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Yield and Transformation Ability of Citrus Protoplasts Derived from Either Cell Suspension Cultures or Embryogenic Callus

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Protoplasts are plant cells that have had their cell walls enzymatically removed. Protoplast transformation incorporates recombinant DNA directly into the genome to produce genetically modified plants, circumventing the traditional *Agrobacterium*-mediated transformation. Citrus cell suspension cultures derived from totipotent embryogenic callus cells and grown in a liquid medium are commonly utilized as source explants for the isolation of citrus protoplasts. However, these rapidly dividing cells are prone to somaclonal variation and point mutations. The objective of this study was to compare yield and transformation efficiency of citrus protoplasts obtained from embryogenic cultures that were maintained on solid medium as callus cultures or in liquid medium as suspension cultures. Microscopic comparison of cells between the two cell culture types was also conducted. Four citrus cultivars were evaluated in this study: two sweet orange ('EV1' and 'Cara Cara'), one tangor ('W Murcott'), and one grapefruit ('Ray Ruby'). Protoplast yield was similar for cell suspension derived protoplasts subcultured on a 14-day interval and protoplasts derived from callus cultures subcultured on a bimonthly interval. The transient transformation rate was also not affected by either cell type or time of harvest in any cultivar. The protoplast transformation rate was not affected by the culture type. We conclude that embryogenic callus maintained on solid medium can be a viable alternative to suspension cultures for protoplast isolation and transformation.

Protoplast technology has been used as an effective cell manipulation tool for rapid germplasm improvement of citrus. Protoplasts are cells with the cell wall enzymatically removed that can regenerate into plants under optimum tissue culture conditions. In citrus, protoplasts have circumvented reproductive barriers by creating somatic hybrids between distantly related cultivars (Grosser et al., 2000). They have also proven to be a viable alternative to the conventional *Agrobacterium*-mediated transformation process through the direct incorporation of recombinant DNA into the citrus genome (Fleming et al., 2000; Guo et al., 2007). Citrus suspension cultures derived from totipotent embryogenic callus cells and grown in a liquid medium are commonly utilized as source explants for the isolation of citrus protoplasts (Fleming et al., 2000; Grosser and Gmitter, 1990; Guo et al., 2005; Kobayashi et al., 1985). Embryogenic callus is obtained from the in vitro culture of unfertilized ovules and maintained in a semi-solid Murashige and Tucker based medium (Vardi et al., 1990; Vardi et al., 1982).

Kobayashi and others (Kobayashi et al., 1985) reported that protoplast yields were higher when obtained from embryogenic callus cultures grown in a liquid medium rather than from callus cultures on solidified medium. However, the cultivar used in their study differs from the ones used in this study. Additionally, there are differences in the protoplast isolation method between that study and ours. Since these early protoplast manipulation experiments, cell suspension cultures have been used primarily as a source of isolating protoplasts. However, these rapidly dividing cells can be prone to somaclonal variation and point mutations (Evans et al., 1984) and can exhibit genetic and epigenetic instability (Tanurdzic et al., 2008). Somaclonal variation has been a primary source of genetic variation in citrus. These variants have been exploited in citrus improvement programs for the development of improved sweet orange cultivars (Grosser, 2017). However, somaclonal variation is not desirable when true to type plants are required (Krishna et al., 2016). Moreover, the maintenance of these rapidly dividing cell suspensions is labor intensive, as these cells require subculturing on specialized liquid medium on a 10–14 day interval, are sensitive to temperature fluctuations and require care to prevent being contaminated (Lambardi et al., 2008). Older cell suspensions frequently result in plants that demonstrate morphological and reproductive abnormalities (Bhatti et al., 1997; Harding, 1996; Shillito et al., 1989). These complications limit the use of liquid cell suspension cultures in many laboratories wanting to do protoplast manipulation and transformation experiments. In contrast, callus cells grow more slowly and can be maintained for 1-2 months before being subcultured on a semi-solid medium and are relatively more stable with less epigenetic changes than the rapidly dividing cell suspensions.

Here, we compare the protoplast yield and transient expression ability of citrus protoplasts obtained from either the rapidly dividing liquid medium derived cell suspension cultures or from the relatively slower embryogenic callus cultures growing on a semi-solid agar medium. Protoplast transformation is becoming increasingly popular as a tool to rapidly transform and evaluate citrus cells in genetic manipulation experiments. Results of this study would be helpful in deciding the reliability of callus cells as a direct source for obtaining protoplasts.

