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Preparation and Fusion of *Citrus* sp. Microprotoplasts

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Abstract. Large-scale production of microprotoplasts from 'Ruby Red' grapefruit (*Citrus paradisi* Macf.) and from the *Citrus* L. sp. relative *Swinglea glutinosa* (Blanco) Merr., was performed after treatment of suspension cells with APM. An average of 75.2% of the microprotoplasts contained a single chromosome, followed by 17.1% with two, 4.6% with three, and 2.0% with four. Only 1.1% had more than five chromosomes. Maximum chromosome number observed was eight and the average yield was 2×10^6 of total microprotoplasts per gram of suspension cells. Flow cytometry analysis confirmed low DNA content. The polyethylene glycol fusion method was used to fuse microprotoplasts from 'Ruby Red' grapefruit with protoplasts of 'Succari' sweet orange [*Citrus sinensis* (L.) Osbeck], and microprotoplasts from *S. glutinosa* with protoplasts from sour orange (*C. aurantium* L.). Embryos or suspension cells from the recipient species with a few additional chromosomes were obtained; however, embryogenesis of the fusion products was reduced or inhibited. Chemical name used: amiprophos-methyl (APM).

Using conventional breeding to develop scion cultivars of *Citrus sinensis* (sweet orange), *Citrus paradisi* (grapefruit), and *C. limon* L. Burn. (lemon), the most important citrus species, is virtually impossible due to the prevalence of nucellar embryony in these species. This asexual form of reproduction inhibits development of large populations for recombination and selection of superior genotypes (Frost and Soost, 1968). Additionally, they are not true biological species; each originated as unique individual interspecific hybrids, and cultivars within each group arose as somatic mutations (Barret and Rhodes, 1976; Bowman and Gmitter, 1990; Scora, 1975). Consequently, there is no genetic diversity per species and a fragile situation is created with respect to biotic stresses.

Advances in plant tissue culture and molecular biology have led to alternative methods for gene transfer in citrus. Plant transformation has been accomplished successfully and plants stably expressing alien genes have been produced (Bond and Roose, 1998; Dominguez et al., 2000; Gutierrez et al., 1997; Yang et al., 2000). The availability of horticulturally important genes, however, is currently scarce. Protoplast fusion methodology has been very successful in citrus, and several somatic hybrids have been produced (Grosser et al., 1998a, 1998b; Louzada et al., 1992, 1993). Even though such hybrids have great potential for rootstock improvement, they may not have direct application as scion cultivars. Thus, there is a need for a new technology capable of transferring a small portion of the genome from one species to another, in one step.

Microcell mediated chromosome transfer (MMCT), developed for mammalian cells (Fournier and Ruddle, 1977) provides a direct way of transferring a single whole chromosome from a donor to a recipient cell. MMCT eliminates dependence on meiosis, reducing the possibility of recombination and allows production of combinations between sexually incompatible species (Kozac et al., 1979). MMCT greatly facilitated mapping of genes and arbitrary DNA sequences to a specific chromosome in the human genome (Johnson-Pais and Leach 1996), and it has been a powerful tool to study regulation of gene expression in human somatic cells (Thayer, 1996).

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Even though the effect of APM in plant chromosomes has been studied for more than a decade, its application to MMCT in plants has been very slow. Morejohn and Fosket (1984) reported disruption of the microtubule dynamics of rose (*Rosa* sp.) cells after treatment with APM. De Laat et al. (1987) observed that treatment of *Nicotiana plumbaginifolia* Viv. suspension cells with APM induced scattering of chromosomes and development of micronuclei. Ramulu et al. (1988) described formation of micronuclei in irish potato (*Solanum tuberosum* L.), carrot (*Daucus carota* L.), and *Haplopappus gracilis* Nutt. after treatment of cell suspensions with APM. More recent studies have lead to isolation *N. plumbaginifolia* microprotoplasts after treatment with APM (Verhoeven and Ramulu, 1991) and synchronization of the cell cycle with hydroxyurea (Ramulu et al., 1993). Sequential filtration of microprotoplasts through nylon sieves of decreasing pore sizes (48-20-15-10-5 μm) was beneficial for producing large quantities of microprotoplasts containing one to four chromosomes (Ramulu et al., 1993). Adaptation of the MMCT procedure to plant cells culminated with production of hybrid plants containing one chromosome of Irish potato plus the complete set of chromosomes of tobacco (*N. Tabacum* L.) or tomato (*Lycopersicon esculentum* Mill.), and tomato plants containing a chromosome from *N. plumbaginifolia* (Ramulu et al., 1995). Recently, Binsfeld et al. (2000) used MMCT to produce common sunflower (*Helianthus annuus* L.) plants with extra chromosomes of giant sunflower (*H. giganteus* L.) or Maximilian sunflower (*H. maximiliani* Schrad.) This paper reports a detailed procedure for mass isolation of microprotoplasts from 'Ruby Red' grapefruit and from the wild citrus relative, *Swinglea glutinosa*, and their use in chromosome transfer experiments.

