Early Events in Agrobacterium-mediated Genetic Transformation of Citrus Explants

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Background and Aims Genetic transformation of plants relies on two independent but concurrent processes: integration of foreign DNA into plant cells and regeneration of whole plants from these transformed cells. Cell competence for regeneration and for transformation does not always fall into the same cell type/developmental stage, and this is one of the main causes of the so-called recalcitrance for transformation of certain plant species.

In this study, a detailed examination of the first steps of morphogenesis from citrus explants after co-cultivation with Agrobacterium tumefaciens was performed, and an investigation into which cells and tissues are competent for regeneration and transformation was carried out. Moreover, the role of phytohormones in the co-cultivation medium as possible enhancers of gene transfer was also studied.

Methods A highly responsive citrus genotype and well-established culture conditions were used to perform a histological analysis of morphogenesis and cell competence for transformation after co-cultivation of citrus epicotyl segments with A. tumefaciens. In addition, the role of phytohormones as transformation enhancers was investigated by flow cytometry.

Key Results It is demonstrated that cells competent for transformation are located in the newly formed callus growing from the cambial ring. Conditions conducive to further development of this callus, such as treatment of explants in a medium rich in auxins, resulted in a more pronounced formation of cambial callus and a slower shoot regeneration process, both in Agrobacterium-inoculated and non-inoculated explants. Furthermore, co-cultivation in a medium rich in auxins caused a significant increase in the rate of actively dividing cells in S-phase, the stage in which cells are more prone to integrate foreign DNA.

Conclusions Use of proper co-cultivation medium and conditions led to a higher number of stably transformed cells and to an increase in the final number of regenerated transgenic plants.

Key words: Agrobacterium tumefaciens, callus, cambium, cell cycle, citrange (Citrus sinensis × Poncirus trifoliata), citrus, competence, genetic transformation, sweet orange (C. sinensis), regeneration, S-phase, transgenic.

INTRODUCTION

Genetic transformation has been achieved for several citrus genotypes, including sweet orange (Citrus sinensis), sour orange (C. aurantium), trifoliate orange (Poncirus trifoliata), citrange (sweet orange × trifoliate orange), lemon (C. limon ‘Fino’), lime (C. aurantifolia), grapefruit (C. paradisi), alemow (C. macrophylla) and Cleopatra mandarin (C. reshni) (reviewed in Peña et al., 2003). However, transformation efficiencies are generally low, and several other economically important citrus species remain non-transformable, as for example clementine and satsuma mandarins (C. clementina and C. unshiu, respectively). The production of transgenic plants involves the stable integration of foreign DNA into the host genome and the subsequent regeneration of whole plants from the transformed cells. These two independent processes are critical for the successful generation of transgenic plants. Competence for regeneration is the first limitation, and indeed many recalcitrant species actually have very low or null regeneration frequencies. In citrus, epicotyl and internodal stem segments have been widely used to regenerate plants via organogenesis. Bud and shoot formation may occur as a result of direct morphogenesis (Moore et al., 1992; Goh et al., 1995; Pérez-Molpe and Ochoa-Alejo, 1997; García-Luis et al., 1999) or from callus tissue formed from the explants (García-Luis et al., 1999; Moreira-Dias et al., 2000). García-Luis and co-workers have also established, in a series of detailed studies, that the citrus genotype, and the culture conditions and media determine the regeneration pathway (García-Luis et al., 1999; Bordón et al., 2000; Moreira-Dias et al., 2000). Moreover, these authors showed that the addition of the cytokinin 6-benzylaminopurine (BAP) was a requisite for optimal shoot regeneration, while the contribution of auxins seemed to be marginal.

