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The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L.Mill.) is genotype and procedure dependent

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Abstract A protocol avoiding the feeder-layer cell system was optimized for *Agrobacterium*-mediated transformation of tomato cotyledonary explants. Over 500 transgenic plants from five tomato cultivars were regenerated in 15 independent experiments. Depending on both genotype and procedure, transformation frequencies ranged from 1.8% to 11.3%. The optimal transformation rate was obtained by inoculating explants with a bacterial suspension in exponential growth (D_{600} = 10^2–10^3 cells/ml) and transferring cotyledon explants to fresh selective regeneration medium every 3 weeks. The ploidy level of both tomato genotypes used as explant source and primary transformants, was studied by flow cytometry. The inbred lines and cultivars were diploid but a polysomatic pattern in the cotyledon explant was confirmed. The rate of tetraploid transgenic plants ranged from 24.5% to 80% and depended on both the genotype and the transformation procedure. Surprisingly, the percentages of transformed plants with higher ploidy levels were not related to the proportion of 4C and 8C nuclei in the cotyledonary tissue. For some genotypes the optimisation of the transformation rate resulted in an increase of tetraploid transgenic plants. Results obtained in this work indicate the convenience of checking the ploidy level of the primary transformants before performing basic studies or introducing tomato transgenic material in a breeding program.

Keywords Tomato · Cotyledonary explant · *Agrobacterium*-mediated transformation · Ploidy level · Fertility

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Introduction

In *Agrobacterium*-mediated transformation of tomato, the transformation efficiency is not a limiting factor as several authors reported transformation rates ranging from 5% for cv ATV847 (Ultzen et al. 1995) to 32.9% for cv Moneymaker (Ling et al. 1998). The method described by Fillatti et al. (1987) for cv UC 82b, gave rise to 70% of explants that produced at least one kanamycin-resistant shoot. In spite of these advances, Birch (1997) emphasized that some practical requirements have to be considered when evaluating genetic transformation as an alternative technique in a breeding program. For the above author, another relevant criterion in achieving practical results with this approach is the simplicity and reproducibility of the transformation method in order to reduce cost and avoid inherently variable manipulations. In this context, the usefulness of the cotyledon as an explant source is of great relevance, considering the availability of seeds, the reproducibility of the sterilization and germination conditions, and the possibility of establishing a defined and controlled developmental state of the explant for each transformation experiment. For these reasons, cotyledons are the favourite explant source in most of the protocols described in the literature.

The major technical challenge facing the practical application of plant transformation is the development of a method that produces a high proportion of transgenic plants without collateral genetic variations. In tomato, Ultzen et al. (1995) transformed cotyledon explants of cv ATV847, and reported that only 60% of the transgenic tomato retained the diploid level. The high frequency of polyploid transgenic plants (40% in this last work) could be due to the mixoploid nature of the cotyledon tissue (Smulders et al. 1994, 1995). Moreover, genetic phenomena like endoreduplication and endomitosis occuring during the cell proliferation and differentiation of the organogenic callus might also explain the appearance of polyploid plants in genetic transformation experiments.

Another constraint to tomato transformation is the limitation of the foreign gene transfer to a reduced num-

ber of genotypes. Some cultivars have been extensively used in transformation studies via *Agrobacterium*. The cultivar "UC 82b", well known for its regenerating capacity, has been transformed by McCormick et al. (1986), Fillatti et al. (1987) and Hamza and Chupeau (1993); cv "Moneymaker" has been used as a receptor by Van Roekel et al. (1993), Frary et al. (1996) and Ling et al. (1998); and the cultivar "Aisla Craig" has been transformed by Bird et al. (1988) and Lipp and Brown (1993). The possibility of applying genetic transformation to other cultivars as well as tomato inbred lines would be of great interest for breeders of both companies and public institutions, as it would increase the spectrum of genetic diversity available for transformation experiments.

The first aim of our work was to establish a simple and reproducible *Agrobacterium*-mediated protocol to transform tomato using the cotyledon as an explant source. Avoiding the feeder-layer cell system, we reduced the demanding and complex manipulation but maintained a high production of independent transformants. Applying this protocol, the transformation of two tomato cultivars and three breeding lines was achieved. We analyzed the ploidy level of 560 primary transformants from 15 independent experiments and observed that the regeneration of diploid primary transformants from cotyledon explants ranged from 20% to 75%, depending on the genotype used as an explant source and the transformation procedure. The screening of the population of primary transformants for their fertility was also performed under greenhouse conditions.