Materials and Methods

Cell culture. Suspension cells of 'Ruby Red' grapefruit, 'Succari' sweet orange, and *S. glutinosa* were produced from callus provided by J.W. Grosser, Citrus Research and Education Center, University of Florida, Lake Alfred. Cells were cultured at 27 °C in half-strength liquid H+H medium (Grosser and Gmitter, 1990) on a gyratory shaker (130 rpm) under two growth lux lamps of 20 W each (GE Lighting, Nela Park, Cleveland, Ohio) and continuous light. Cells used to produce microprotoplasts were subcultured every 3 to 4 d to maintain logarithmic growth as suggested by

Verhoeven and Ramulu (1991), while the ones used for protoplasts were subcultured on a 14-d cycle.

Isolation of microprotoplasts. Early log-phase suspension cells (1.0 g fresh weight drained) of 'Ruby Red' grapefruit and *S. glutinosa* were harvested 1 d after subculture and treated with a fleshly prepared solution of 10 mM hydroxyurea (HU) for 24 h. Nontreated suspension cells were used as a control. Treated and control cells were washed four times with H+H medium (Grosser and Gmitter, 1990) and incubated with 0, 16, 24, 32, or 48 μM APM (Bayer Corp., Agr. Division, Kansas City, Mo.) for 24 h. After the initial APM treatment, suspension cells were incubated for 24 h in a cell wall digesting mixture containing equal parts of enzyme solution and 0.6 M BH3 medium (Grosser and Gmitter, 1990) supplemented with 0, 16, 24, 32, or 48 μM APM, and 20 μM cytochalasin B (CB-Sigma, St. Louis). Protoplasts were then filtered through a 45-μm stainless steel mesh screen and pelleted at 100 g_n to remove the enzyme and purified by centrifugation through a 25% sucrose–13% mannitol gradient and washed once with 0.4 M mannitol. During the protoplast manipulations, all solutions contained 0, 16, 24, 32, or 48 μM APM, and 40 μM CB. A continuous isoosmotic gradient of percoll was prepared by adding 7.2% (w/v) mannitol to a percoll solution (Amersham Pharmacia Biotech, Piscataway, N.J.) followed by centrifugation for 30 min at 100,000 g_n in a swinging bucket rotor (SW 41 Ti; Beckman Instruments, Inc., Fullerton, Calif.). The protoplast suspension was layered in the preformed gradient and centrifuged for 2 h using the same conditions. After centrifugation, the bands were collected in 15 mL of 0.6 M BH3 (Grosser and Gmitter, 1990) and filtered sequentially through a 20-μm nylon mesh screen (Small Parts, Inc., Miami Lakes, Fla.), 14-μm nucleopore membrane (Corning, Action, Mass.), and a 10- and 5-μm nylon mesh screen (Small Parts, Inc.). Filtration was performed by gravity flow. If necessary, a light pressure was applied. Microprotoplasts were collected by two rounds of centrifugation at 80 g_n, and 100 g_n using a table top centrifuge and resuspended in an appropriate volume of 0.6 M BH3 medium (Grosser and Gmitter, 1990). Microprotoplasts were stained with one drop of acridine orange solution at 10 $\mu g \cdot mL^{-1}$ and checked for integrity under fluorescence. To determine yield, microprotoplasts were counted under a light microscope using a hemacytometer.