Another limitation of the process could be the competence for transformation of citrus cells and tissues. Apart from the influence of the genetic component, it is known that explant wounding and proper phytohormone treatments enhance host cell susceptibility to Agrobacterium (Potrykus, 1991; Sangwan et al., 1992). Although citrus species are not natural hosts of Agrobacterium tumefaciens, it has been
demonstrated that this bacterium is able to transform citrus cells both in vivo and in vitro, and that the disarmed succinamopine strain EHA 105 is a very efficient vector to introduce foreign DNA into citrus cells (Cervera et al., 1998b; Ghorbel et al., 2001). However, it should be emphasized that not every transformed cell can be regenerated into plants, and that competent cells for regeneration are not necessarily transformable. Therefore, transgenic plants can be obtained from those cells that are competent for both transformation and regeneration, and transformation efficiency is dependent on the degree of overlap between both types of cells (Potrykus, 1991). In spite of the high number of studies showing organogenesis and attempting genetic transformation in citrus, little is known on how these two events together contribute to the success of the entire transgenic plant production process. The use of the gfp reporter gene allowed us to localize competent cells for transformation in callus presumably formed from the cambium tissue of citrus explants in early steps after cocultivation with Agrobacterium (Ghorbel et al., 1999). Furthermore, treatments favouring the development of such callus tissue, such as co-cultivation in a culture medium rich in auxins and exposure of the explants to darkness during the first 2–4 weeks after bacterial inoculation, greatly increased transformation frequencies and consequently regeneration of transgenic shoots (Cervera et al., 1998a; Ghorbel et al., 1999). In this work, a highly responsive citrus genotype and well-defined tissue culture conditions have been used to perform a histological examination of morphogenesis from citrus explants after co-cultivation with Agrobacterium in a medium rich in auxins. For the first time, experimental proof of the cambial origin of competent cells for transformation in citrus is provided. Moreover, flow cytometry has been used to investigate the role of auxins in the co-cultivation medium as possible enhancers of transformation. It gives evidence that the higher rate of actively dividing cells in S-phase during co-cultivation in a medium rich in auxins coincides with much higher stable integration of the foreign DNA, and shows that the proper combination of phytohormones in the co-cultivation medium shifts the cells at the cut ends of the explants into a competent state for integrative transformation.

MATERIALS AND METHODS

Plant materials, bacterial strain and culture media

Five-week-old Carrizo citrange [Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.] seedlings, germinated and grown in vitro as described in Peña et al. (1995), were used as a source of tissue for transformation. Agrobacterium tumefaciens EHA 105 (Hood et al., 1993) carrying the binary vector p35SGUSINT (Vancanneyt et al., 1990) was used as vector system for transformation. The uidA gene driven by the CaMV 35S promoter and terminator sequences served as reporter gene. The intron in the uidA gene of p35SGUSINT blocks its expression in A. tumefaciens. The NPTII gene driven by the NOS promoter and terminator sequences was used as the selectable marker gene. Bacteria were cultured overnight at 28 °C in LB medium containing 25 mg L⁻¹ kanamycin and 25 mg L⁻¹ naldixic acid. Bacterial cells were pelleted at 2000 g for 10 min, resuspended, and diluted to 4 × 10⁷ cell mL⁻¹ in inoculation medium (IM). This medium consisted of MS salts (Murashige and Skoog, 1962), 100 mg L⁻¹ myo-inositol, 0.2 mg L⁻¹ thiamine–HCl, 1 mg L⁻¹ pyridoxine–HCl, 1 mg L⁻¹ nicotinic acid and 3 % (w/v) sucrose, pH 5.7.

Three different co-cultivation media (CM) were tested: CM1 consisted of IM plus 8 g L⁻¹ agar; CM2 consisted of IM plus 1 mg L⁻¹ BAP and 8 g L⁻¹ agar; and CM3 consisted of IM plus 2 mg L⁻¹ indole-3-acetic acid (IAA), 1 mg L⁻¹ 2-isopentenyl-adenine (2-ip), 2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 8 g L⁻¹ agar. Shoot regeneration medium (SRM) contained the components of IM plus 3 mg L⁻¹ BAP and 10 g L⁻¹ agar, and was also supplemented with 100 mg L⁻¹ kanamycin, for selection of transgenic events, and 250 mg L⁻¹ vancomycin plus 500 mg L⁻¹ cefotaxime to control bacterial growth.

Transformation and regeneration

Carrizo citrange epicotyl segments (about 1 cm long) were cut transversely and incubated for 15 min with the bacterial suspension, blotted dry on sterile filter paper, and placed horizontally on CM plates. After 3 d of co-cultivation, the explants were transferred to SRM. Cultures were maintained in darkness for 2–4 weeks at 26 °C and then transferred to the following conditions: 16-h photoperiod, 45 μE m⁻² s⁻¹ illumination, 60 % relative humidity and 26 °C. Explants were subcultured to fresh medium every 3 weeks.