Materials and methods

Plant regeneration

Seeds from cv p73 (kindly provided by Dr. Mª José Diez, UPV, Valencia, Spain), cv UC82b (Intersemillas S.A. company) and inbred lines LP0, LP2 and LP4 (Nunhems Zaden B.V. company) were used as starting material.

Seeds were surface-sterilized by immersion for 30 min in a diluted commercial bleach solution (5% w/v sodium hipoclorite) with 0.1% (v/v) of 7X-O-matic (Flow laboratories). The treated seeds were then rinsed three times with sterile deionized-distilled water. After sterilization, seeds were germinated in the darkness (190 mm × 20 mm test tubes) on solid medium (GM, Table 1) consisting of Murashige and Skoog's (1962) inorganic basal salts, supplemented with 1% (w/v) sucrose. This medium was solidified with 0.8% (w/v) Agar Industrial (Pronadisa) and its pH adjusted to 5.7 before autoclaving at 115 °C for 30 min.

After 3 to 4 days, when the radicle emerged and curved into the medium, test tubes were transferred to a tissue-culture chamber at 24 °C ± 2 °C under cool-white fluorescent and Gro-Lux light (60–100 μE m^{-2} s^{-1}; 16-h light/8-h dark photoperiod). All subsequent in vitro culture steps were conducted under the same conditions. Cotyledons from 12 to15 day old seedlings were transversally cut into two segments (when the first primary leaf was expanded). Aproximately 24 cotyledon explants (proximal and distal parts) were cultured in a 90-mm Petri dish. The adaxial parts were placed onto the pre-culture medium (PCM, Table 1) in a 15 mm × 90 mm plastic Petri dish for 2 days of pre-incubation. This preculture system was previously described in tomato by McCormick et al. (1986), Hamza and Chupeau (1993) and Van Roekel et al. (1993).

Bacterial strain and plasmids

The *Agrobacterium tumefaciens* LBA 4404 strain was used in all the genetic transformation experiments. All binary vectors described in this study contained the nptII gene as a selection marker and three different plasmids were used in 12 transformation experiments: plasmid pBin19 harbouring a salt-tolerance related gene (HAL3, provided by Dr. Serrano; IBMCP Valencia) from *Saccharomyces cerevisiae*, under control of the 35S Cauliflower Mosaic Virus promotor; plasmid pBin19 harbouring a salt-stress induced gene (TAS14, provided by Dr. Pintor Toro; IRNA Sevilla) from tomato (*Lycopersicon esculentum* Mill.); and plasmid pVDH303 (provided by Van Der Have company) harbouring, from the left to the right border, the nptII gene as a selection marker (under control of the constitutive 35S Cauliflower Mosaic Virus promotor), the *asnA* gene from *Escherichia coli* encoding an asparagin synthetase (under control of the constitutive *PCpea* promotor) and the reporter gene GUS (under control of the constitutive 35S Cauliflower Mosaic Virus promotor). Details of these constructions will be published elsewhere.

Bacteria were grown overnight in LB medium (100 ml flask) with antibiotics (rifampicin 40 $mg \cdot l^{-1}$ and kanamycin 100 $mg \cdot l^{-1}$). In transformation experiments with method A, the overnight cul-

Table 1 Composition of culture media in transformation experiments of *L. esculentum* L. Mill. mediated by *A. tumefaciens*

Item	GM[b]	PCM[c]	CCM[d]	WM[e]	SIM[f] not sel.	SIM[g] sel.	RM[h] not sel.	RM[i] sel.
MS[a]	100%	100%	100%	100%	100%	100%	100%	100%
Sucrose (g)	10	30	30	20	30	30	20	20
Myo-inositol (mg)	–	100	100	100	100	100	100	100
Vitamins SH (ml)	–	10	10	–	10	10	–	–
IAA (mg)	–	4	4	–	4	4	0.1	0.1
Kinetin (mg)	–	4	4	–	4	4	–	–
Zeatin (mg)	–	–	–	–	1	1	–	–
Kanamycin (mg)	–	–	–	–	–	100	–	50
Cefotaxim (mg)	–	–	–	600	400	300	–	–
Acetosyringone (ml)	–	–	2	–	–	–	–	–
Agar Industrial (g)	8	–	–	–	–	–	8	8
Agar Bacteriol. (g)	–	8	8	–	8	8	–	–