Protoplast isolation, fusion, and culture of fusion products. Protoplasts were isolated from 'Ruby Red' grapefruit and 'Succari' sweet orange embryogenic suspension cells, and from sour orange leaves according to Grosser and Gmitter (1990). Fusion of protoplasts and microprotoplasts was performed by the polyethylene glycol method (Grosser and Gmitter, 1990) in a ratio of 1 protoplasts : 3 microprotoplasts. The following fusion combinations were performed: 'Ruby Red' grapefruit microprotoplasts with 'Succari' sweet orange protoplasts and *S. glutinosa* microprotoplasts with sour orange protoplasts. The fusion combination of 'Ruby Red' grapefruit microprotoplasts with 'Succari' sweet orange protoplasts was cultured in the dark on 0.6 M EME medium (Grosser and Gmitter, 1990) and *S. glutinosa* microprotoplasts with sour orange protoplasts was cultured in 0.6 M BH3 medium (Grosser and Gmitter, 1990). Osmolarity reduction was performed by adding a few drops of 0.6 M BH3 + 0.6 M EME + 0.15 M EME in the ratio of 1:1:1 (v/v) when microcalli were at the five-cell stage. A second osmolarity reduction was performed 4 weeks later by adding a few drops of 0.6 M BH3 + 0.15 M EME in the proportion of 1:2 (v/v). Resulting embryos were transferred to solid 0.15 M EME medium for further growth, and then to B germination medium (Grosser and Gmitter, 1990). In instances when embryos fail to form, suspension cell cultures were established with the fusion products and maintained in log growth as described previously. Protoplasts from the recipient species and microprotoplasts from the donor species were grown individually for comparison.

Cytology. For chromosome scattering and micronuclei visualization, APM-treated suspension cells were treated for 2 h with 1,4-dicholorobenzene (Aldrich, Milwaukee, Wis.) followed by fixation in 3 ethanol : 1 acetic acid (v/v) solution for 24 h and digested with 1 M HCl at 60 °C for 10 min. Digested cells were washed in phosphate-buffered saline (PBS, pH7.4), stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) at 0.4 $\mu g \cdot mL^{-1}$ and visualized under a fluorescence microscope (Olympus America Inc., Melville, N.Y.) using a B filter (bright lines at 405 and 435 nm). Chromosome counts of the fusion products were performed in suspension cells or in embryo roots using the same method.

Protoplasts and microprotoplasts were treated with 12% formaldehyde in 0.2 M phosphate buffer (pH 6.0) for 4 h, followed by washing and fixation with a 3 ethanol : 1 acetic acid (v/v) solution for 24 h (Ramulu et al., 1993). Chromosome counts were performed by staining protoplasts and microprotoplasts with DAPI at 0.4 $\mu g \cdot mL^{-1}$. The mounted slides were partially dehydrated and visualized under fluorescence with B filter. Chromosome counts were performed in three independent microprotoplasts isolation experiments, before and after sequential filtration, with five slides per sample. Only slides with well spread microprotoplasts or protoplasts were counted. An average of 100 microprotoplasts were counted per slide. A visual scan of the whole slide was made to search for microprotoplasts with more than four chromosomes.

Flow cytometry. Fixed nuclei were prepared according to Yu et al. (1993) from a suspension of protoplasts or microprotoplasts with a density of 1×10^6 mL^{-1} and stained with of propidium iodide at 150 $\mu g \cdot mL^{-1}$. Nuclei suspensions were aspirated with a needle to break clumps, and diluted with isoflow sheath fluid (Beckman Coulter, Inc., Fullerton, Calif.) before analysis. Flow cytometry was performed using a flow cytometer (FACScan; Becton, Dickinson and Co., Franklin Lakes, N.J.) and the signals were gated to eliminate debris. Diploid nuclei were analyzed for comparison.

Results and Discussion

To study the feasibility of MMCT as a breeding method for citrus we examined the microprotoplast isolation procedure using cell suspensions of 'Ruby Red' grapefruit and *S. glutinosa*. Considering that Ramulu et al. (1993) obtained large quantities of microprotoplasts from a cell suspension of *N. plumbaginifolia* using 32 μM of APM, we evaluated concentrations of 0, 16, 24, 32, or 48 μM of APM with and without synchronization of the cell cycle by 10 mM of HU. APM and APM + HU treated suspension cells had cells with chromosomes scattered in groups of one or more (Fig. 1); however, most were dispersed as one chromosome. No chromosome scattering was observed in the control (HU nontreated). Multinucleated cells (Fig. 2) were observed in all the treatments containing APM, however, it was more easily observed after incubation with APM and cytochalasin B plus cell wall digesting solution.

