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High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus

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Summary In preliminary experiments on *Agrobacterium*-mediated transformation of citrus, we found transformation events occurring in callus formed from the cambium. Factors affecting *Agrobacterium*–sour orange (*Citrus aurantium* L.) interactions, such as culture medium, explant source and culture conditions, were studied to assess competence for transformation in such callus and to improve transformation frequency. Cell divisions and redifferentiation from the transgenic cells leading to transformation events were stimulated more by a combination of benzylaminopurine (BAP) + naphthalene-acetic acid (NAA) in the regeneration–selection medium than by BAP alone. Both age and source of the sour orange plant material affected transformation frequency. Explants from 4-month-old seedlings grown in the greenhouse showed higher transformation frequency than younger and older plant materials, indicating that they had a more suitable balance between dedifferentiated cells competent for transformation and *Agrobacterium* virulence. Enhancement of transformation frequency enabled us to incorporate the coat protein gene of citrus tristeza virus (CTV) in a sufficient number of sour orange plants to be able to evaluate this strategy for producing CTV-resistant plants.

Keywords: Agrobacterium tumefaciens, coat protein-mediated resistance, competence, transformation frequency, woody plant transformation.

Introduction

Citrus is the most extensively grown fruit tree crop in the world (FAOSTAT database results 1998: http://apps.fao.org/lim500/nph-wrap.pl). Sour orange (*Citrus aurantium* L.) was the predominant rootstock in most citrus-growing areas for many years because of its good agronomic attributes, particularly fruit yield, quality, rusticity, and tolerance to various pathogens and abiotic stresses. However, a major weakness is its sensitivity to decline caused by citrus tristeza virus (CTV). This closterovirus affects all citrus species and varieties grafted on sour orange, with the exception of lemons (*C. limon* L. Burm.), and has caused the death of millions of trees propagated on this rootstock (Bar-Joseph et al. 1981). This has led to the substitution of sour orange with other rootstocks that are resistant or tolerant to CTV. However, none has all the desirable characteristics of sour orange, particularly in the Mediterranean citrus-growing area in which many soils are calcareous or saline. In Spain and other Mediterranean countries, sour orange has been substituted mainly with trifoliate hybrids such as citranges (*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.); however, these hybrids perform less well than sour orange in these regions. Genetic improvement of the sour orange rootstock to overcome its sensitivity to CTV would be desirable, and it remains an important objective for the citrus industry.

The expression of coat protein (*CP*) genes in transgenic plants is an example of pathogen-derived resistance and has been widely used to develop virus-resistant plants. Since the first report using this strategy to protect tobacco plants against tobacco mosaic virus (TMV) infection (Powell-Abel et al. 1986), there have been many reports of transgenic plants resistant to virus diseases (Beachy 1997).

Genetic transformation and recovery of transgenic citrus trees has been achieved in various species, hybrids and *Citrus* relatives such as rough lemon, sweet orange, Carrizo citrange, *Poncirus trifoliata* and Mexican lime (reviewed in Peña and Navarro 1999). Previous studies on genetic transformation of sour orange demonstrated that this species is highly recalcitrant to transformation. Thus, production of transgenic plants was very low (less than 0.1% transformation efficiency) and circumstantial (Gutiérrez-E. et al. 1997). The method used was not reliable or efficient enough to be routinely used to introduce genes of possible interest and to recover a sufficient number of plants to be evaluated for the novel incorporated trait.

Recently, we have used the green fluorescent protein gene (*gfp*) as a vital marker to localize the sites of transgene expression at early stages after transformation in sour orange and other citrus genotypes (Ghorbel et al. 1999). Here, we report a

detailed study of factors affecting the transformation and regeneration of sour orange. We used the information to improve the method for producing transgenic sour orange plants. Transgenic trees of sour orange with the *CP* gene of CTV were obtained with the improved procedure.