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Materials and Methods

Plant materials. Four citrus cultivars were evaluated in this study—two sweet orange, *Citrus sinensis* ('EV1' and 'Cara Cara'), one tangor, *C. reticulata* ×*sinensis* ('W Murcott') and one grapefruit *C.* ×*paradisi* ('Ray Ruby'). Callus lines from each of these cultivars were initiated during Oct. to Dec. 2016 from unfertilized ovules as described previously (Grosser and Gmitter, 1990). Embryogenic callus cultures were maintained in semi-solid EME medium supplemented with 5 mg·L^{-1} kinetin and subcultured at a 60-day interval onto fresh medium. One gram of embryogenic callus was seeded into 25 mL of modified H+H liquid medium in a 125-mL sterile polycarbonate Erlenmeyer flask with a vented (0.22 μm PTFE pore) screw closure (ThermoFisher Scientific, Waltham, Mass.) to produce the cell suspension cultures used in this study. The H+H medium described by Grosser and Gmitter (1990) was utilized in this study and modified by increasing the amount of sucrose from 35 g·L^{-1} to 50 g·L^{-1}, and with the addition of 100 mg·L^{-1} myo-inositol and 500 mg·L^{-1} MES to the base medium. The autoclaved medium was filter sterilized through a 0.2 μm filter to remove suspended particles. Cell suspensions were subcultured on a bi-weekly interval. For our protoplast transformation studies, these cells were evaluated on the seventh and tenth days following subculturing while callus cells were evaluated at one and two months after subculturing.

Protoplast isolation and transformation. One gram each of callus or cell suspension cultures was used for protoplast isolation as described by Grosser and Gmitter (1990). Protoplasts were isolated from the same weight of cells either suspended in the liquid suspension media or from callus proliferating in semi-solid medium. Protoplast isolation time was determined based on prior observations and isolation times differed between fast growing cell suspension liquid cultures and the relatively slow growing callus cultures. Protoplast yield was calculated with a hemacytometer as number of protoplasts/mL of the solution for both callus and cell suspension derived protoplasts of each cultivar. Protoplast transformation was carried out using a cationic-lipid transfection reagent (Lipofectamine LTX with Plus ™ reagent; ThermoFisher Scientific, Waltham, Mass.). Isolated protoplasts were resuspended in 1:1 (v:v) protoplast solution (Grosser and Gmitter, 1990) at 2 × 10^6 protoplasts/mL and plated in Thermo Scientific™ BioLite 6-well plates. The LTX reagent (18 μL) was diluted 10-fold in the 1:1 protoplast medium. A mixture of 90 μL plasmid DNA (1 μg·μL^{-1}) was diluted in an equal amount of the Plus™ reagent (total 180 μL) and added to 180 μL of the LTX reagent. The plasmid DNA (pUER-EGFP) contained an endoplasmic reticulum targeted *egfp* gene driven by a 35S promoter. This mixture was incubated for 10–12 min at room temperature for DNA lipid complex (lipoplex) formation. This DNA-lipid complex was mixed with the isolated protoplasts and kept in dark for 48 h.

Fluorescence microscopy. Visualization of EGFP expression to assess transformation efficiencies was performed using a Carl Zeiss Axio Scope A1 fluorescent microscope with a Rhodamine filter (Ex: BP 545/25, Em: BP 525/50) (Carl Zeiss Microscopy GmbH, Gottingen, Germany). Images were captured using a Zeiss ICc1 Axio camera.

Scanning electron microscopy. Callus cells were fixed in 4% paraformaldehyde overnight at 4 °C. Cells were rinsed three times in 1xPBS before dehydration using an ethanol series (30%, 50%, 70%, 80% 95%, 100%). Cells were rinsed in three washes of 100% ethanol. Capsules were prepared by placing a mesh with a small pore size on one end and the capsules were submerged in 100% ethanol. Cells were placed inside the capsules and a cap with matching mesh was secured on top. Capsules were placed in baskets. Samples were critical point dried using a Ladd 28000 critical point dryer (Ladd Research Industries, Williston, VT), mounted on stubs on a double-sided 12 mm Carbon sticker (Electron Microscopy Sciences, Hatfield, PA), and sputter coated with palladium/gold using a Ladd 30800 sputter Coater (Ladd Research Industries, Williston, VT). Samples were observed, and images were captured using a Hitachi S4000 SEM (Hitachi, Tokyo, Japan).

Statistical analyses. Statistical analyses were performed using JMP® Pro version 13.2.0 (SAS Institute Inc.). Analysis of variance (ANOVA), followed by post hoc pairwise comparisons with the Tukey-Kramer honestly significant different test (Tukey HSD) was used to compare the protoplast yield of each citrus cultivar at different time periods.

