Agrobacterium-mediated genetic transformation of 'Hamlin' sweet orange[1]

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Abstract – The development and optimization of efficient transformation protocols is essential in new citrus breeding programs, not only for rootstock, but also for scion improvement. Transgenic 'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) plants were obtained by *Agrobacterium tumefaciens*-mediated transformation of epicotyl segments collected from seedlings germinated *in vitro*. Factors influencing genetic transformation efficiency were evaluated including seedling incubation conditions, time of inoculation with *Agrobacterium* and co-culture conditions. Epicotyl segments were adequate explants for transformation, regenerating plants by direct organogenesis. Higher percentage of transformation was obtained with explants collected from seedlings germinated in darkness, transferred to 16 hours photoperiod for 2-3 weeks, and inoculated with *Agrobacterium* for 15-45 min. The best co-culture condition was the incubation of the explants in darkness, for three days in culture medium supplemented with 100 μM of acetosyringone. Genetic transformation was confirmed by performing ß-glucoronidase (GUS) assays and, subsequently, by PCR amplification for the *nptII* and GUS genes.

Index terms: *Citrus sinensis*, epicotyls, seedlings, transgenics, breeding methods.

Transformação genética de laranja 'Hamlin' via *Agrobacterium*

Resumo – O desenvolvimento e otimização de protocolos eficientes de transformação genética é essencial nos programas atuais de melhoramento de citros, tanto para porta-enxertos, como para copas de valor comercial. Plantas transgênicas de laranja 'Hamlin' (*Citrus sinensis* (L.) Osbeck) foram obtidas pela transformação genética de segmentos de epicótilo, coletados de plântulas germinadas *in vitro*, com *Agrobacterium tumefaciens*. Foram avaliados fatores que influenciam a eficiência da transformação genética, como: condições de incubação das plântulas utilizadas para coleta de explantes, tempo de inoculação com *Agrobacterium* e condições de co-cultivo. A regeneração de plantas a partir de segmentos de epicótilo ocorreu em alta freqüência, por organogênese direta. A maior porcentagem de plantas transgênicas foi obtida utilizando-se explantes coletados de plântulas germinadas no escuro e posteriormente transferidas, por 2-3 semanas, para condições de 16 horas de fotoperíodo, e infectados com *Agrobacterium* por um período de 15-45 minutos. As melhores condições de co-cultivo foram a incubação dos explantes no escuro, por três dias, em meio de cultura suplementado com 100 μM de acetoseringona. A transformação genética foi confirmada pelo teste histoquímico para ß-glucoronidase (GUS) e, posteriormente, pela amplificação de DNA, por PCR, para detecção dos genes *nptII* e GUS.

Termos para indexação: *Citrus sinensis*, epicótilo, plântulas, transgênicos, métodos de melhoramento.

Introduction

Conventional citrus breeding programs have been carried out since the end of the 19th century. After the pioneer work of Swingle and Webber in Florida/USA, several other citrus breeding and cultivar improvement programs have been developed, not only in that State, but also in California, and other countries besides the USA, such as Italy, Spain, South Africa, Brazil, France, Java, Philippines and Japan (Soost & Cameron, 1975). The lack of good results in these programs cannot be attributed to poor research, but, instead to different natural barriers to conventional breeding related to citrus reproductive biology. Among those limitations, nucellar polyembryony (apomixis), high heterozygosity, in-

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breeding depression and the long juvenile period can be cited as the most important limitations to citrus cultivar improvement.

The integration of new technologies, including molecular methods and cell and tissue culture techniques have offered alternatives to genetic improvement and some of these barriers can be overcome. Among these new methods, somatic hybridization through protoplast fusion and genetic transformation appear as important tools to be incorporated in citrus breeding programs. Genetic manipulation of citrus using biotechnological methods has been developed since the 80's, after the report of the first intergeneric somatic hybrid *Citrus sinensis* + *Poncirus trifoliata* (Ohgawara et al., 1985) and with the first attempt to produce a transgenic citrus plant (Kobayashi & Uchimiya, 1989). The development and optimization of efficient protocols for DNA transfer to produce transgenic plants can offer great advantages to citrus rootstock and scion breeding programs, allowing the introduction of specific traits to a known variety, without the risk of segregation (Vardi et al., 1990; Kaneyoshi et al., 1994; Peña et al., 1995a).