Materials and methods

Bacterial strain and vectors

Agrobacterium tumefaciens EHA 105 (Hood et al. 1993), containing the binary plasmid p35SGUSINT (Vancanneyt et al. 1990), was used as a vector system for transformation. *Agrobacterium tumefaciens* EHA 105 is a disarmed derivative of *A. tumefaciens* A281 (Hood et al. 1993), which is supervirulent in citrus (Cervera et al. 1998*c*). The T-DNA contains the NOSpro-*nptII*-NOSter cassette as a selectable marker, and the 35Spro-GUSINT-35Ster cassette as a reporter gene and selectable marker. To recover transgenic plants with the CTV *CP* gene, we used the transformation vector *A. tumefaciens* EHA 105 containing the binary plasmid pBI121/*CTV-CP* (Domínguez et al. 2000), in which the *CTV-CP* expression cassette (35Spro-*CTV-CP*-NOSter) is flanked by the NOSpro-*nptII*-NOSter and 35Spro-*uidA*-NOSter cassettes.

Transformation and regeneration

Agrobacterium was cultured overnight in an orbital shaker at 200 rpm and 28 ºC in LB medium (Sambrook et al. 1989) containing 25 mg l^{-1} each of kanamycin and nalidixic acid. Bacterial cells were pelleted at 5000 *g* for 10 min, resuspended and diluted to 4×10^7 cells ml^{-1} in liquid inoculation medium, consisting of MS salt solution of Murashige and Skoog (1962), 0.2 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} pyridoxine hydrochloride, 1 mg l^{-1} nicotinic acid and 3% (w/v) sucrose, pH 5.7. Twelve-month-old greenhouse-grown sour orange seedlings were used as the source of tissue for transformation. Stem pieces (20 cm long) were stripped of leaves and thorns, disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile water. Internodal stem segments were incubated with gentle shaking for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension in inoculation medium. The infected explants were blotted dry on sterile filter paper and placed horizontally on plates containing cocultivation medium (CM) for a 3-day cocultivation period. The CM consisted of MS salts, 1 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} pyridoxine hydrochloride, 1 mg l^{-1} nicotinic acid, 3% (w/v) sucrose, 2 mg l^{-1} indole-3-acetic acid, 1 mg l^{-1} 2-isopentenyl-adenine, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% (w/v) agar, pH 5.7. After the 3-day cocultivation period, the explants were blotted dry with sterile filter paper and transferred to shoot regeneration medium (SRM).

To assess factors affecting transformation frequency, four tests were performed sequentially and the most efficient transformation conditions were then used to produce transgenic sour orange plants.

(1) Three shoot regeneration media (SRM: SRM1, SRM2 and SRM3) were tested. The SRM consisted of MS salts, 0.2 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} pyridoxine hydrochloride, 1 mg l^{-1} nicotinic acid, 3% (w/v) sucrose, 1% (w/v) agar, pH 5.7, plus 100 mg l^{-1} kanamycin for the selection of transgenic events and 250 mg l^{-1} vancomycin and 500 mg l^{-1} cefotaxime to control bacterial growth. The SRM was supplemented with 1 mg l^{-1} benzylaminopurine (BAP) (SRM1), or with 1 mg l^{-1} BAP plus 0.3 mg l^{-1} naphthalene-acetic acid (NAA) (SRM2), or with 2 mg l^{-1} BAP (SRM3).

(2) Cocultivation and regeneration–selection were tested at two temperatures. Cocultivation was done in low irradiance (20 µmol m^{-2} s^{-1}), a 16-h photoperiod, 60% relative humidity and 26 or 22 ºC. The explants were transferred to selective medium and maintained in the dark for 4 weeks at 26 or 22 ºC. The cultures were then transferred to a 16-h photoperiod, 45 µmol m^{-2} s^{-1} illumination, 60% RH and 26 ºC.

(3) Four- and 12-month-old greenhouse-grown seedlings, and 2-week-old seedlings germinated and grown *in vitro*, were tested as sources of tissue for transformation.

(4) Pre-cultivation of explants for 1 day before inoculation with *Agrobacterium* was also tested. Pre-cultivation was done in a culture medium similar to SRM, but without antibiotics, and supplemented with 1 mg l^{-1} BAP and 0.3 mg l^{-1} NAA (PC1) or with 1 mg l^{-1} BAP and 0.3 mg l^{-1} 2,4-D (PC2).