[a] MS: Murashige and Skoog (1962)
[b] GM, germination medium
[c] PCM, preculture medium
[d] CCM, co-culture medium
[e] WM, washing medium
SIM, shoot induction medium: [f] not sel.: not selective; [g] sel.: selective
RM, rooting medium: [h] not sel.: not selective; [i] sel.: selective

ture was re-suspended and diluted with LB medium to D_{660} = 0.70 to 0.80 for inoculation with 200 μM of acetosyringone; the final kanamycin concentration was 25 mg·l^{-1}. In transformation experiments with method B, the overnight culture was diluted to D_{660} = 0.10 to 0.15 and grown to D_{660} = 0.20 to 0.30 in LB medium, supplemented with kanamycin 25 mg·l^{-1}, approximately for 4–5 h. Bacteria were re-suspended and directly used at this concentration and acetosyringone concentration adjusted to 200 μM before inoculation.

Transformation procedure

After 2 days in the dark on PCM, the explants were carefully submerged in the *Agrobacterium* inoculum (30 ml) in a 380-ml sterile glass vessel for 8–10 min (swirling the jar gently). They were blotted dry on sterile filter paper and transferred to the co-culture medium (CCM, Table 1). After 24 to 48 h in the dark at 26 °C, the explants were transferred to sterile glass jars containing 150 ml of the washing medium (WM, Table 1) for 10 min. They were blotted dry on sterile filter paper and cultured on the organogenic medium without selective pressure (SIM not selective, Table 1) in a 15 mm × 90 mm plastic Petri dish. After 48 h in photoperiodic conditions, explants were transferred to the selective shoot-induction medium with 100 mg·l^{-1} of kanamycin (SIM selective, Table 1). In transformation experiments with method A, 12 explants were cultivated in 380-ml glass jars containing approximately 40 ml of the SIM selective, and every 4 weeks the explants were subcultured in the same conditions. In transformation experiments with method B, 24 explants were cultivated in a 25 mm × 90 mm plastic plate containing approximately 20 ml of the selective SIM and every 3 weeks the explants were subcultured under the same conditions.

Individual shoots were excised and transferred to rooting medium (RM not selective) without kanamycin or cefotaxim. Each elongated shoot was transversally cut for further propagation: one part of the axilary shoot was cultured on the antibiotic-free RM, and the other axilary shoots of the same genotype were used to perform a rooting test on RM supplemented with 50 mg·l^{-1} of kanamycin (RM selective, Table 1).

Analysis of transgenic plants

The collection of the putative transgenic tomato plants proceeding from each independent experiment was analyzed by a PCR assay in order to detect the presence of the *nptII* gene. This result and the copy number of the integrated *nptII* gene were confirmed by Southern analysis.

PCR analysis

Two hundred milligrams of genomic DNA from in vitro plant leaves were prepared according to Rogers and Bendich (1994). The yield was 30 μg/200 mg of fresh tissue. PCR detection of the neomycin phosphotransferase gene (*nptII*) was performed with standard methodologies (Taylor 1991). For each sample, 1 μg of DNA was incubated in a final volume of 10 μl with 0.25 μl of 5′ and 3′ primers, 0.25 μl of reaction buffer (2 mM $MgCl_2$), 2 mM each of dNTP and 0.25 units of thermostable DNA polymerase from *Thermus brockianus* (Dynazyme, Finnzymes, Finland). The reaction consisted of 5 min at 94 °C for denaturation, and 30 cycles including 30 s at 94 °C for denaturation, 60 s at 55 °C for annealing and 60 s at 72 °C for extension. The forward and reverse primers for the *nptII* gene were 5′-AAGATGGATTGCACGCAGGTTC and 5′-GAAGAACTCGTCAAGAAGGCGA, respectively, amplifying a fragment of 781 bp from position 161 to position 942 of the sequence (Beck et al. 1982).

All the molecular analysis of the integration (Southern analysis) and expression of the putative agronomically interesting transgenes (Northern analysis for HAL3 and asnA, and Western analysis for TAS14) will be published elsewhere.