The first demonstration of genetic transformation in *Citrus* was reported by Kobayashi & Uchimiya (1989), as already mentioned, with the direct DNA uptake induced by PEG in protoplasts. However, successful plant regeneration was not achieved. Since then, other protocols have been described using co-cultivation of *Agrobacterium* with internodal segments of plants cultivated *in vitro* (Moore et al., 1992; Peña et al., 1995b; Gutiérrez et al., 1997; Pérez-Molphe-Balch & Ochoa-Alejo, 1998), or in the greenhouse (Peña et al., 1995a, 1997), with cell suspension cultures (Hidaka et al., 1990), with epicotyl segments (Kaneyoshi et al., 1994; Cervera et al., 2000; Yang et al., 2000), or using biolistic in cell suspension cultures (Yao et al., 1996).

The genetic transformation works reported on citrus have used GUS and *nptII*, as reporter and selection genes, respectively (Hidaka et al., 1990; Peña et al., 1995a; Gutiérrez et al., 1997). So far, there are few reports of the introduction of agronomical important genes in citrus: encoding for the citrus tristeza virus coat protein (Gutiérrez et al., 1997; Domínguez et al., 2000; Yang et al., 2000), *HAL2* gene, for tolerance-to-salinity (Cervera et al., 2000) and *LEAFY* and *APETALA1* genes which promote flower initiation (Peña et al., 2001).

Genetic manipulation of citrus in Brazil has focused on the production of somatic hybrids through protoplast fusion (Glória et al., 2000a, 2000b; Mendes et al., 2001).

The objective of this work was to study the influence of different factors in citrus genetic transformation efficiency using epicotyl segments of *in vitro* germinated seedlings of 'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck).

Material and Methods

Seeds were extracted from mature fruits of 'Hamlin' sweet orange of a citrus germoplasm collection, Piracicaba, SP, Brazil, and dried at room temperature for 24 hours. After that, seed coat was removed and seeds were treated with sodium hypochloride solution (0.5%), for 15 minutes. Seeds were then transferred to test tubes (25 x 150 mm), with 10 mL of MS medium (Murashige & Skoog, 1962) and incubated at 27ºC, in darkness, for 10-15 days. Plants with 12-15 cm high were then cultured at 16 hours photoperiod for 7-10 days. Epicotyl segments were excised with 0.8-1.0 cm long.

Agrobacterium tumefaciens strain EHA-105, carrying the plasmid p35SGUSINT was used. Bacteria was cultivated in YEP solid medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L sodium chloride, 15 g/L agar), containing kanamycin (100 mg/L) and rifampicin (50 mg/L), for 48 hours. After that, a single colony was transferred to a 250 mL erlenmeyer, with 50 mL of YEP liquid medium, supplemented with the antibiotics and cultivated under 180 rpm, 28ºC, for 16 hours. Bacterial suspension was centrifuged at 5,000 rpm (15ºC/15 min) and resuspended in liquid medium MS. The antibiotics were filter sterilized and added to the autoclaved medium.

Epicotyl segments were incubated in bacteria solution for 20 min. Following incubation, explants were blotted dry and plated on EME medium (Grosser & Gmitter Junior, 1990) supplemented with sucrose (25 g/L), BA (1 mg/L) and acetosyringone (100 μM), in darkness, at 27ºC, for a three day period. After co-culture, segments were transferred to EME medium supplemented with BA (1 mg/L), kanamycin (100 mg/L) and cefotaxime (500 mg/L). Explants were subcultured every two weeks. Well developed shoots were transferred to EME medium supplemented with GA_3 (1 mg/L), kanamycin (100 mg/L) and cefotaxime (500 mg/L) for elongation. For rooting, GUS^+ plantlets were transferred to EME medium supplemented

with IBA (1 mg/L) and cefotaxime (500 mg/L). Plantlets that did not root had their apical meristem grafted onto *in vitro* rootstock seedlings. For acclimatization, plantlets were transferred to the commercial substrate mixture Rendmax Citrus TM - Eucatex and kept under high relative humidity for 30 days. Different experiments were performed in order to evaluate the factors influencing the transformation efficiency, as follows: 1) epicotyl segments were excised from seedlings cultivated for 0, 1, 2, 3 or 4 weeks in a 16 hours photoperiod after germination in darkness; 2) epicotyl segments were incubated with *Agrobacterium* suspension for 0, 5, 15, 30, 45 or 60 min; 3) epicotyl segments were co-cultivated at 16 hours photoperiod or in darkness; 4) co-cultivation medium was supplemented with acetosyringone at 0, 100 or 200 µM; 5) explants were co-cultivated with *Agrobacterium* for 0, 1, 3 or 5 days. All experiments had five replications per treatment. Each replication consisted of a petri dish with 30 explants, in a total of 150 explants per treatment. The experiments were repeated at least twice.