The explants were subcultured to fresh SRM every 4 weeks. Transformation frequency was evaluated after 6 weeks on SRM as the total number of transformation events (β-glucuronidase (GUS)-expressing cell clusters at the cut ends of the explants, visible under a stereo microscope at 50× magnification) per 50 inoculated explants, and also as the number of explants with transformation events per 50 inoculated explants × 100. All experiments were repeated at least twice.

To recover whole transgenic plants, emerging shoots were assayed for GUS activity and then shoot-tip grafted *in vitro* onto Troyer citrange seedlings. Grafting of the *in-vitro*-growing plants on vigorous rootstocks allowed the rapid acclimatization and development of plants under greenhouse conditions. In these experiments, transformation efficiency was defined as the number of GUS-positive regenerated plants per total number of inoculated explants.

Effects of treatments were tested by analysis of variance ($P < 0.05$). Differences among means were tested by the Duncan range test ($P < 0.05$) (Steel and Torrie 1997). Percentage data were arcsine–square root-transformed before analysis for homogeneity of variances.

GUS, PCR, Southern and Western analyses

Explants and leaf and stem pieces from the regenerated shoots were tested for histochemical GUS expression as previously described (Peña et al. 1995). The GUS positive plantlets were screened by PCR for the presence of the *nptII* gene when p35SGUSINT was used as vector, and for the *CTV-CP* transgene when pBI121/*CTV-CP* was used as vector. The DNA was extracted from leaf pieces according to McGarvey and Kaper (1991). Standard PCR techniques were used to detect

the transgenes. The *nptII* fragment was amplified with primers 5′-GACGAGGCAGCGCGGCTAT-3′ and 5′-AAGAAGGCGATAGAAGGCGA-3′, and the *CTV-CP* fragment was amplified with primers 5′-GGATCCATGGACGGAAAC-3′ and 5′-GGATCCTCAACGTGTGTTG-3′. For *nptII* detection, reactions were performed under the following conditions: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, for 35 cycles. For *CTV-CP* amplification, reactions were carried out at: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, for 30 cycles, plus a final extension at 72 ºC for 2 min. Amplified DNA was detected by ultraviolet illumination after electrophoresis on 2% (w/v) agarose–ethidium bromide gels.

For Southern analyses, DNA was isolated from leaves according to Dellaporta et al. (1983). Twenty µg of *Hin*dIII-, *Dra*I-, *Pst*I- and *Eco*RI-digested DNA samples was separated on 1% (w/v) agarose gels and blotted to positively charged nylon membranes (Boehringer Mannheim GmbH, Mannheim, Germany). Filters were probed with digoxigenin (DIG)-labeled (Boehringer Mannheim) fragments of the coding region of *nptII* or *CTV-CP* prepared by PCR, following the supplier's instructions.

Leaf tissue from transgenic plants was used to obtain crude protein extracts for Western analyses. Extraction buffer comprised 0.1 M Tris-HCl, pH 6.8, and 1 mM phenylmethylsulfonyl fluoride. Protein extracts were fractionated by electrophoresis on SDS-PAGE (14% polyacrylamide) and electroblotted to Immobilon-PVDF membranes (Millipore, Bedford, MA) by means of a semidry transfer system (Bio-Rad, Hercules, CA), following the supplier's instructions. Immunodetection was performed with the monoclonal antibody MCA-13 (kindly supplied by Dr. S. Garnsey, Horticultural Research Laboratory, Orlando, FL) to the CTV coat protein (Permar et al. 1990) as primary antibody, and goat anti-mouse IgG (Boehringer-Mannheim) conjugated with alkaline phosphatase as secondary antibody.

Results

Improvement of transformation frequency in sour orange

In preliminary experiments, we established that CM was the best medium for cocultivation of sour orange explants with *Agrobacterium* (results not shown). To increase genetic transformation frequency in sour orange after cocultivation, several treatments were performed and evaluated.