Following high-speed centrifugation, nine or less bands were observed in gradients, depending on the amount of protoplasts loaded initially, the concentration of APM, and the synchronization of the cells by HU. The banding pattern was directly correlated with final yield. All treatments without HU had very poor banding formation and a negligible amount of microprotoplasts were produced. From the treatments with cell synchronization, 24 and 32 μM of APM had the best banding formation. Treatments of 16 or 48 μM APM + HU had thinner bands and low yield. The best microprotoplast

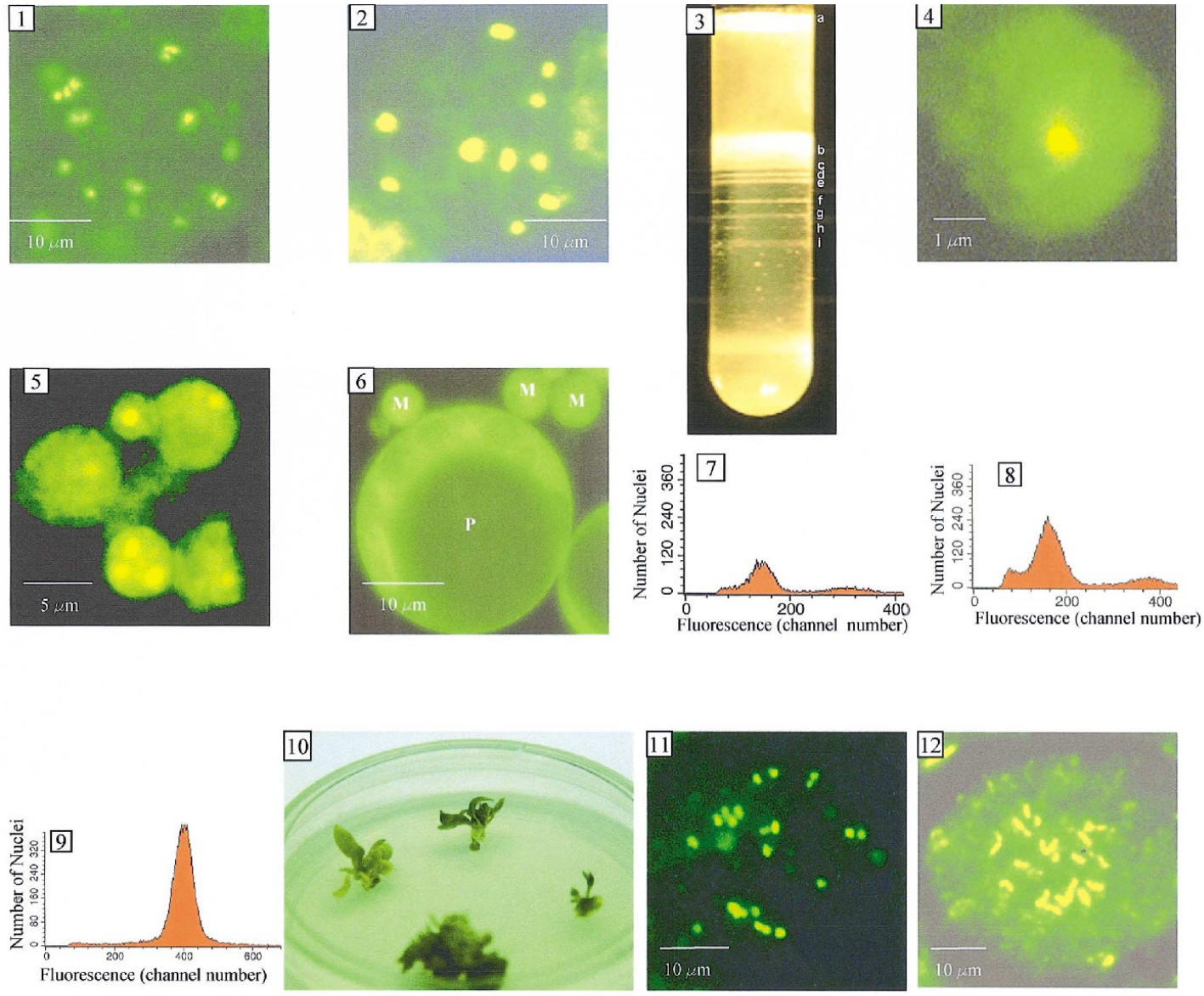

Fig. 1. Diploid cell (2n = 18) of *Swinglea glutinosa* with chromosomes scattered.

Fig. 2. Multinucleated protoplast of 'Ruby Red' grapefruit.

Fig. 3. Isoosmotic percoll-mannitol gradient after centrifugation at 100,000 g_n showing separation of microprotoplasts. Band a contains sticky material, bands b–i contain microprotoplasts of different sizes.

Fig. 4. Microprotoplasts containing a single chromosome.