To recover whole transgenic plants, emerging shoots were shoot-tip grafted in vitro onto Troyer citrange [C. sinensis (L.) Osb. × Poncirus trifoliata (L.) Raf.] seedlings. A new grafting of the plants grown in vitro onto vigorous rootstocks allowed the rapid acclimatization and development of plants under glasshouse conditions (Peña et al., 1995; Peña and Navarro, 1999). Southern blot analyses were performed to confirm the stable integration of the uidA transgene in the regenerated plants, as reported in Domínguez et al. (2002).

GUS assays

Explants transformed with Agrobacterium and basal portions from regenerating shoots were assayed for GUS activity 1–2 and 6 weeks, and 4 months after bacterial inoculations, respectively. GUS assays were performed by overnight incubation of the plant materials at 37 °C in 2 mM X-Gluc solution as described by Jefferson et al. (1987). Explants were observed under a stereomicroscope and each blue spot was considered as an independent transformation event. Likewise, shoots showing solid blue colour after GUS analysis of their basal ends were considered putatively transgenic.

Histological analysis

Cut ends of Agrobacterium-inoculated and non-inoculated explants (0.5 cm long) were treated for histological studies following standard procedures (Jensen, 1962).
Explant pieces were fixed by immersion in FAA (commercial formalin: glacial acetic acid: 50% ethyl alcohol; 1:1:18). Tissue dehydration was accomplished by submerging samples in a series of ethanol: tertiary butyl alcohol, and embedding them in small blocks of solid paraffin. Paraffin-embedded tissues were then trimmed on a rotary microtome in 10-μm thick sections. Sections were affixed to slides and stained by using the periodic acid–Schiff’s reaction, in which tissue cellulose, hemicellulose, and starch become red. A cover slip was mounted over the preparations using Canada balsam. After drying in the dark for at least 1 d, samples were ready to be observed under the microscope.

Flow cytometry

Nuclei were isolated from 1-mm-thick sections cut from the apical and basal ends of 1-cm-long epicotyl segments. Samples were taken from Agrobacterium-inoculated and non-inoculated control epicotyl segments the day of inoculation (day 0) and 1, 2, 3, 4 and 7 d after inoculation. Plant material was chopped with a razor blade in presence of a nuclei isolation solution (high resolution DNA kit type P solution A; Partec, Münster, Germany). The suspension was filtered through a 30-μm nylon mesh and nuclei were stained with DAPI solution (4’,6-diamidino-2-phenylindol; high resolution DNA kit type P solution B; Partec). Flow analysis was performed in a one channel flow cytometer ploidy analyser (Partec) (λmaxex = 340 nm, λmaxem = 465 nm). The flow cytometer was equipped with a high-pressure HBO 100 W mercury lamp and KG1 and BG38 filters. Flow analyses were performed with at least five samples per treatment with at least 5000 nuclei per histogram. Each experiment was repeated three times. The fluorescence histograms were evaluated by means of DPAC V2/0 Software (Partec), which calculates the absolute values and the percentage of cell cycle phases (G1, S and G2/M) from the histogram data. Trout erythrocytes were used as internal DNA standard.

RESULTS

Histological analysis reveals that cells competent for regeneration are located in callus tissue formed from the cambium

After culture in CM3 for 3 d, histological sections showed that cell division was first seen in the cambium, and several cell layers were formed along the whole cut surface of the explants (Fig. 1A). On the seventh day of culture in SRM, these explants showed an intense cell proliferation in the cambium that resulted from periclinal divisions of the original cambial cells (Fig. 1B). Then, a characteristic ring of callus tissue was visible between the phloem and the xylem from an upper view of the cut end of the explant. One week later, not only periclinal but also anticlinal divisions were occurring in the vascular cambium, and the callus ring became conspicuous macroscopically (Fig. 2C and D). Interestingly, callus proliferation was much higher at one of the cut ends of the explants. At that time (14 d in SRM), most callus cells were big and vacuolated but meristematic cells were also observed, being typically small and relatively isodiametric, and containing dense cytoplasms and prominent nuclei (Fig. 1C). These meristoids differentiated and developed into adventitious buds after 4 weeks of culture in SRM (Fig. 1D).

