Production of Transgenic Valencia Orange Suspension Cells to Be Used as Donors for Chromosome Transfer

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ABSTRACT

The method for plant transformation and antibiotic selection has been well established for several citrus varieties. Transgenic citrus suspension cells, however, have never been produced and maintained, thus, a procedure for transformation, antibiotic selection, and maintenance of transgenic suspension cells is still required. Transgenic citrus suspension cells containing the npt II gene for resistance to an aminoglycoside antibiotic would be very valuable for chromosome transfer experiments. To determine the lethal antibiotic concentration where growth inhibition of suspension cells would occur, the antibiotics kanamycin sulfate, geneticin, and paromomycin sulfate were used. Paromomycin sulfate at 50 µg/ml was found to be the best antibiotic for selection. Geneticin and kanamycin were not appropriate for selection of transgenic Valencia sweet orange suspension cells. For protoplast transformation, the electroporation parameters and incubation procedures were established. Pulses of 400, 500, and 700 V/cm were evaluated. The best electroporation settings were pulse of 500 V/cm and capacitance of 1000 µF. To secure high survival rates of the protoplasts, it was essential to add BH3 medium immediately after electroporation. Transgenic suspension cells of Valencia sweet orange containing the npt II and the ß-glucuronidase (Gus) gene were produced and maintained in 14 day subculture cycles in medium containing 50 µg/ml of paromomycin sulfate. This concentration was also very efficient for selection of transgenic Valencia embryos, since non-transgenic embryos were bleached.

RESUMEN

Existen métodos bien establecidos para la transformación de plantas y la selección con antibióticos en diferentes variedades de cítricos. Sin embargo, nunca se han producido y mantenido células en suspensión de cítricos transgénicos, por lo tanto, aun se requiere de un procedimiento para la transformación, selección con antibióticos, y mantenimiento de células transgénicas en suspensión. Las células en suspensión de cítricos transgénicos conteniendo el gen npt II para resistencia a un antibiótico aminoglicosídico serían muy valiosas para los experimentos de transferencia de cromosomas. Se utilizaron los antibióticos sulfato de kanamicina, geneticina y sulfato de paromomicina para determinar la concentración letal de antibióticos en la que ocurriría la detención del crecimiento de las células en suspensión. Se encontró que el sulfato de paromomicina a 50 µg/ml fue el mejor antibiótico para la selección. Ni geneticina o kanamicina fueron apropiados para la selección de células transgénicas en suspensión de naranja dulce Valencia. Se establecieron los parámetros de electroporación y procedimientos de incubación para la transformación de protoplastos. Se evaluaron pulsos de 400, 500, y 700 V/cm. Las mejores condiciones fueron pulsos de 500 V/cm y capacitancia de 1000 µF. Para asegurar altos niveles de supervivencia de protoplastos, fue esencial incorporar medio BH3 inmediatamente después de la electroporación. Se produjeron células transgénicas en suspensión de naranja dulce cultivar Valencia conteniendo npt II y el gen ß-glucuronidasa (GUS) en medio conteniendo 50 µg/ml de sulfato de paromomicina y se mantuvieron en ciclos de subcultivo de 14 días. Esta concentración fue también bastante efectiva para la selección de embriones transgénicos de naranja Valencia, ya que los embriones no transgénicos no desarrollaron y murieron.

Additional Index Key words: Electroporation, paromomycin, chromosome transfer

Conventional methods of plant breeding have been inefficient in producing new improved varieties of citrus due to the long juvenility period, high heterozygosity, sexual incompatibility, sterility and the high degree of apomixis in several species. Most of the currently grown citrus scion varieties originated as chance seedlings, limb or bud sport mutations (Hodgson 1967) or via induced mutation. Sweet oranges (*Citrus sinensis* (L) Osb.), and grapefruits (*Citrus paradisi* Macf.), the most important commercial species, are highly apomictic, producing one or several asexual embryos per seed. The sexual embryos in most cases die for lack of nourishment. Alternative breeding methods like somatic hybridization via protoplast fusion have been successful in citrus, and several somatic hybrids has been