After cocultivation, stem segments from 1-year-old sour orange seedlings were transferred to three selection media: SRM1, SRM2 and SRM3. The highest transformation frequency was obtained when the explants were cultured in SRM2 (Table 1). This medium favoured callus formation at the cut ends of the explants from which shoot regeneration occurred. In SRM1 or SRM3, explants showed direct shoot regeneration with little callus formation at the wounds. We used SRM2 as the selection medium in further experiments.

We compared transformation frequency following cocultivation and selection at 26 ºC with cocultivation at 22 ºC and the first month of selection at 22 ºC. Cocultivation or selection at 22 ºC did not significantly alter transformation frequency compared with cocultivation or selection at 26 ºC (Table 2). A temperature of 26 ºC was used for cocultivation and selection in further experiments.

Age of the plant material used as the source of explants significantly affected sour orange transformation (Table 3). Among the explants examined, explants from 4-month-old seedlings grown in the greenhouse showed the highest transformation frequency, whereas the lowest transformation frequency was observed in 2-week-old epicotyl segments from *in-vitro*-grown seedlings. However, these data were not significantly different from those obtained with explants from 12-month-old seedlings grown in the greenhouse. After 1 month in SRM2, however, most epicotyl segments became necrotic and did not develop callus or shoots. Four-month-old stem segments were used as the source of tissue for transformation in further experiments.

Pre-culture of explants in two media (PC1 and PC2) prior to inoculation and cocultivation with *Agrobacterium* significantly decreased genetic transformation frequency in sour orange (Table 4). Transformation frequency was lower in explants pre-cultured in PC2 than in PC1, and this was directly related to the development of abnormal callus in explants pre-cultured in PC2. Formation of abnormal callus could be

Table 1. Influence of selection medium on transformation of sour orange explants.

Selection medium	Number of transformation events in 50 explants ± SE[1,2]	Percentage of explants with transformation events (%)[1,3]
SRM1 (1 mg l^{-1} BAP)	14 ± 1 a	16.25 a
SRM2 (1 mg l^{-1} BAP + 0.3 mg l^{-1} NAA)	26 ± 3 b	28 b
SRM3 (2 mg l^{-1} BAP)	8.5 ± 1.5 a	12.5 a

[1] Different letters denote significant differences ($P < 0.05$) in a Duncan range test.
[2] The data correspond to the mean of two experiments.
[3] Defined as the number of explants with transformation events (blue spots) per 50 *Agrobacterium*-inoculated explants × 100. The data correspond to the mean of two experiments.

Table 2. Effect of temperature during cocultivation and selection on transformation of sour orange explants.

Treatment	Number of transformation events in 50 explants ± SE[1,2]	Percentage of explants with transformation events (%)[1,3]
Coculture and selection at 26 °C	28.5 ± 0.5 a	28 a
Coculture at 26 °C, selection at 22 °C	28 ± 2 a	26 a
Coculture at 22 °C, selection at 26 °C	24 ± 1 a	23 a

[1] Different letters denote significant differences ($P < 0.05$) in a Duncan range test.
[2] The data correspond to the mean of two experiments.
[3] Defined as the number of explants with transformation events (blue spots) per 50 *Agrobacterium*-inoculated explants × 100. The data correspond to the mean of two experiments.

Table 3. Influence of age and type of explant source on transformation of sour orange.

Type and age of explants source	Number of transformation events in 50 explants ± SE[1,2]	Percentage of explants with transformation events (%)[1,3]
Two-week-old *in vitro*-germinated seedlings	12.3 ± 4.3 a	15 a
Four-month-old greenhouse-grown seedlings	93.3 ± 14.2 b	42.6 b
Twelve-month-old greenhouse-grown seedlings	24.6 ± 2.1 a	24 a

[1] Different letters denote significant differences ($P < 0.05$) in a Duncan range test.
[2] The data correspond to the mean of three experiments.
[3] Defined as the number of explants with transformation events (blue spots) per 50 *Agrobacterium*-inoculated explants × 100. The data correspond to the mean of three experiments.

attributed to excessive exposure of the explants to 2,4-D in the pre-culture, inoculation and cocultivation media.