Fig. 5. Microprotoplasts with one or three chromosomes.

Fig. 6. Size comparison of protoplast (P) and microprotoplast (M).

Fig. 7. Histogram of nuclear DNA content of *Swinglea glutinosa* microprotoplasts.

Fig. 8. Histogram of nuclear DNA content of 'Ruby Red' grapefruit microprotoplasts.

Fig. 9. Histogram of nuclear DNA content of *Swinglea glutinosa* protoplast.

Fig. 10. Embryos from 'Ruby Red' grapefruit + 'Succari' sweet orange attempting germination.

Fig. 11. Cell from 'Ruby Red' grapefruit + 'Succari' sweet orange with 22 chromosomes.

Fig. 12. Cell from *Swinglea glutinosa* + sour orange with 22 chromosomes.

yield occurred for cells treated with 32 μM APM + HU, therefore we selected this treatment as the optimum. No differences were observed between the two species studied. We performed at least 10 more microprotoplast isolations for the two species using the APM concentration selected and the yields were always very consistent in the range of 2×10^6 microprotoplasts per gram of suspension cells.

Band a, formed at the surface of the gradient, and contained a sticky material with the appearance of plastid (Fig. 3). When collected together with the aforementioned bands, it contaminated the microprotoplast suspension. Part of the material could be removed with the tip of a Pasteur pipette or a glass rod, and the other bands could then be collected. Band a was stickier in the *S. glutinosa* microprotoplast isolation. Band b was very thick and contained a large amount of microprotoplasts of various sizes, and it was always collected together with band c, which was also rich in microprotoplasts. The volume of these two bands was diluted with 15 mL of BH3 medium (Grosser and Gmitter, 1990) and used for mass production of microprotoplasts. The percoll precipitated to the bottom of the tube.

The original method described by Ramulu et al. (1995) used 0.4

M mannitol to dilute the bands, however, this did not work for citrus. The microprotoplasts could not be precipitated when using 0.4 M mannitol or when a large volume of 0.4 M mannitol from the gradient was collected with the bands. At times a gelatinous material was present in band b; nonetheless, it did not interfere with the isolation, since it decanted to the bottom of the tube (Fig. 3). Bands d to i were composed primarily of very small, clean microprotoplasts; however, it was difficult to precipitate these due to the large volume of mannitol collected. When bands d and e were dense enough, they were collected together with b and c, and diluted in a higher volume of BH3 medium (Grosser and Gmitter, 1990). When bands b to g were collected together, the volume of mannitol collected was too high, which prevented precipitation of the microprotoplasts. Since bands b and c would produce enough microprotoplasts, we preferred, in most cases, to work only with these bands. We attempted initially to filter sequentially the microprotoplasts through Nucleopore membranes (Corning) of 14, 8, and 5 μm, to enrich the suspension with small microprotoplasts. However, the yield was low due to the high pressure needed mainly with the 8 and 5 μm membranes which damaged the microprotoplasts. The cloth screen of 20, 10 and 5 μm with the 14-μm Nucleopore membrane used after the 20-μm screen was very efficient for purifying the microprotoplasts without applying high pressure. After filtration, centrifugation, and resuspension in BH3 medium (Grosser and Gmitter, 1990), the microprotoplast suspension was very clean and contained high numbers of microprotoplasts with single chromosomes (Figs. 4 and 5).

Chromosome counts before and after filtration of microprotoplasts from the treatment containing 32 μM APM + 10 mM HU revealed that most of the microprotoplasts contained a single chromosome (Figs. 4 and 5). Before filtration, 57.5% contained one chromosome, 22.3% had two, 8.0% had three, 4.7% had four, and 7.5% had more than five chromosomes, with a maximum of 12 chromosomes. After filtration, 75.2% contained one chromosome, 17.1 % two, 4.6% three, 2.0% four, and 1.1% contained more than five chromosomes with a maximum of eight. All the slides were scanned visually over the whole surface in an attempt to find microprotoplasts with more than four chromosomes. A comparison of sizes between microprotoplasts and protoplasts is illustrated in Fig. 6.

To confirm the low chromosome number in the microprotoplasts, flow cytometry analysis was performed in nuclei from microprotoplasts and compared with nuclei from diploid 'Ruby Red' grapefruit and *S. glutinosa*, which contains 18 chromosomes. The chromosome number from the diploid was confirmed by cytology (unpublished data). Under fluorescence microscopy we observed that nuclei from microprotoplasts were very small and had a tendency to clump in groups of three or more. Flow cytometry analysis confirmed the low DNA content of microprotoplasts from *S. glutinosa* (Fig. 7) and 'Ruby Red' grapefruit (Fig. 8) with peaks below channel 200. The G1 peak for intact protoplasts had peaks in channel 400 (Fig. 9).