Southern analysis

Fifteen micrograms of total genomic DNA from primary transformed and control plants were extracted by standard procedures (Rogers and Bendich 1994). DNA was digested with *Hin*dIII and separated by electrophoresis in a 0.7% agarose gel. DNA was transferred to a nylon membrane and hybridized with a *nptII* radiactive probe (Feinberg and Vogelstein 1983). For the *nptII* probe an internal fragment of the gene was obtained by PCR amplification, by using the primers described above.

Analysis of ploidy level

Twelve to fifteen day old cotyledons (from cultivars and inbred line seedlings), and leaves proceeding from primary transformants cultivated on antibiotic-free rooting medium, were used for nuclei isolation. Pieces of tissue (1 cm^2) were chopped individually on a 50 mm glass plate with a sharp razor blade in 200 μl of nucleus isolation buffer (Partec). After chopping, the re-suspended sample was passed through a 50 μm nylon filter (Nybolt), and we added 800 μl of coloration solution (Partec) containing 1 mg·l^{-1} of DAPI (4,6-diamino-2-phenyl-indole) for fluorescent DNA staining. The DNA content of the isolated nuclei was measured using a Partec PAS-II flow cytometer, equipped with a mercury lamp. The data were plotted on a semi-logarithmic scale, so that the histogram peaks from 2C to 32C were evenly distributed along the abscissa. For calibration, the 2C-peak of nuclei of young leaves from diploid tomato seedlings of each cultivar was used. About 5,000 to 10,000 nuclei were measured per sample.

Results

Regeneration and transformation

The culture medium used to regenerate plants (SIM, Table 1) promoted both induction of caulogenesis and elongation of adventitious shoots for further individualization. In control experiments, the regeneration efficiency (Table 2) ranged from 95.5% (LP4) to 26.3% (LP2). Cultivars p73 and UC 82b exhibited a high regeneration rate (86.9% and 86.0% respectively) and produced several shoots on the organogenic callus after the second subculture in SIM (mean number of 3.1 and 2.0 per explant, respectively).

A total of 15 genetic transformation experiments were carried out and the effect of the genotype and transformation procedure examined in five tomato cultivars (p73, UC 82b, LP0, LP2 and LP4). Green kanamycin-resistant calli began to form on the cut-edge of the explant 2 to 3 weeks after transformation. After approximately 7–8 weeks (second transfer to SIM), clusters of adventitious shoot-buds, leafy structures and individual shoots developed on the organogenic calli. At the third transfer to SIM, calli were cut and separated from the explant; the brown necrotic fragments were discarded. Compact and green calli were transferred to SIM every 3 or 4 weeks, depending on the transformation procedure. The ability to regenerate shoots increased along the different subcultures. For example in the experiment with cv p73 and Method B, the percentages of organogenic kanamycin-resistant calli were 20%, 42% and 55% respectively at the 1st, 2nd and 3rd subcultures. First shoots were ex-

Table 2 Summary of the regeneration efficiencies cultivating half-cotyledon explants of five cultivars of tomato on SIM without antibiotic. Results presented in percentage (%) were calculated as the number of explants with an organogenic response (i.e. adventitious shoots) per total number of explants cultured ± standard error

Tomato cultivar	Number of cultivated explants	Explants with organogenic response		Mean number of shoot per explant in SIM at	
		no.	%	1st subculture	2nd subculture
p73	84	73	86.9 ± 3.7	0.83 ± 0.10	3.14 ± 0.10
UC 82b	86	73	86.0 ± 3.7	0.52 ± 0.10	2.08 ± 0.10
LP0	86	70	81.4 ± 4.2	0.48 ± 0.10	1.09 ± 0.10
LP2	76	20	26.3 ± 5.1	0.30 ± 0.11	0.97 ± 0.11
LP4	88	84	95.5 ± 2.2	0.35 ± 0.10	2.82 ± 0.10