Production of transgenic plants

Based on the above experiments we used a transformation procedure for sour orange that included cocultivation in CM medium of internodal stem segments from 4-month-old greenhouse-grown seedlings, and regeneration–selection in SRM2. Under these conditions, a transformation efficiency of 6.6 ± 1.1% was obtained when we used the binary vector p35SGUSINT, and 3.6 ± 1% when pBI121/*CTV-CP* was used as the vector system. Histochemical GUS assays allowed us to identify regenerated transgenic shoots, and PCR assays served to determine which regenerants carried the *CTV-CP* or *nptII* transgenes.

When plants were about 20–30 cm in height, Southern anal-

Table 4. Influence of pre-culturing explants on transformation of sour orange.

Treatment	Number of transformation events in 50 explants ± SE[1,2]	Percentage of explants with transformation events (%)[1,3]
No pre-culture	142 ± 6 c	82 c
Pre-culture of explants on PC1 medium (1 mg l^{-1} BAP + 0.3 mg l^{-1} NAA)	46.5 ± 3.5 b	40 b
Pre-culture of explants on PC2 medium (1 mg l^{-1} BAP + 0.3 mg l^{-1} 2,4-D)	3.5 ± 1.5 a	7 a

[1] Different letters denote significant differences ($P < 0.05$) in a Duncan range test.
[2] The data correspond to the mean of three experiments.
[3] Defined as the number of explants with transformation events (blue spots) per 50 *Agrobacterium*-inoculated explants × 100. The data correspond to the mean of three experiments.

yses were performed to confirm the stable integration of the *CTV-CP* (Figure 1) or *nptII* (results not shown) gene cassettes in the plant genome. Digestion with *Hin*dIII produced a 1.76-kb fragment corresponding to the *CTV-CP* cassette (Figures 1b and 1c). Digestion with *Dra*I, which has a unique restriction site in pBI121/*CTV-CP* (Figure 1c), generated hybridizing fragments that were composed of T-DNA and plant DNA flanking the integration site. As expected, each transgenic line revealed different integration patterns depending on the number of copies of the transgene integrated in the plant genome (Figure 1a). The length of the fragments varied depending on the location of the nearest *Dra*I site in the flanking plant DNA and the integrity of the inserted T-DNA (Figure 1a).

Expression of the *CTV-CP* gene was investigated by Western analysis, which demonstrated that, in most samples, a protein product of 25 kDa, corresponding to the CTV coat protein, was immunoreactive with the monoclonal antibody MCA-13 (Figure 2).

After 9 months growing in the greenhouse, all transgenic plants showed a normal phenotype, identical to that of control non-transformed sour orange plants (Figure 3).

Discussion

Phytohormones (mainly auxins) in the cocultivation medium seem to play an important role in inducing the proliferation of dedifferentiated cells competent for transformation in newly formed callus tissue from citrus explants (Peña et al. 1997). We showed that the presence of an auxin in the regeneration–selection medium further increased transformation frequency. Villemont et al. (1997) have postulated that T-DNA transfer or integration, or both, is a host cell cycle phase-dependent phenomenon and that only DNA-duplicating cells can integrate the T-DNA. Furthermore, they have reported that, for stable expression, further cell divisions and cell proliferation from the original transformed cell is essential. For sour orange, we speculate that the combination of BAP + NAA in the regeneration–selection medium is more favorable than BAP alone in stimulating cell divisions and re-differentiation from the transgenic competent cell to form a transformation event.