Results and Discussion

Protoplast transformation has several advantages over *Agrobacterium* mediated transformation. It is not time limited and provides a seemingly unlimited supply of cells for transformation throughout the year. Additionally, bypassing the use of *Agrobacterium* allows the transformation of citrus cultivars that are recalcitrant to *Agrobacterium* infection. Direct gene transfer using the protoplast system also allows for quick transient gene expression studies and can potentially allow for linear DNA incorporation into the genome (Davey et al., 2005). In this study, the protoplast yield and transient transformation efficiency from callus and cell suspension cultures were studied. Our results suggested that both were comparable for each cultivar within their respective growth times.

Protoplast yield from the 'W Murcott' cells ranged from 3.2 × 10^6 cells in the one-month-old callus cultures to 2.5 × 10^6 cells in the two-month-old callus cultures. The yield of both suspension cultures (7 day and 10 day) were in-between this range (Fig. 1). A similar trend was also observed in 'Cara Cara'. Protoplast yield in the 'EV1' cells ranged from 2.8 × 10^6 cells in the seven-day old suspension cells to 1.9 × 10^6 cells in the two-month-old cal-

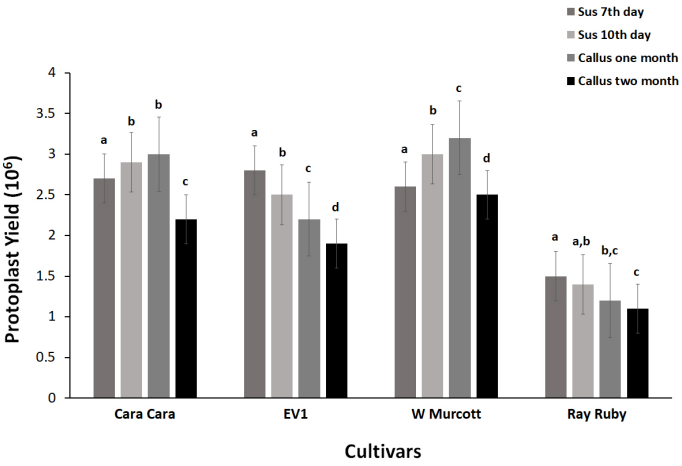

Fig. 1. The average protoplast yield for cell suspensions and callus cultures at different time points. Protoplast yield is the number of viable protoplasts per gram of cells. Statistical significance is represented by letters. Those that share the same letter were not statistically significant from one another.

lus cells. 'Ray Ruby' cells produced the lowest protoplast yield with the most being produced from the seven days old suspension cells (1.5×10^6 cells). Most of the suspension cells are usually in the S-phase of mitotic cycle within 7 days from subculture and optimum amounts of protoplasts can be isolated then (Dutt et al., 2010; Zhang et al., 2007). However, it is evident from our results that the 7- to 10-day window can provide adequate protoplast yield from cell suspensions of most cultivars. Protoplast yields could be directly correlated with the growth of cell suspension cultures in *Solanum chilense* (*syn*. *Lycopersicon chilense*) in which the highest cell growth was observed 8 days after subculture (Patil et al., 2003). Similar observations have also been made in other suspension cell systems (Hakman and von Arnold, 1988; Taylor et al., 1992; Vasil and Vasil, 1982) where higher protoplast yields were usually obtained from homogeneous cell suspension cultures (Taylor et al., 1992). Our suspension cells were compact, richly cytoplasmic with starch granules compared to the more loosely clumped and friable callus cells similar to that observed earlier by others (Pérez et al., 1998; Vardi et al., 1982). It is indeed possible that cellular density could have played an important role in the ability of the suspension cells to provide more protoplasts when compared to a similar amount of the callus.

Observations were made to examine the morphology of suspension cells derived from 'Cara Cara' and 'Ray Ruby' since there were significant differences in their protoplast yields. We observed 'Cara Cara' cells derived from suspension cultures (Fig. 2 A and C) were smaller in size and aggregated in small clumps compared to cells derived from 'Ray Ruby' suspensions (Fig. 2 B and D). When compared at a higher resolution, majority of 'Ray Ruby' clumps consisted of larger cell aggregates that were multiple cells deep (Fig. 2 E) compared to the smaller 'Cara Cara' clumps that appeared smoother with very few cell clusters (Fig. 2 F). The protoplast yield could be correlated to the physical morphology of the cellular clumps since the smaller 'Cara Cara' clumps would be more amenable to digestion with the protoplast enzyme solution than the corresponding larger clumps. Protoplast yield can also be affected by cells with a high starch content that can result in higher levels of protoplast breakage (Grosser and Gmitter, 2011). Future studies will evaluate the long-term yield from these cells.