Microprotoplasts produced from 'Ruby Red' grapefruit were used in fusion experiments with protoplasts of 'Succari' sweet orange, while microprotoplasts from *S. glutinosa* were fused with protoplasts of sour orange. The fusion products from all combinations grew faster than nonfused protoplasts cultured separately, while the microprotoplasts cultured separately did not grow. Protoplasts isolated from sour orange leaves did not divide, whereas protoplasts from 'Succari' sweet orange cultured in 0.6 M EME medium (Grosser and Gmitter, 1990) divided but did not produce embryos. The protoplast culture of 'Ruby Red' grapefruit produced normal embryos and regenerated into plants.

The combination of 'Ruby Red' grapefruit microprotoplasts with 'Succari' protoplasts produced three embryos. When transferred to germination medium, they failed to regenerate into plants, even though the embryos grew and produced secondary embryos (Fig. 10). One of the embryos produced roots. The chromosome number was determined to be 22; four extra chromosomes compared to 18 found in 'Succari' (Fig. 11). The embryogenic capacity of sweet orange is known to be inhibited in media with concentrations of sucrose higher than 0.3 M (Ohgawara et al., 1985) such as 0.6 M EME medium (Grosser and Gmitter, 1990). Therefore, it was expected that no embryos should be formed unless fusion with the other parent occurs, since the fusion combination was cultured in 0.6 M sucrose. For the *S. glutinosa* + sour orange combination, no cell division should take place unless the chromosome from *S. glutinosa* was transferred to sour orange because sour orange leaf protoplasts do not divide by themselves nor do the microprotoplasts. Use of nonregenerative mesophyll protoplasts in combination with protoplasts from an embryogenic source for fusion experiments has been a widely used strategy in citrus (Grosser et al., 1996; Louzada et al., 1992, 1993). More than 100 somatic hybrids of citrus have been reported to date, and most were produced using this strategy. In a few cases where diploid plants were obtained from the mesophyll protoplast parents they were characterized as cybrids using molecular techniques (Grosser et al., 1996). Several attempts in our laboratory to produce callus from mesophyll protoplasts cocultured with embryogenic callus, suspension cells, or protoplasts from different citrus species failed (unpublished data), however, diploid cybrid callus has been produced previously from mesophyll protoplasts by protoplast fusion (Saito et al., 1994). Cytology performed for the callus and suspension cells produced from the combination *S. glutinosa* + sour orange revealed several cells with extra chromosomes (Fig. 12). It is very unlikely that the cells harboring extra chromosomes were from *S. glutinosa* since most of the microprotoplasts contain single chromosomes which would require the microprotoplasts to fuse in an orderly fashion to support cell division. Additionally, the microprotoplasts are probably devoid of cytoskeleton because the protoplasts used to produce them were treated with CB during the protoplast isolation and the percoll gradient. According to Ramulu et al. (1993) CB treatment destroys the protoplast cytoskeleton.

The reduction/inhibition of embryogenesis observed in the fusion combinations performed was probably induced by CB, because it was found that this substance has a negative effect on the regeneration of subprotoplasts (Lorz et al., 1981). Cytochalasin B is a very important component of the microprotoplast isolation, because it destroys the protoplast microfilaments releasing cleaner microprotoplasts with less adhering organelles (Verhoeven et al., 1991). We are currently testing different concentrations of CB, which could increase formation of clean microprotoplasts without interfering with plant regeneration.

The procedure of microprotoplast isolation and its use for transferring a limited number of chromosomes has great potential for creation of a new breeding method for citrus. Such a method would allow for production of a series of unique individuals from one fusion event. Considering that the haploid number of citrus is nine, at least nine different individuals could be produced. Adding the combinations of two by two, or three by three, or more different chromosomes, the amount of new hybrids that can be produced at one time is very high. Seedless cultivars could probably be produced by creating aneuploid hybrids with odd numbers of chromosomes. It is unlikely that the traits would be significantly altered if the donor and recipient are from the same species, and a few chromosomes are added. Additionally, hybrids of three cultivars could also be pro-

duced by fusion of microprotoplasts from two donor species with one recipient. This technique would create a breeding method of broad applicability for citrus.

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