Pre-culture of explants in a medium with phytohormones prior to bacterial inoculation has been shown to increase genetic transformation frequency in many plants (Birch 1997), because it stimulates cell dedifferentiation and division, and thus competence for transformation. In sour orange, such preconditioning of the explants resulted in a stress response that was detrimental to transformation. Possibly, the endogenous content of phytohormones (together with the exogenous supply during cocultivation) was sufficient to activate cells at the cut ends of the explants. Further exposure to auxins during pre-culture in PC2 seemed to have a negative effect on the physiology of the explants. Moreover, the wound response was enhanced when bacterial infection was performed soon after cutting the explants, because production of phenolic compounds by the wounded cells and corresponding induction

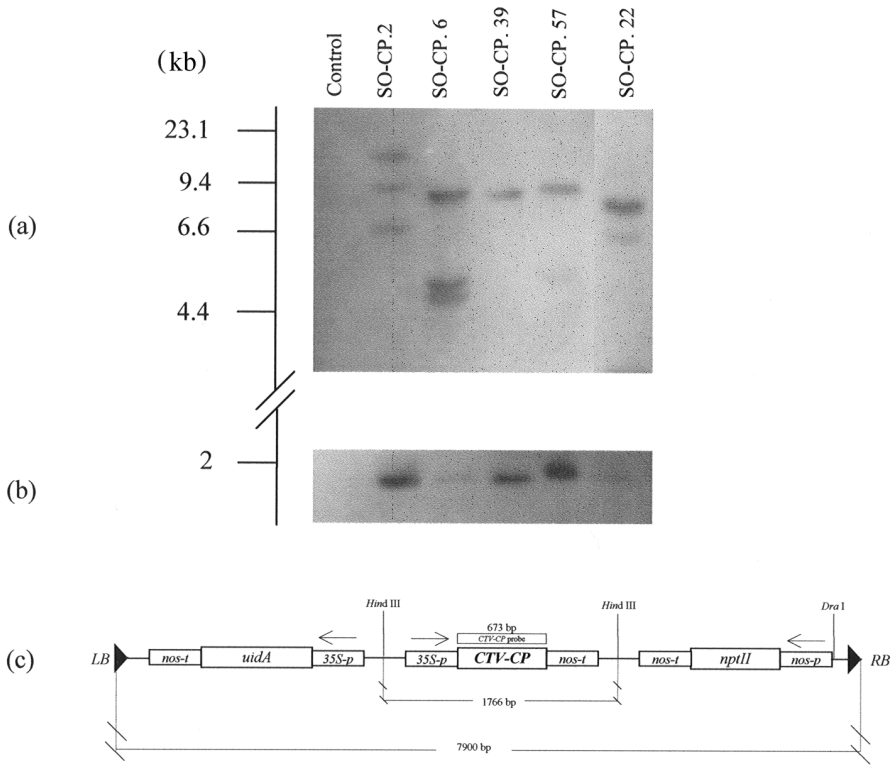

Figure 1. Southern blot hybridization of DNA extracted from leaves of five transformed plants carrying the *CTV-CP* gene cassette. Control is DNA extracted from a non-transformed sour orange plant. Molecular weight is indicated in kilobases (kb). (a) Southern blot from DNA digested with *Dra*I. The SO-CP.2, SO-CP.6, SO-CP.57 and SO-CP.22 transgenic plants have several incomplete T-DNA integrations, because the length of some fragments is lower than 7.9 kb. (b) Southern blot from DNA digested with *Hin*dIII. (c) Schematic representation of the T-DNA from pBI121/*CTV-CP*. Restriction sites for *Hin*dIII and *Dra*I, generated fragments, and position of the *CTV-CP* probe are indicated.

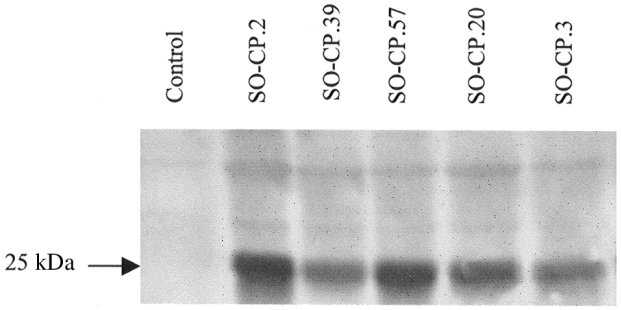

Figure 2. Western blot assay using MCA-13 monoclonal antibody against CTV coat protein (25 kDa). Crude protein extracts from a non-transformed plant (control) and from five transgenic lines.

of the virulence genes from *Agrobacterium* occurs almost simultaneously.