produced (Louzada et al., 1992, 1993; Louzada and Grosser, 1994; Grosser et al., 1996). These somatic hybrids are very valuable for rootstock improvement, and as breeding parents in interploid crosses to produce seedless mandarin varieties. However, for sweet orange and grapefruit improvement there are no significant benefits. Genetic transformation could also be an alternative method to breed citrus, since several reports on the production of transgenic citrus plants have been reported (Yang et al., 2000; Gutierrez et al., 1997; Peña et al., 1995). However, many traits of horticultural importance are quantitative, and therefore less likely to be manipulated by genetic transformation. A new approach for citrus breeding could be Microprotoplast Mediated Chromosome Transfer (MMCT), as reported by Ramulu et al. (1996), which enabled the transfer of a single chromosome, from potato to tomato and tobacco. This methodology consist of the production of microprotoplasts containing one or a few chromosomes from a transgenic donor suspension cell, fusion with a non-transgenic recipient protoplast, and regeneration of hybrid plants in selective medium. The single requirement of this methodology is that the donor suspension cells must contain a selectable marker such as the npt-II gene for resistance to an aminoglycoside antibiotic. Citrus transgenic suspension cells have never been produced and maintained in continuous growth; therefore, no transformation method or antibiotic concentration has yet been set up. The objective of this research was to establish the procedure to produce and maintain citrus transgenic donor suspension cells for use in chromosome transfer experiments.

MATERIALS AND METHODS

Establishment of the Lethal Antibiotic Concentration. To establish the antibiotic concentrations where growth inhibition of suspension cells would occur (ie lethal concentrations), embryogenic suspension cells of *Microcitrus papuana*, and Ruby Red grapefruit (*Citrus paradisi* Macf.) were used. Embryogenic suspension cells were maintained in 125 ml Erlenmeyer flasks in a rotary shaker (130 rpm) in two week subculture cycles in H+H medium (Grosser and Gmitter, 1990) under diffuse light. The initial drained weight of the suspension cells was measured and 30 ml of H+H medium containing the antibiotics paromomycin sulfate (10, 25, 50, 100, and 200 µg/ml), kanamycin sulfate (10, 25, 50, 100, 200 µg/ml), or Geneticin (1, 2, 3, and 5 µg/.ml) were added. For each species a control (no antibiotic) was also used. After two weeks of growth, the final drained weight was determined and subtracted from the initial weight to calculate the cell growth and the lethal concentration. Each treatment was replicated three times. The choice of the antibiotic concentrations above was based on previous experiments (not published). After the determination of the lethal concentration for the best antibiotic, a test was performed for Valencia sweet orange suspension cells to validate the results for this species. The validation was performed at the lethal concentration, above and below.

Genetic Transformation. Protoplasts from Valencia sweet orange embryogenic suspension cells were isolated and purified according to Louzada et al. (1993), washed in HEPES electroporation buffer (Fromm et al. 1985), and the density adjusted to 1 x 10^6 protoplasts/ml in the same buffer. Thirty µl (1µg/ml) of the plasmid DNA pBin34SGUS (Yang et al., 2000) were added to 800 µl of the protoplast suspension in a 1.5 ml microcentrifuge tube, mixed gently, transferred to a 0.4 cm gap cuvette and electroporated immediately. Protoplasts

Table 1. Average of drained weight and percentage of growth or inhibition of suspension cells of *M. papuana* and Ruby Red grapefruit cultured in medium containing Paramomycin sulfate.

	M. papauna			Ruby Red		
Paromomycin	Initial Weight	Final Weight	% of Growth	Initial Weight	Final Weight	% of Growth
µg/ml	---------g---------			---------g---------		
0	3.36	7.74	130	3.90	9.24	137
10	2.89	6.31	118	3.27	4.54	39
25	3.04	5.35	76	3.21	4.56	42
50	2.36	2.95	25	3.31	2.27	-31
100	2.69	2.15	-20	2.63	1.36	-48
200	3.25	2.33	-28	3.57	1.42	-60

Table 2. Average of drained weight and percentage of growth or inhibition of suspension cells of *M. papuana* and Ruby Red grapefruit cultured in medium containing Kanamycin sulfate.

	M. papauna			Ruby Red		
Paromomycin	Initial Weight	Final Weight	% of Growth	Initial Weight	Final Weight	% of Growth
µg/ml	---------g---------			---------g---------		
0	3.36	7.74	130	3.90	9.24	137
10	2.48	5.75	131	3.14	7.00	123
25	2.83	6.23	120	3.39	7.22	113
50	2.31	4.89	112	3.92	7.43	90
100	2.48	5.13	106	3.23	6.28	94
200	2.86	5.67	98	3.25	6.68	105

Fig. 1. Non-transgenic embryos bleached by exposure to 50µg/ml of Paromomycin.