Protoplast transformation efficiency could not be correlated to the protoplast yield in our current study. We obtained protoplasts of varying sizes from each of the cultivars (Fig. 3). While a lower protoplast yield resulted in a lower rate of transformation ('Ray Ruby'), there was no statistical difference between the protoplasts obtained from the different cell type and time for each cultivar (Table 1). All protoplasts were viable transformants indicating that the transformation efficiency was dependent on the cultivar transformed as indicated by EGFP expression (Fig 4A). We used a cationic-lipid transfection reagent to aid in integrating DNA in the nucleus, which is different from the conventional PEG-mediated method used for citrus transformation (Fleming et al., 2000; Guo et al., 2005). Transfection reagents are designed to escape the endosomal pathway and degradation by nucleases and allow the DNA to be more efficiently transported into the nucleus (Khalil et al., 2006; Varkouhi et al., 2011). These transfection reagents

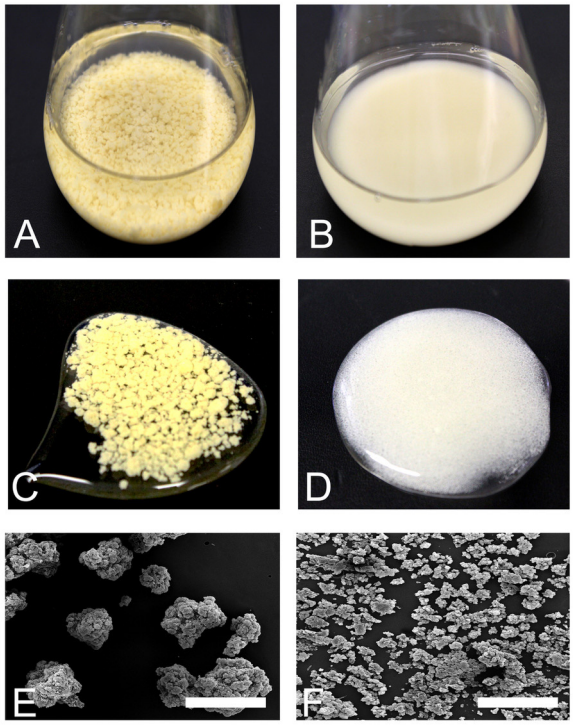

Fig. 2. Variations in texture, shape and size as observed between the cell suspension cultures of *Citrus* ×*paradisi* 'Ray Ruby' (**A, C, E**) and *C. sinensis* 'Cara Cara' cultivars (**B, D, F**). Cell suspensions of 'Ray Ruby' (**A**) and 'Cara Cara' (**B**) as observed in the 125 mL Erlenmeyer flask; close-up of the 'Ray Ruby' (**C**) and 'Cara Cara' (**D**) cell clumps; Scanning Electron Microscopy (SEM) of the 'Ray Ruby' (**E**) and 'Cara Cara' (**F**) cell clumps. Bar indicates 1 mm length.