When explants were cultured in CM1 or CM2, the process of morphogenesis was different. Mitotic activity at the cambium started soon after transferring the epicotyl segments to SRM, and adventitious buds formed onto the cambial zone near to the cut surface, though also through indirect organogenesis (Fig. 1E). Most of these buds differentiated within the first 2–4 weeks of culture in SRM, as reported by García-Luis et al. (1999). In this case the prominent callus coming from the cambium was not clearly visible at the cut surface of the explants.

Cells competent for transformation are also located in callus tissue formed from the cambium

When explants were inoculated with Agrobacterium and co-cultivated in any of the three CM, the regeneration process was similar to that described above but cell division and callus formation proceeded more slowly, and organogenesis started to be seen several weeks later. This could be attributed to the detrimental effect of the bacteria over citrus cells and tissues. After 21 d of incubation in SRM, histological examination of GUS-assayed explants revealed that a variable proportion of callus cells coming from the cambium showed GUS activity, indicating that they were genetically transformed. Furthermore, only cells from this callus tissue showed GUS staining (Fig. 2A and B). Attempts to localize GUS expression in meristematic cells were unsuccessful, maybe because of difficulties for substrate penetration, the low concentration of the reaction product, and the tendency of the substrate to accumulate in xillary elements formed in the cambial callus, as it has been reported by other authors (Geier and Sangwan, 1996; Guivarc’h et al., 1996). The presence of an intron in the uidA gene of p35SGUSINT prevented GUS expression in Agrobacterium cells, which could possibly remain in citrus tissues 2 weeks after co-cultivation. GUS activity was never detected in control explants.

Macroscopic observation of the cut ends of the explants at 14 d of incubation in SRM also showed that GUS spots were restricted to callus tissue coming from the cambial ring (Fig. 2C). Moreover, transformation events were mainly located in one cut surface, which was always the one developing more cambial callus (Fig. 2D).

Cell-cycle stage of cells at the cut surface of the explants was influenced by phytohormones in the co-cultivation medium

Flow cytometric analysis was performed to investigate entry into the cell cycle and ratio of cells in S-phase at the cut ends of the explants co-cultivated in the different culture media from 0 to 7 d in culture. Cells located at the cut surface were stained with a DNA-specific fluorochrome and examined with a ploidy analyser, that calculated the rate of
cells in G₁, S and G₂+M phases depending on the nuclear DNA content of each sample. Cells gave characteristic peaks of fluorescence emission corresponding to 2C nuclei (G₁ phase) and 4C (G₂+M nuclei) with an S phase transition between these two peaks (Fig. 3). Only data on frequency of citrus cells at S-phase were analysed here, because interest was only in T-DNA transfer. The absolute requirement of S-phase (DNA duplication) for Agrobacterium-mediated transformation has been demonstrated (Villemont et al., 1997). In previous experiments, it was observed that one of the cut ends of the explants was developing more callus than the other. To assess whether one of the cut ends was more prone to entry into the cell cycle, explants were transferred to CM3 for 3 d and then to SRM, pointing out the basal and apical orientation of each end. At day 0 most nuclei were at G₁-phase (72.6 ± 0.4 %), though a small proportion was at S-phase (about 15 %) and at G₂+M (about 10 %), probably due to a wound response effect that was detected by the analyser. The ratio of nuclei in S-phase progressively increased from 0 to 3 d, and decreased after transfer to SRM in both cases, but basal ends had a significantly higher rate of nuclei in S-phase during culture in CM3, as shown in Fig. 4, indicating that basal ends were the main contributors to the higher rate of nuclei in S-phase observed when explants were co-cultivated in the medium rich in auxins. Similar differences were found in the rate of nuclei in S-phase between basal and apical ends when explants were co-cultivated in CM1 or CM2 (results not shown).