electroporated without plasmid DNA served as control. Electroporation was carried out at room temperature using a Gene Pulser II electroporation system (Bio Rad Laboratories). A single pulse of 400, 500 or 700 V/cm field strength, at 1000 µF of capacitance was applied. Immediately after electroporation, 1 ml of 0.6 M BH3 liquid medium (Grosser and Gmitter, 1990) was added, and the protoplasts were incubated at room temperature for 30 min. After centrifugation at 100 x g, the protoplasts were resuspended and cultured at 2 x 10^5 protoplast/ml in plastic Petri dishes (60x15 mm, Falcon, Lincoln Park, N.J.) in 0.6 M BH3 medium in the dark. Fifteen days after culture, paromomycin sulfate was added to 10 µg/ml in half of the plates including half of the controls, and maintained in the dark for an additional 15 days. Thirty and 40 days after electroporation the osmolarities of all plates were reduced as described by Grosser and Gmitter (1990) and maintained in low light. At the 60 day stage the plate contents were transferred to solid EME medium (Grosser and Gmitter, 1990) containing 25 or 50 µg/ml of paromomycin sulfate with an overlay of liquid medium with the same antibiotic concentration. As the somatic embryos started to be produced, they were transferred to solid EME with paromomycin sulfate at the same concentration as the plates that they originated from. When the embryos reached about 1 cm of diameter they were assayed histochemicaly for GUS activity as described by Jefferson et al. (1987). The GUS positive embryos were transferred successively to EME medium or left longer in the same medium for callus induction. An additional electroporation experiment was performed as described above; however, no selection was performed until all the embryos were about 1 cm in diameter, after which they were transferred to solid EME medium containing 25 and 50 µg/ml for selection. GUS positive callus was transferred to 125 ml Erlenmeyer flasks containing 50 ml of H+H medium with 50 µg/ml of paromomycin sulfate. The transgenic suspension cells were maintained in a 14 day subculture cycle at the same conditions described previously. The transgenic suspension cells are now being used in chromosome transfer experiments.

Southern Analysis. Total DNA was extracted from transgenic and non-transgenic suspension cells using the modified CTAB method as described by Porebski et al. (1997). Ten µg DNA was digested with Bam HI and Sma I restriction enzymes, separated by electrophoresis using 1% agarose gel and transferred to a nylon membrane filter (Hybond, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Southern analysis was performed using the non radioactive AIK-Phos Direct Labeling and Detection System according to the manufacturer instructions (Ammersham Pharmacia Biotech Inc, Piscataway, NJ, USA).

RESULTS AND DISCUSSION

Establishment of the Lethal Concentration of Antibiotic. To determine the lethal concentration of the antibiotics paromomycin sulfate, kanamycin sulfate and geneticin, for suspension cells of Valencia sweet orange, an indirect growth study was performed using *Microcitrus papuana* and Ruby Red grapefruit. We assumed that these two species would be preferable instead of Valencia due to their rapid growth rates, as a large amount of cells are necessary to conduct such a study. After the establishment of the lethal concentration for the two species, the data was validated with Valencia sweet orange suspension cells by examining their growth in the lethal concentration, and in concentrations above and below it.

Table 3. Average of drained weight and percentage of growth or inhibition of suspension cells of *M. papuana* and Ruby Red grapefruit cultured in medium containing Geneticin.

	M. papauna			Ruby Red		
Paromomycin	Initial Weight	Final Weight	% of Growth	Initial Weight	Final Weight	% of Growth
µg/ml	---------g---------			---------g---------		
0	3.36	7.74	130	3.90	9.24	137
1	2.55	4.20	65	2.57	3.66	42
2	2.66	2.82	6	3.34	2.60	-22
3	2.83	2.17	-23	4.02	3.47	-14
5	3.07	2.30	-2	3.62	3.02	-17

