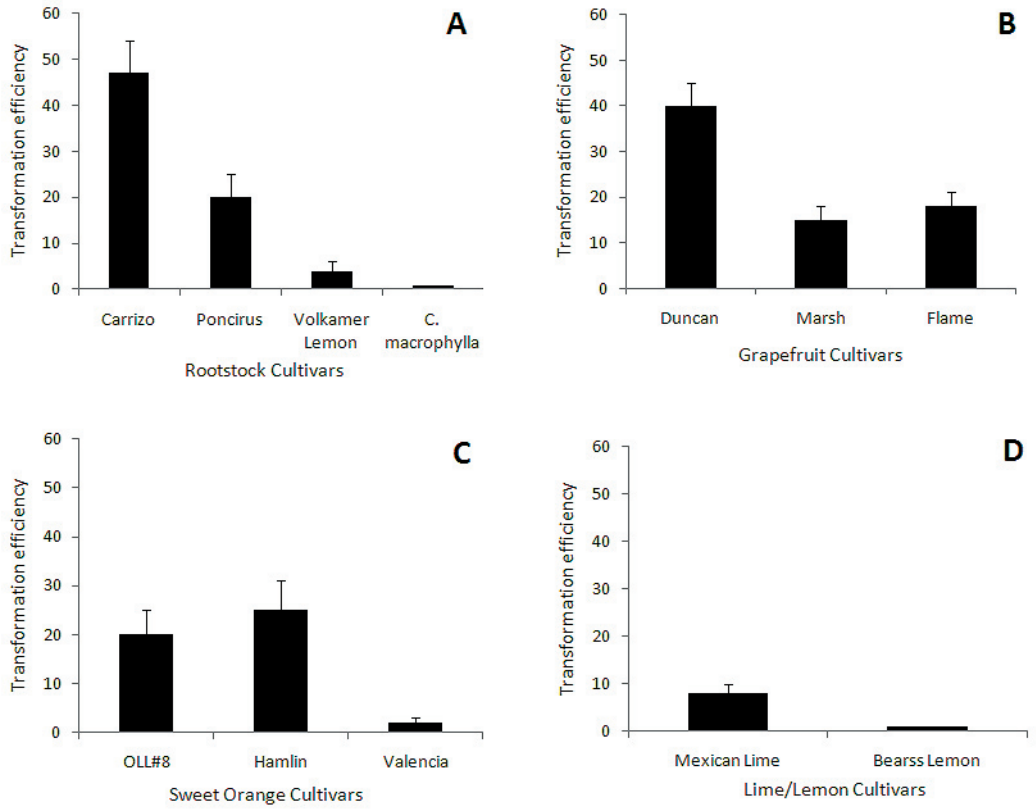

Fig 2. Transformation efficiency of different citrus rootstock and scion cultivars. Vertical lines represent standard errors.

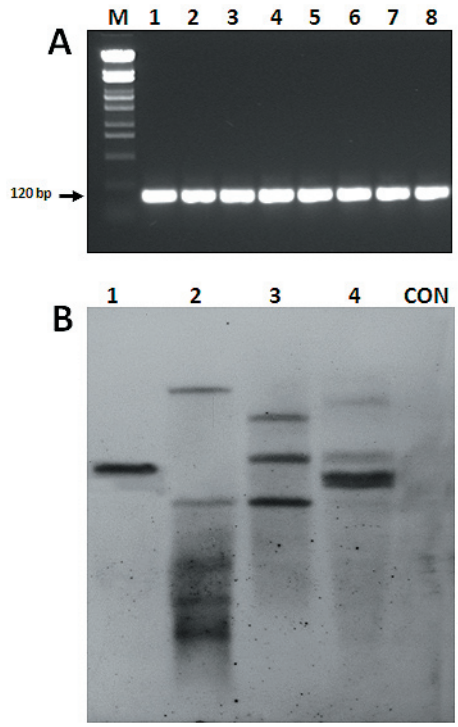

Fig. 3. Amplification products obtained from (**A**) PCR of genomic DNA of transgenic citrus plants with transgene specific primers. M, λ-Sty1 marker; 1–8 are 8 individual transgenic lines. (**B**) Southern hybridization analysis of total DNA from leaf tissue of four randomly selected transgenic plants (Lane 1, Hamlin; lane 2, Duncan; lane 3, Carrizo and lane 4; Mexican Lime) and a non-transgenic control plant (CON).

plant, all rootstock cultivars were very easy to graft in vitro, resulting in a high (90%) success rate.

Among the grapefruit cultivars (Fig. 1B), 'Duncan' grapefruit had the highest transformation efficiency, which was comparable to levels obtained by Carrizo. The two other cultivars, i.e., 'Marsh' and 'Flame', were similar in response, which ranged from 15% to 18%. Although 'Duncan' was easy to transform, it was moderately difficult to keep alive after in vitro grafting, reducing the final number of transgenic plants available for analysis. 'Marsh' and 'Flame' behaved similar to 'Duncan'. Our results suggest an improvement in the transformation efficiency over results reported by Moore et al. (1992) or Luth and Moore (1999) for the cultivar 'Duncan' and results of Ananthakrishnan et al. (2007) for the cultivars 'Flame' and 'Marsh'.