The age and source of the plant material were critical for the improvement of sour orange transformation. Age is strongly correlated with cell competence for transformation because higher concentrations of auxins are found in young tissues than in old tissues. Furthermore, aging decreases the susceptibility of plant cells to *Agrobacterium* infection (Huang et al. 1993, Cervera et al. 1998*b*). Both factors could explain the higher transformation frequency found in explants from 4-month-old greenhouse-grown seedlings than from 12-month-old seedlings. This finding seems to contradict the observation that the lowest transformation frequency was observed in explants from 2-week-old *in-vitro*-grown seedlings. In this case, however, necrosis was observed at the cut ends of the epicotyl explants just a few weeks after bacterial infection. Sangwan et al. (1992) proposed that the presence of newly synthesized cell wall is required for successful attachment of *Agrobacterium* to plant cells preceding transformation. Because juvenile tissues show a high percentage of actively dividing cells, it can be argued that *Agrobacterium* attachment to plant cells is more efficient in 2-week-old epicotyl segments than in older tissues. Consequently, *Agrobacterium* virulence would also be much higher, and this could be the cause of the necrotic response. A similar phenomenon has been observed when 5-week-old seedlings were used as the source of tissue for transformation of sweet orange (L. Peña et al., unpublished results). However, this effect was not observed when we used epicotyl segments of similar age for transformation of Carrizo citrange (Peña et al. 1995, Cervera et al. 1998a). The influence of the citrus genotype and its specific relationship with *Agrobacterium* could account for these differences.

Culture conditions affect *Agrobacterium*–plant cell interactions. Temperature greatly affects *Agrobacterium*-mediated gene transfer in *Phaseolus acutifolius* A. Gray and *Nicotiana tabacum* L. (Dillen et al. 1997). Dillen et al. (1997) concluded that the optimum temperature for cocultivation of *Phaseolus* callus with *Agrobacterium* was 22 ºC, because highest transient expression of the *uidA* transgene in tobacco leaves was obtained when cocultivation was performed between 19 and 22 ºC. Furthermore, they suggested that many transformation systems could be improved by optimizing the temperature. However, this seems not to be the case for citrus, or at least sour orange, because cocultivation or selection during the first month at 22 ºC did not increase transformation frequency. Alt-Moerbe et al. (1988) proposed that activation of the virulence genes of *Agrobacterium* is temperature-sensitive, and the same type of regulation has been suggested by Dillen et al. (1997) to explain their results. We have found that a critical step in the super-virulence of *A. tumefaciens* EHA 105 in citrus is *virG* induction (Ghorbel et al. unpublished results). However, Alt-Moerbe et al. (1989) have reported that substantial amounts of *virG* were detected even at 28 ºC. We conclude that temperature, at least between 22 and 26 ºC, is not a limiting factor in sour orange transformation mediated by *Agrobacterium*.

Investigation of factors affecting sour orange transformation has allowed us to enhance transformation efficiency about 50-fold compared with that obtained by Gutiérrez-E. et al. (1997). Differences in transformation efficiency between experiments performed with pBI121/*CTV-CP* and with p35SGUSINT vectors might be attributable to differences in T-DNA sizes. In conclusion, we have developed a reliable and efficient transformation system for sour orange that has allowed us to incorporate the coat protein gene of CTV in a sufficient number of plants and with variable levels of expression. We are now investigating the effect of coat protein accumulation in protection against CTV. To evaluate the efficiency of this strategy to control the virus, we have inoculated transgenic plants with severe CTV isolates producing the seedling yellows syndrome. We plan to examine the decline syndrome in varieties such as sweet oranges, grapefruits and mandarins, grafted on sour orange transgenic rootstocks and grown under field conditions.

Figure 3. A transgenic sour orange plant carrying the *CTV-CP* gene cassette shows a normal phenotype (right), similar to that shown by a control non-transformed plant (left).

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