Control suspension cells (no antibiotic) of *M. papuana* and Ruby Red grapefruit grew 130% and 137% of their initial weight, respectively (Tables 1, 2 and 3). At the concentration of 25 µg/ml of paromomycin, *M papuana* grew 76% of the initial weight and Ruby Red grapefruit only 42% (Table 1), which is a drastic reduction compared to the control. At 50 µg/ml of paromomycin, *M papuana* had a growth of 25% of the initial weight (Table 1), which is 105% lower than the control, and Ruby Red grapefruit cells died. The negative number in Tables 1 and 3 means that the cells lost their cellular content because of death, causing a reduction in dry weight, compared to the initial weight. At a concentration of 100 µg/ml of paromomycin all cells were dead. Kanamycin sulfate on the other hand had little effect on the growth of the suspension cells. Even in concentrations as high as 200 µg/ml the growth rates were approximately 100% for both species compared to the initial weight (Table 2), only 32% below the control (Table 2). In contrast, geneticin in its lowest concentration (1 µg/ml) induced a drastic growth reduction of 65% and 95% for *M papuana* and Ruby Red respectively when compared to the control growth rates (Table 3). No growth was observed at 2 µg/ml (Table 3). Therefore paromomycin sulfate was chosen as the best antibiotic for selection and maintenance of transgenic suspension cells of *M papuana* and Ruby Red grapefruit. Kanamycin did not suppress the growth adequately, and geneticin was too strong. We estimated 50 µg/ml of paromomycin sulfate to be the lethal concentration. To validate the data with Valencia sweet orange we examined its growth at 25, 50, 75, and 100 µg/ml of paromomycin for three independent experiments, with three replications. Approximately 50% growth was observed at 25 µg/ml, but no growth occurred from 50 to 100 µg/ml. We therefore established 50 µg/ml as the lethal concentration of paromomycin for the maintenance of Valencia suspension cells. We also examined the effect of concentrations of 10, 15, 25, and 50 µg/ml of paromomycin in Valencia protoplasts and found that 10 µg/ml were enough to block its growth. After the regeneration of the cell wall, at the stage of microcallus (about 5 cells), 15 µg/ml was more efficient, but at later stages, closer to embryo formation, 25 or 50 µg/ml was more adequate to select for transgenic embryos. In the experiment performed without selection, several embryos were obtained from the control, and electroporated samples. We observed that all the embryos from the control bleached when transferred to medium containing 50 µg/ml of paromomycin. Fig. 1 shows control embryos of various sizes grown in non-selective medium and transferred to medium containing 50 µg/ml of paromomycin. Fig. 2 shows transgenic embryos in the same medium.

Genetic transformation and production of transgenic suspension cells. The electroporation parameters used in this research were based on experiments previously performed for transient gene expression (unpublished data). In that study we observed that the field strength of 500 V/cm, and capacitance of 1000 µF produced the highest transient expression. Higher capacitance or higher field strength increased the damage of the protoplasts.

Besides the electroporation parameters, the handling of the protoplasts after transformation played an important role in the viability of the protoplasts. The addition of 1 ml of BH3 medium immediately after electroporation was the most important factor, preventing the protoplasts from agglutinating or getting damaged. Additionally, it was important to maintain the protoplasts for 30 min in BH3 medium before centrifugation and culturing.

For the production of transgenic suspension cells we relied on the observation that callus is easily obtained from embryos originated from protoplasts (unpublished data). Since the embryos come from single cells, a mass of transgenic callus would be produced. We evaluated the field strength of 400, 500 or 700 V/cm and capacitance of 1000 µF for the production of transgenic embryos. Stable transgenic embryos could be

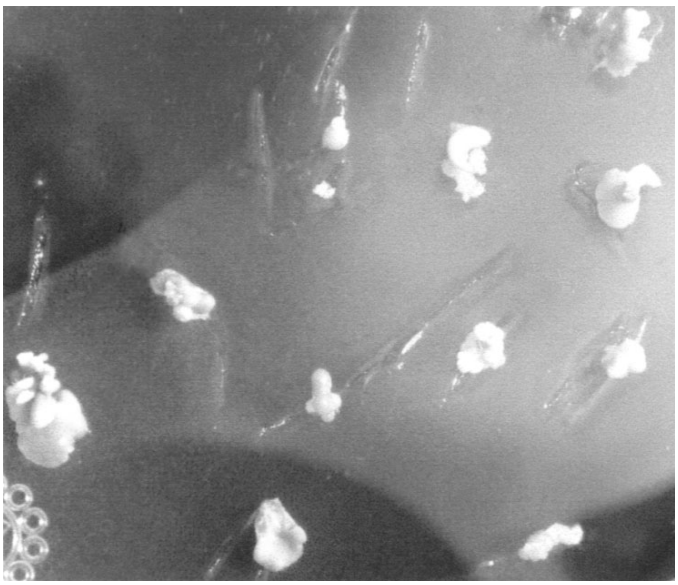

Fig. 2. Transgenic embryos exposed to 50µg/ml of paromomycin.