For the analysis of putatively transformed tissue leaves or stem segments were excised from 1-2 cm long plants. Segments were incubated in darkness, at 37°C, for 24 hours, in a X-GLUC solution (Jefferson, 1987) for ß-glucoronidase assays (GUS). GUS^+ plants were transferred to EME medium supplemented with GA_3 (1 mg/L) and cefotaxime. DNA was extracted according to Doyle & Doyle (1990). PCR amplification was performed using 50-100 ng of DNA, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 2U Taq DNA polymerase and 0.25 µM of the GUS primer or 0.1 µM of the kanamycin primer. The primers 5'GGT GGG AAA GCG CGT TAC AAG3' and 5'TGG ATC CCG GCA TAG TTA AA3', described by Peña et al. (1997), were used to amplify a 600 bp specific fragment of GUS gene, in a thermal cycler (MJ Research). The PCR was performed at setting of 35 cycles of 1 min at 92°C, 1 min at 55°C and 1.5 min at 72°C. The primers 5'GAG GCT ATT CGG CTA TGA CTG G3' and 5'ATC GGG AGC GGC GAT ACC GTA A3', described by Bond & Roose (1998), were used to amplify a 700 bp specific fragment of *nptII* gene using the program of 4 min at 94°C followed by 30 cycles of 2 min at 96°C, 2 min at 50°C and 3 min at 72°C.

Results and Discussion

The most common genetic transformation system utilized in citrus with explants collected from seedlings has been the *Agrobacterium* mediated system. These explants can be epicotyl segments excised from seedlings germinated in darkness (Bond & Roose, 1998) and later transferred to culture conditions involving a 16 hours photoperiod, varying from one to three weeks (Cervera et al., 1998) up to three months (Pérez-Molphe-Balch & Ochoa-Alejo, 1998) or even excised from seedlings germinated under light conditions (Moore et al., 1992) or internodal segments excised from seedlings cultivated in greenhouse (Peña et al., 1995a, 1997). In this work it was chosen to start using explants excised from seedlings germinated *in vitro* because of the efficiency in regenerating shoots (Moura et al., 2001) and the good control of contamination. The explants responded very well to the *in vitro* culture and the germination in darkness for two weeks and after that, transferring to 16 hours photoperiod allows the regeneration of GUS^+ plants (Table 1). Explants from plants cultivated in fulltime darkness resulted in a slower bud development and in a lower number of plants than when the seedlings were kept in light. The best results were obtained using explants collected from plants cultivated for 1-3 weeks in 16 hours photoperiod, when it was possible to regenerate a higher number of plants and a better percentage of GUS^+ plants compared with other treatments.

Citrus genetic transformation protocols present variations in different steps of the process and different culture conditions can be necessary for different species (Peña et al., 1997). Explant inoculation

Table 1. Seedling cultivation period in 16 hours photoperiod, or fulltime darkness (four weeks) at the moment of *Agrobacterium* explant inoculation, versus bud differentiation and regeneration of GUS^+ plants of 'Hamlin' sweet orange. Piracicaba, SP, 2000.

Seedling cultivation (week)	Explants with buds/total explants (%)	GUS^+/analyzed plants (%)
Four	55/107 (51.4)	15/22 (68.2)
Three	61/112 (54.5)	18/25 (72.0)
Two	82/145 (56.6)	28/43 (65.1)
One	70/110 (63.6)	38/53 (71.7)
In darkness	65/149 (43.6)	22/27 (81.5)

method with *Agrobacterium* also varies from placing the drops of bacterial suspension on explant surface (Moore et al., 1992) to different times of incubation, varying from 15 (Cevera et al., 1998) to 45 minutes (Pérez-Molphe-Balch & Ochoa-Alejo, 1998). In this study, the longer the time of inoculation, the greater the number of explants with shoots (Table 2). However, the percentage of GUS$^+$ plants decreases with the increase of the time of inoculation. The increase in number of scapes, the regeneration of non transformed plants, can be explained by an inefficient selection due to the protection of non transformed cells from the selection agent by surrounding transformed cells (Ghorbel et al., 1999). It can be concluded that time of inoculation between 15 and 45 minutes allows adequate bud differentiation and a good percentage of GUS$^+$ plants.