In subsequent experiments, the frequency of cells in S-phase was compared in basal ends co-cultivated in CM1,
CM2 and CM3. Explants were either inoculated with *Agrobacterium* and transferred to CM1, CM2 or CM3, or directly cultured in CM1, CM2 or CM3 for the following 3 d. At day 1, no significant differences were shown in the frequency of nuclei at S-phase when explants were cultivated in any of the three CM. However, drastic differences were observed after 2 d in CM; whereas the ratio of S-phase cells did not change too much in CM1 and CM2, a significant increase of nuclei at S-phase was shown in CM3. There were about twice as many S-phase cells in CM3 as there were in CM1 or CM2. This tendency remained at day 3 in non-inoculated explants but not in *Agrobacterium*-inoculated ones, and it started to decrease after transferring the explants to SRM at day 4 and beyond (Fig. 5). The differences observed at day 3 in the response of inoculated and non-inoculated explants can be attributed to the detrimental effect of the bacteria over citrus cells.
Stable transformation of cells at the cut end of the explants was also influenced by phytohormones in the co-cultivation medium.

To determine whether transformation was influenced by the co-cultivation medium used, *Agrobacterium*-inoculated explants were subjected to histochemical GUS assays after co-cultivation in CM1, CM2 or CM3 for 3 d and selection in SRM for 6 weeks. As shown in Table 1, the highest number of stable transformation events was found in basal ends from the explants co-cultivated in CM3. In general, basal ends had many more GUS spots than the corresponding apical ends in the three culture media used. It can be noted that apical ends from explants co-cultivated in CM3 had as many GUS events as basal ends from the other two culture media. This coincided with a similar rate of cells at S-phase in apical ends co-cultivated in CM3 and basal ends co-cultivated either in CM1 or CM2. For instance, at 3 d of culture, apical ends in CM3 showed 44.7 ± 6.9 % nuclei at S-phase, and basal ends in CM2 showed 41.0 ± 5.9 % nuclei at S-phase. These results strengthen the correlation between the ratio of cells at S-phase and stable transformation.

At 6 weeks post-inoculation, it was possible to observe bud and shoot regeneration in certain cases, but most shoots were escapes (non-transformed regenerants). Escape regeneration was especially relevant from apical ends of explants co-cultivated in CM1 and CM2, which coincided with the lowest transformation frequency (Table 1) and scarce callus production. Four months after *Agrobacterium*-inoculation, 40 GUS+ shoots had been recovered from the 30 explants co-cultivated in CM3. Stable integration of the uidA transgene was demonstrated by Southern blot analysis (results not shown). At that time only 15 and 12 GUS+ shoots were recovered from the same number of explants co-cultivated in CM1 and CM2, respectively.

### DISCUSSION

In recent years, several laboratories have been involved in trying to establish genetic transformation systems for economically important citrus genotypes, because it could be an essential tool to investigate gene function and for genetic improvement of the crop. In spite of the efforts invested, transformation efficiencies were usually low. This has been probably due to the lack of studies attempting to dissect the important events necessary to make citrus cells...
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competent for both transformation and regeneration. In previous studies it had been observed that transgenic cells in citrus epicotyl and internodal segments were localized in callus tissue that seemed to be of cambial origin, suggesting that treatments favouring the development of such callus could enhance transformation (Cervera et al., 1998a; Ghorbel et al., 1999). Here cell competence and the role of phytohormones for transformation and regeneration of shoots from citrus explants have been specifically investigated.

Although in all culture media tested here regeneration proceeded through indirect organogenesis, co-cultivation in media without phytohormones or with BAP at 1 mg L⁻¹ and subsequent culture in medium with BAP at 3 mg L⁻¹ promoted a faster differentiation response and bud formation. A conspicuous callus was formed from the cambium cells in explants co-cultivated in the medium rich in auxins and bud differentiation did not occur during the first weeks after Agrobacterium-inoculation. This is consistent with the totipotent state of cambial cells that were able to rapidly respond to external stimuli during culture in vitro. Thus competence for regeneration and cell division were strongly related in citrus epicotyl segments. Interestingly, GUS-assayed explants showed that transgenic sectors were only localized in callus cells coming from the cambium, clearly indicating that the development of the cambial callus was also essential to obtain transformation from citrus explants. Therefore, cells competent for transformation and for regeneration were localized in the same callus tissue.