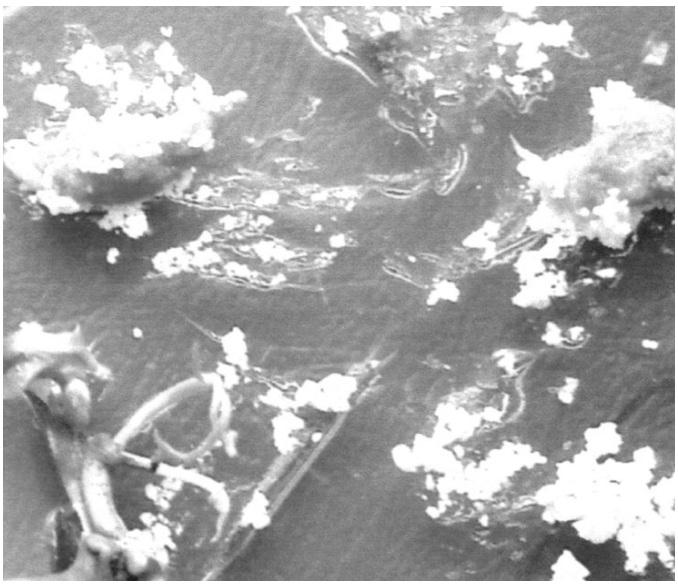

Fig. 3. Transgenic embryos producing callus in medium containing 50µg/ml of paromomycin

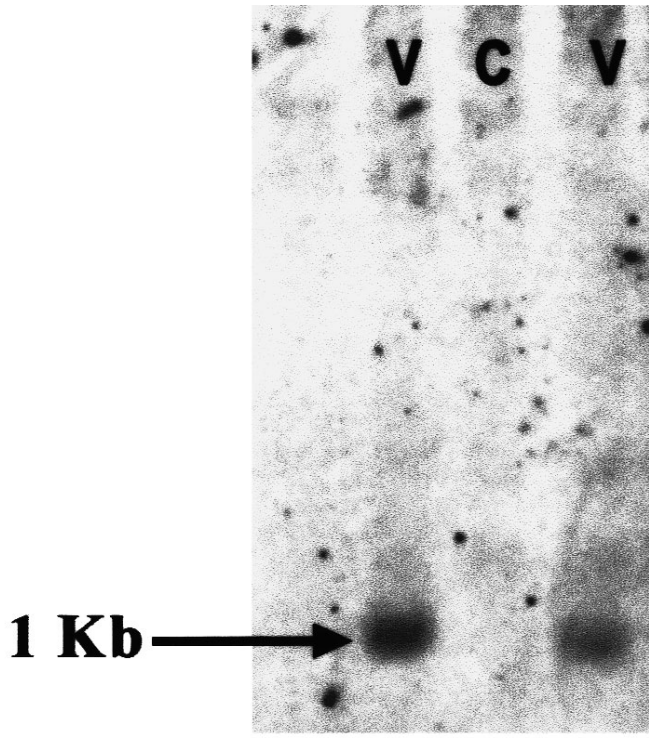

Fig. 4. Northern blot analysis of total RNA digested with Bam HI and Sma I restriction enzymes, to release a 1KB fragment containing part of the npt II gene. V-trangenic Valencia suspension cells C-Control.

produced in all field strengths, however, 400 and 700 V/cm produced just a few, and 500 V/cm produced the largest numbers of transgenic embryos. The low voltage probably could not deliver the gene inside of the protoplasts efficiently, and the high voltage induced more damage to the protoplasts. All the embryos were assayed for ß-glucuronidase (GUS) activity, and no escapes were detected. Some embryos developed a bleached area when transferred to solid medium containing antibiotic, indicating a probable chimera, and they were thus discarded. A large number of transgenic embryos produced callus (Fig. 3). We noticed that round or brownish embryos produced more callus than the ones with normal shape. The callus were maintained in medium containing 50 µg/ml of paromomycin and subcultured monthly. Additionally, GUS activity assays were performed periodically. The callus lines were transferred to liquid medium containing 50 µg/ml of paromomycin and subcultured every 14 days. Fig. 4 show a southern blot analysis of DNA isolated from transgenic and control Valencia suspension cells. The DNA was digested with Bam HI and Sma I restriction enzymes, to release a 1KB fragment containing part of the npt II gene, and hybridized with alkaline phosphatase-labeled probe specific for the npt II fragment. A single band is present in the transgenic suspension cells, but not in the control. Even though we were not interested in plant regeneration, several transgenic plants were obtained. The procedure described in this manuscript will facilitate the selection of transgenic citrus suspension cells or plants, independent of the transformation procedure used. In addition it offers a reliable electroporation protocol for citrus protoplasts.

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