During the co-cultivation period, many factors can influence the efficiency of the genetic transformation process. Explants incubation in absence of light favored the regeneration of a higher number of GUS$^+$ plants (Table 3). Regarding the co-cultivation time it was observed that simple inoculation of the explants with *Agrobacterium* and its transfer to selection medium did not allow the regeneration of transgenic plants as also demonstrated by Cervera et al. (1998) (Table 4). The co-culture for three days increased the percentage of explants with buds, as it was possible to obtain a higher number of plants than with one day co-cultivation and a higher percentage of GUS$^+$ plants than with five days co-cultivation. An overgrowth of the bacteria with five days co-culture was not detected as reported by Cervera et al. (1998).

The supplementation of the co-culture medium with products that stimulate the infection with *Agrobacterium* has been reported by other authors. Acetosyringone has been the preferential product with the concentration of 100 µM as the most adequate. Bond & Roose (1998) supplemented the co-culture medium with 200 µM of acetosyringone. Domínguez et al. (2000) mentioned the use of tomato cell suspension culture tomato medium containing auxins, to improve the efficiency of citrus genetic transformation. The addition of acetosyringone did not improve the results as expected, the percentage of GUS$^+$ plants had only a slight increase at 200 µM concentration (Table 5).

Table 2. Inoculation period of epicotyl segments with *Agrobacterium* versus bud differentiation and regeneration of GUS$^+$ plants of 'Hamlin' sweet orange. Piracicaba, SP, 2000.

Period of inoculation (min)	Explants with buds/total explants (%)	GUS$^+$/analyzed plants (%)
0	168/150 (112.0)	-
5	62/141 (44.0)	15/46 (32.6)
15	47/143 (33.9)	10/22 (45.4)
30	41/150 (27.3)	24/31 (77.4)
45	35/125 (28.0)	22/40 (55.0)
60	73/130 (56.2)	24/40 (60.0)

Table 3. Conditions of incubation at explant co-culture with *Agrobacterium* versus bud differentiation and regeneration of GUS$^+$ plants of 'Hamlin' sweet orange. Piracicaba, SP, 2000.

Conditions of incubation	Explants with buds/total explants (%)	GUS$^+$/analyzed plants (%)
Control	168/150 (112.0)	-
Light	81/117 (69.2)	17/34 (50.0)
Dark	83/150 (55.3)	31/49 (63.3)

Table 4. Period of co-culture of the explants with *Agrobacterium* versus bud differentiation and regeneration of GUS$^+$ plants of 'Hamlin' sweet orange. Piracicaba, SP, 2000.

Days of co-culture	Explants with buds/total explants (%)	GUS$^+$/analyzed plants (%)
0	9/144 (6.2)	0/1 (0.0)
1	6/133 (4.5)	2/2 (100.0)
3	49/114 (43.0)	16/26 (65.5)
5	65/118 (55.1)	10/41 (24.4)

Table 5. Acetosyringone concentration in the co-culture medium versus bud differentiation and regeneration of GUS$^+$ plants of 'Hamlin' sweet orange. Piracicaba, SP, 2000.

Acetosyringone concentration (µM)	Explants with buds/total explants (%)	GUS$^+$/analyzed plants (%)
Control	147/150 (98.0)	-
0	79/138 (57.2)	19/36 (53.0)
100	92/139 (66.2)	23/42 (55.0)
200	90/146 (61.7)	21/32(65.6)

The results related to the explants response to *in vitro* culture and genetic transformation were also registered. Figure 1 shows various phases of the experiments. The genetic transformation was confirmed by DNA analysis on GUS$^+$ shoots, when it was possible to verify the presence of a 600 bp DNA fragment corresponding to GUS gene and a 700 bp DNA fragment corresponding to gene *nptII*.