There was no significant difference in the transformation efficiency among the sweet orange cultivars 'OLL8' and 'Hamlin' (Fig. 1C). OLL8 is a 'Valencia'-like somaclone that produces fruit with exceptional juice color. It was, however, significantly different from 'Valencia'. 'OLL8' and 'Hamlin' had a transformation efficiency that ranged from 20% to 25% with a comparable ease of culture in vitro and in responsiveness to in vitro grafting. 'Valencia' proved recalcitrant, both in its ability to incorporate the transgene of interest following transformation and also in its ability to form a successful in vitro graft union with either of the two rootstocks. Other authors have reported that sweet orange cultivars are recalcitrant to *Agrobacterium* mediated transformation with the exception of 'Hamlin' (Almeida et al., 2003; Pena et al., 1995b).

Mexican lime was moderately difficult to transform with a maximum efficiency of 8% (Fig. 1D). However, unlike 'Valencia', this cultivar responded very well to in vitro grafting and more than 90% of the transgenic lines produced could be successfully grafted. However, lemon ('Bearss') was recalcitrant both to in vitro regeneration as well as subsequent micrografting. Mexican lime has been reported to be recalcitrant to *Agrobacterium* mediated transformation by other researchers. Fourteen transgenic lime plants out of 304 explants were obtained by Pena et al. (1997) and 42 transgenic plants from 1200 explants were obtained by Dominguez et al. (2000). In both cases, 6- to 12-month-old greenhouse-grown Mexican lime seedlings were used as explants for transformation. However, our report details the production of juvenile transgenic plants using epicotyl explants in contrast to the source of explants used by other researchers.

Since no graft incompatibility has been reported between the cultivars evaluated in this study and the rootsock(s) used, we can assume that the rate of success in transgenic plant recovery could depend on the skill or the micrografter or the ability of the transgenic shoots to form a successful graft union. None of the tangerine or tangelo cultivars evaluated ('Murcott', 'Dancy', or 'Orlando') could be successfully regenerated after *Agrobacterium* mediated transformation (Table 1). The main factor affecting this was a general recalcitrance to transformation using epicotyl segments. Also, like 'Volkamer' lemon or *C. macrophylla*, the epicotyl segments were thin and proved to be very susceptible to *Agrobacterium* overgrowth, even under very low levels of inoculum.

The constructed binary vectors contained a bifunctional *egfp/nptII* fusion gene that acted as a dual-purpose selectable marker and reporter gene (Li et al., 2001). The presence of GFP enabled us to perform a non-destructive assay by shining blue light to identify putative transformed shoots. Under blue light, transformed shoots appeared bright green while non-transformed shoots appeared red due to chlorophyll autofluorescence.

Following production of transgenic plants, the putative transgenic lines were tested for the presence of transgene using PCR (Fig. 3A). Each PCR positive transgenic line was subsequently acclimated and hardened in our greenhouses. To determine stable integration of transgene into the plant's genome, Southern blot analysis was performed on four randomly selected transgenic plants and a non-transgenic control using a DIG-labeled DNA probe corresponding to the *egfp* gene. Among the transgenic plants analyzed, line 1 had a single copy, two independent lines (lines 3 and 4) had three, and the other (line 2) contained five copies while the non-transgenic control did not show any bands. However, none of the transgenic lines exhibited transgene silencing as there was no visual difference in GFP expression in any of the transgenic plants. This suggested that even in plants with multiple copy numbers, there was no gene silencing as has been observed by Muskens et al. (2000).

Ex vitro micrografting is a useful technique when a population of clones is needed in a short period of time (Skaria, 2000). After acclimation, in vitro citrus grows slowly and requires time to establish a good root system before it can thrive. In order to rapidly multiply select transgenic lines, we modified the micrografting technique as described by Skaria (2000) to make it suitable for ex vitro micrografting of tender transgenic shoots. The use of wedge grafting and wrapping of union with nescofilm reduced mortality from 50% in initial studies without nescofilm to less than 5% with nescofilm. Nescofilm is waterproof and flexible. It is also self-adhering. This combination was found suitable since it provided a barrier that retained moisture in while allow-

ing gaseous exchange of oxygen (<http://www.karlan.com/images/pdf/NescofilmComparisonData.pdf>). The use of a 200-μL pipette tip as a "cap" provided the young bud with high humidity and prevented it from drying. The grafted plant was placed under low light and room temperature conditions for 2 weeks following grafting. Lower light and room temperature conditions proved ideal since unions healed better under low light levels. Stronger light levels coupled with higher temperature as that found under greenhouse conditions tend to dry out grafts quickly. After the graft union had healed, the plants were transferred to higher light conditions and the pipette tip removed, allowing the bud to establish and develop. Using this technique it was possible to generate plants that could be used for disease resistance studies within 4 to 6 months of grafting.

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