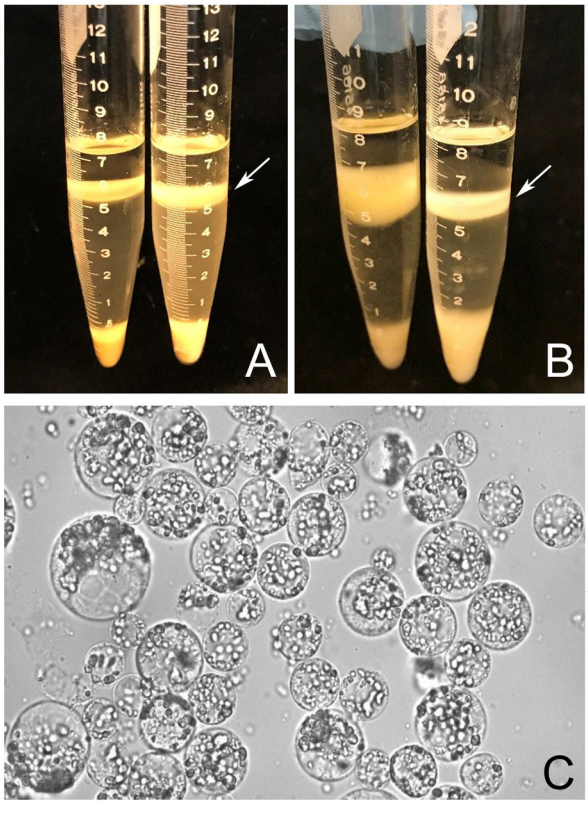

Fig. 3. Representative protoplast rings of *Citrus reticulata* ×*sinensis* 'W Murcott' from the different samples. Protoplast ring obtained from a seven-day suspension (left) compared to ten-day suspension cells (right) (**A**) and one-month-old callus (left) compared to two-month-old callus (right) (**B**). Arrow indicates the protoplast ring. An image of the isolated protoplasts from 'W Murcott' (**C**). Cells were observed to be heterogeneous in size.

Table 1. The mean number and standard deviation of GFP positive protoplasts derived from cells in suspension and callus showed that the transformation efficiency was similar for their respective cultivars, two sweet orange, *Citrus sinensis* ('EV1' and 'Cara Cara'), one tangor, *C. reticulata* ×*sinensis* ('W Murcott') and one grapefruit *C.* ×*paradisi* ('Ray Ruby'). The mean number of EGFP cells were observed at seventh- and tenth-day cell suspension cultures and one- and two-months callus cultures for each cultivar.

	Mean number of EGFP positive cells observed after transformation			
	Suspension culture		Callus culture	
Cultivar	Day 7	Day 10	1 month	2 months
Cara Cara	20 ± 1.7	19 ± 2.1	21 ± 1.4	20 ± 2.8
EV1	16 ± 1.7	18 ± 1.1	15 ± 2.8	16 ± 1.7
W. Murcott	16 ± 3.3	18 ± 1.7	13 ± 3.5	15 ± 0.8
Ray Ruby	6 ± 0.8	4 ± 1.7	8 ± 0.7	9 ± 2.8

spontaneously make complexes with the plasmid DNA when they are simply mixed via electrostatic interactions between the negatively charged DNA and the positively charged head group of lipid reagent (Bhatia, 2016; Felgner et al., 1994). Subsequent release of the plasmid DNA from the complex is required in order to be expressed in the nucleus (Okuda et al., 2004; Xu and Szoka, 1996). Although transient expression is usually several fold higher than stable gene expression, we observed several EGFP positive cells in a state of mitotic division (Fig. 4) that could indicate stable transgene incorporation. However, as this study was restricted to transient gene expression, we cannot conclude how these cells will behave following transfer to somatic embryogenesis medium.

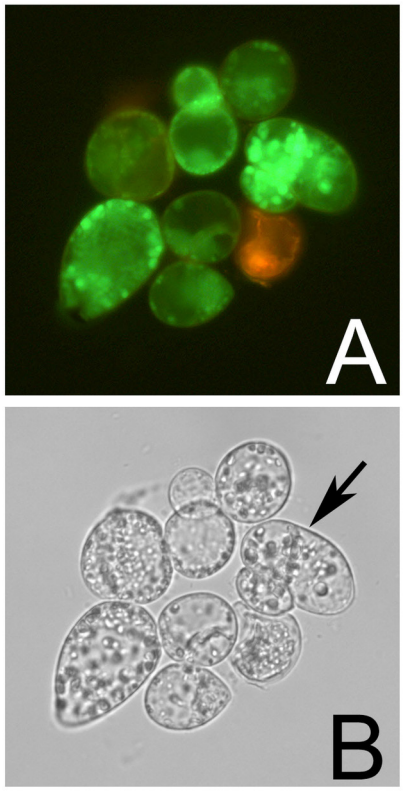

Fig. 4. Transformed *Citrus reticulata* ×*sinensis* 'W Murcott' protoplasts expressing EGFP (**A**) with the same group of cells photographed under Brightfield light (**B**). Arrow indicates a group of mitotic cells.

Conclusions

Citrus protoplasts derived from either suspension cells or callus from each of the four cultivars could be transformed utilizing our modified protoplast transformation technique. The protoplast yields obtained from either suspension cells or callus were comparable for each cultivar, however the transformation efficiency was dependent on the cultivar. While cell suspensions could result in a high protoplast yield, they also require a more rigorous maintenance schedule, whereas the callus cultures require less maintenance. Callus cultures may be a better option for research programs that do not have the time and man power that it takes to maintain cell suspensions or where there could be concerns with the development of somaclonal variations and point mutations occurring due to the rapid rate of cell division as observed in the suspension cells.

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