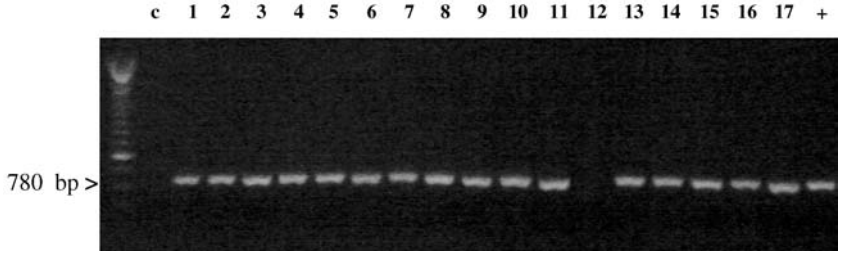

Fig. 1 Electrophoretic analysis of the PCR products of R_0 tomato plants. The ethidium bromide-stained agarose gel showed amplification by the polymerase chain reaction (PCR) of the neomycin phosphotransferase gene (781-bp fragment; *arrow* on the left) in transgenic tomato plants. Aliquots of genomic DNA (1 μg) from the different samples were analyzed. *Lane c*: genomic DNA from an untransformed tomato plant (control, *c*). *Lane 1*: positive control of DNA from the transformed tomato plant previously charcterized by PCR and Southern analysis for the *nptII* gene. *Lanes 2 to 11 and 13 to 17*: putative primary transformants; these tomato plants were kanamycin-resistant. *Lane 12*: putative false transformant; regenerated tomato plant sensitive to kanamycin. *Lane +*: positive control of plasmid DNA

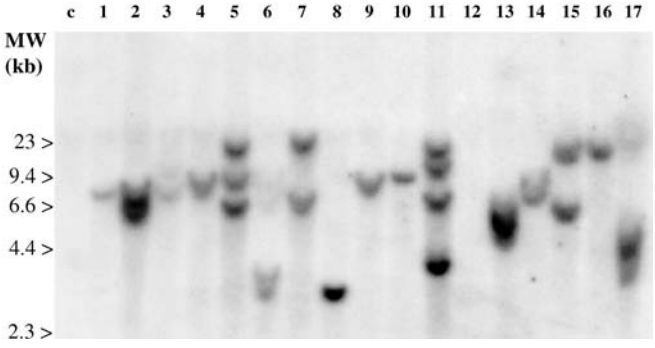

Fig. 2 Southern-blot analysis of genomic DNAs from R_0 tomato plants. *Lane c*: genomic DNA from an untransformed tomato plant (control, c). *Lane 1*: genomic DNA from a transformed tomato plant previously charcterized by Southern analysis (control). *Lanes 2 to 11 and 13 to 17*: tomato primary transformants (R_0) kanamycin-resistant and PCR positive for *nptII* (Fig. 1). *Lane 12*: genomic DNA from false transformant; tomato plant kanamycin-sensitive and PCR negative for nptII (Fig. 1). *Arrows* on the left indicate the size standards; from the bottom to the top: 2.3, 4.4, 6.6, 9.4 and 23 kb

cised from the callus at the third transfer to SIM and placed on RM without antibiotic (Table 1). Individual shoots formed roots within 10 days, and 2-weeks later plantlets were elongated enough to provide several axilary shoots. Some of them were used for the rooting test on RM supplemented with kanamycin, others were conserved on RM to provide leaf tissue for cytometry analysis and DNA extraction.

The time required before transferring acclimatized plants to the greenhouse was 6 months for cvs P73 and LP0, 7–8 months for cv UC 82b and 9–10 months for cvs LP2 and LP4. This variation in the period of time required to achieve transgenic plants depended on the cultivars ability to form shoots, as previously observed by McCormick et al. (1986).

All the plants rooting on the selective RM medium were found to be nptII PCR positive with the appropriate controls (Fig. 1). Southern-hybridization (with the nptII probe) of DNA from PCR positive plants for the selectable marker gene did not give a false positive (Fig. 2). On the basis of these data, we define a primary transformant as an individualized shoot (i.e. an independent genotype), rooting on selective RM medium. For all experiments, the transformation efficiency was calculated as the percentage of explants producing at least one transgenic plant. Obviously, the estimation of the transformation frequencies recovered in this work may be lower than the real ones, as several independent transformation events might occur in the same explant (confirmed by Southern analysis; data not shown).

Table 3 Summary of the transformation efficiency[a] with two commercial cultivars and three different inbred lines of tomato

Tomato cultivar	Number of inoculated explants	Number of transgenic calli	Transformation efficiency (%)[b]
p73[c]	1,370	120	11.2 ± 0.96
UC82b[c]	930	97	10.4 ± 0.98
LP0	220	25	11.3 ± 2.13
LP2	270	15	5.5 ± 1.38
LP4	280	5	1.8 ± 0.79

[a] Transformation experiments with method B (see Materials and methods)
[b] Transformation efficiency was calculated as the percentage of explants producing at least one transgenic plant ± SE
[c] Each data is the mean value of three independent experiments (300–450 explants for each transformation experiment) ± SE

Tomato genotype and transformation procedure

Avoiding the feeder-cells system, we have obtained over 500 transgenic plants in 15 independent experiments, where the average transformation frequency ranged from 1.8% to 11.3%, depending on the cultivar and the method of transformation.