Flow cytometry analysis revealed that co-cultivation in the medium rich in auxins rapidly favoured cell entry into the cell cycle because cells at the cut ends were actively dividing at a higher rate and duplicating their DNA after 2 d of culture in CM3. Thus, addition of auxins to the culture medium promoted active cell division and dedifferentiation. This coincided with a much higher transformation frequency in the cut ends of such explants. On the other hand, a lower ratio of S-phase cells was found in the explants during co-cultivation in CM1 and CM2, and much lower stable transformation was obtained. Taken together, our data suggest that dedifferentiation is crucial for transformation of citrus cells. This corroborates the results reported by Villemont et al. (1997) who showed that the host cell S-phase is necessary for T-DNA transformation in Petunia leaf mesophyll cells.

Another remarkable observation was that callus from the cambium and therefore stable transformation was preferentially produced at the basal end of the explants, probably reflecting an increase in auxin concentration at the basal ends due to the basipetal auxin transport. This polar transport of auxins has been previously reported in in vitro cultures of flower buds from tobacco (Smulders et al., 1988), hypocotyl segments from sunflower (Suttle, 1991) and epicotyl segments from pea (DeKathen and Jacobsen, 1995). Then, it could be hypothesized that citrus cells at the explants would be highly responsive not only to an exogenous auxin supplement but also to endogenous auxin accumulation.

García-Luis and co-workers have assessed critically a number of factors that contribute to morphogenesis from citrus explants, such as explant orientation and polarity, hormone requirements, illumination conditions and genotype (García-Luis et al., 1999; Bordón et al., 2000; Moreira-Dias et al., 2000). They have shown that, in the absence of added BAP, no callus was formed at the apical end of the explants, very few buds were visible under the dissecting microscope, and most of them remained quiescent without becoming a shoot, indicating that cytokinin application was critical for callus formation and bud differentiation, with auxins only having a marginal effect (García-Luis et al., 1999). However, the best conditions for regeneration are not necessarily the best for transformation. In fact, the results of the work presented here demonstrate that auxins play an essential role in the competence of the cell to transform in citrus. The promotive effect of phytohormones in shifting cells to a competent state for transformation is well known, especially in the case of auxins (Sangwan et al., 1992). It is shown here that the addition of BAP promoted cell division and rapid differentiation of buds, as shown by García-Luis et al. (1999), but most of these buds were escapes, indicating that cell division and callus formation was not enough to ensure efficient transformation. Only buds regenerating later, after more prominent callus formation, were transgenic. This result could explain, at least in part, the recalcitrance for transgenic plant production of most citrus genotypes. In recently published works on transformation of different sweet orange and grapefruit varieties, regeneration of escape shoots varied between 60 % and >90 % (Costa et al., 2002; Yu et al., 2002; Almeida et al., 2003). The results of the work presented here show that dedifferentiation was required to shift citrus cells to a competent state for stable transformation, and that dedifferentiation was especially triggered by the addition of auxins (mainly 2,4-D; authors’ unpublished results) to the co-cultivation medium and possibly by their endogenous accumulation in specific explant sectors. At the same time, co-cultivation in the medium rich in auxins could prevent early differentiation of citrus cells into meristemoids that would result in regeneration of escapes. Once transformation at high frequency was achieved in the proper co-cultivation medium, addition of BAP to the regeneration medium could promote differentiation of the transgenic events into buds and shoots.

Villemont et al. (1997) reported that pre-cultivation of Petunia leaf explants in a medium with 2,4-D and BAP during 2–3 d before co-cultivation with Agrobacterium drastically enhanced genetic transformation. During pre-culture, cells were actively dividing and duplicating DNA, and at the moment of bacterial inoculation phytohormone-activated cell nuclei were being recruited into S-phase. In the experiments reported here, co-cultivation in a medium rich in auxins had the same effect. Proper phytohormone treatments have been used to overcome the reduced transformation susceptibility of the Arabidopsis mutant uvh1, which was previously considered resistant to Agrobacterium-mediated transformation (Chateau et al., 2000). It would be interesting to investigate how phytohormone treatments (especially 2,4-D) affect cell dedifferentiation and T-DNA transfer in explant cells from citrus genotypes that remain recalcitrant to genetic transformation.
LITERATURE CITED