Based on results presented and the protocols already reported in literature, epicotyl segments from seedlings germinated *in vitro* for two weeks in darkness and transferred to light for 1-3 weeks proved to be a good source of explants for the citrus genetic transformation experiments. Period of inoculation

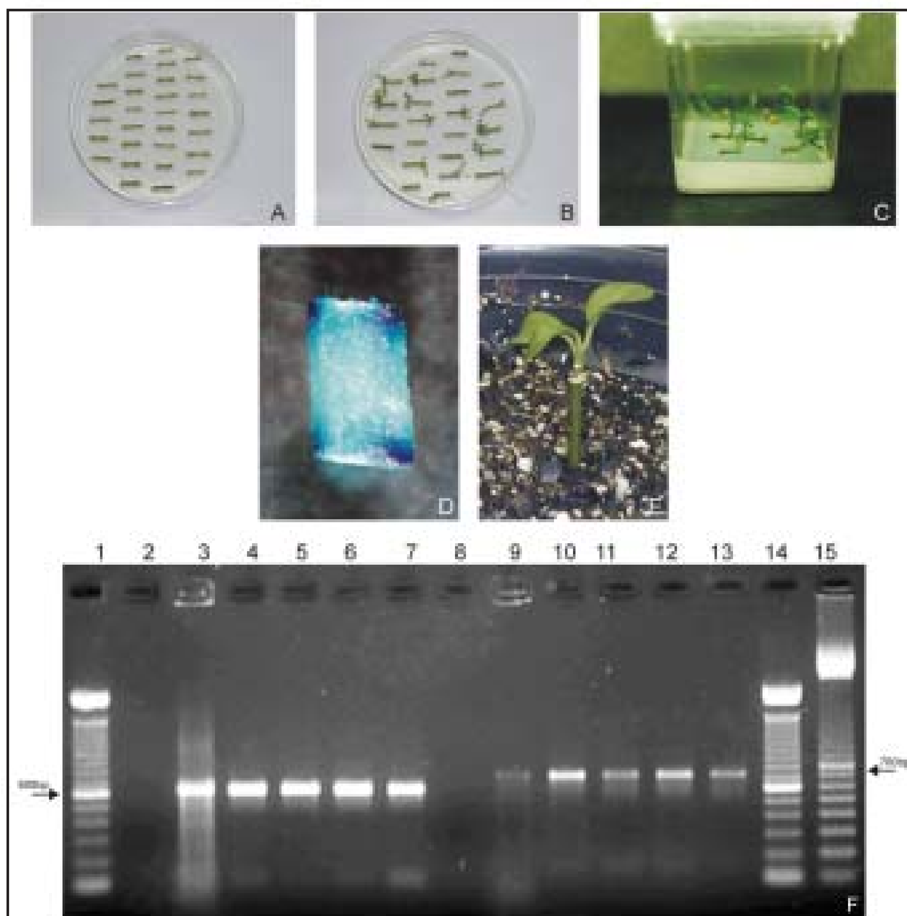

Figure 1. Production of transgenic 'Hamlin' sweet orange plants. A: epicotyl segments after *Agrobacterium* inoculation; B: shoot regeneration from epicotyl segments cultivated on selective medium; C: shoots cultivated on elongation medium; D: stem segment tissue histochemically stained for ß-glucoronidase activity; E: plantlet acclimatized to soil conditions; F: PCR analysis of DNA from GUS$^+$ plantlets. Lanes 1 and 14: ladder 100 bp (GIBCO BRL); Lane 15: ladder 100 bp (Pharmacia); Lanes 2 and 8: negative control consisting of non-transformed 'Hamlin' DNA; Lanes 3 and 9: positive control consisting of the transformation plasmid; Lanes 4-7: represent GUS$^+$ plants; Lanes 10-13: represent *npt*II$^+$ plants.

with *Agrobacterium* for 15 minutes, co-culture for three days in darkness, with culture medium supplemented with acetosyringone was adequate for recovering GUS$^+$ plants.

Conclusion

'Hamlin' sweet orange can be successfully transformed through *Agrobacterium tumefaciens*.

Acknowledgements

To Fapesp and Fundecitrus for financial support; to CNPq for a research fellowship (B. M. J. Mendes and F. de A. A. Mourão Filho); to Fapesp and Capes/PICDT for doctoral fellowship, respectively (R. de L. Boscariol and W. A. B. de Almeida).

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