The transformation rates between cvs p73 and UC82b were compared by applying a transformation protocol previously developed in our laboratory (Method A). In the control experiments, both cultivars achieved a regeneration rate of up to 85% (Table 2). However, in the transformation experiments with method A, the efficiency of transformation for UC82b (6.2%) was approximately 2.5-fold compared with p73 (2.5%), confirming the high competence of UC82b for *Agrobacterium*-mediated transformation. On the basis of the transformation rates obtained with the p73 cultivar, we decided to optimise the transformation protocol.

When these cultivars were transformed with Method B (Table 3) the transformation efficiency increased 4.5-fold (11.2%) and 1.7-fold (10.4%) for p73 and UC82b respectively. Method B involved several changes including a modification in the bacterial concentration (D_{600} = 10^2–10^3 cells/ml) compared to Method A (D_{600} = 10^7–10^8 cells/ml). The higher virulence of the bacteria in exponential growth might be the first factor involved in the increased efficiency. Additionally, in procedure B, the frequency of the subculture was also modified and explants were transferred onto fresh medium every 3 weeks. Both conditions, the bacterial concentration of D_{600} = 10^2–10^3 cells/ml and increased subculture frequency, were used by Van Roekel et al. (1993) to transform cotyledons of cv Moneymaker. The authors reported transformation frequencies averaging 9%, but their method involved the use of feeder cells and other conditions which also differ from our work: namely *Agrobacterium* strain (C58), shoot induction medium (I/Z 0.1/2.0 mg·l^{-1}) and 3 days without selection pressure before cultivating explants with kanamycin.

Method B described in our study, permitted us to enhance the transformation rate up to 11.2% for the cultivar with low competence for transformation (p73), avoiding a demanding feeder-cell culture and using the less-virulent LBA 4404 *Agrobacterium* strain. Additionally, with this procedure we were able to obtain transgenic plants in three tomato inbred lines: LP0, LP2 and LP4 (Table 3). In the experiments performed with inbred lines, we observed that the transformation rate was strongly dependent on genotype: LP0 and LP2 gave 11.3% and 5.5% of explants with transgenic plants, while LP4 only reached 1.8% with the same procedure. Interestingly, cultivar LP4, the most competent for regeneration (95.5% of explants with shoots, Table 2), was the most recalcitrant for transformation. By contrast, the LP2 cultivar (the less competent for regeneration) gave a relatively high frequency of transformation.

Analysis of the ploidy level

The five cultivars used in our study were all confirmed to be diploid by flow cytometry (Table 4). The LP0 line showed the lowest percentage of 2C nuclei (39.61%) and gave the highest rate of 8C nuclei (10.96%). Cultivars p73 and UC 82b have more 2C nuclei (44.44 and 48.79% respectively), and a similar level of 8C nuclei (5.3%). With the LP2 and LP4 cultivars, the highest rate of 2C nuclei (53.67 and 60.44% respectively) and the lowest percentage of 8C nuclei (1.83 and 3.02% respectively) was observed. As reported by Smulders et al. (1994, 1995), these results confirmed that cotyledonary tissues are mixed populations of polysomatic cells. However, the polysomatic pattern of tomato cotyledons appears to be dependent on the cultivar.

Analysis of transgenic plants by flow cytometry indicated that the distribution of tomato plants with a different ploidy level depended on both genotype and the

Table 4 Analysis of the ploidy level of the cotyledon explants from seedlings of five tomato cultivars used as an explant source in *Agrobacterium*-mediated transformations. Values are expressed as mean ± SE of the mean. Data were obtained from 20 independent plantlets per tomato genotype

Tomato cultivars	Debris	Percentage of nuclei with		
		2C	4C	8C
LP0	9.52 (±0.10)	39.61 (±0.10)	39.90 (±0.10)	10.96 (±0.10)
p73	8.76 (±0.09)	44.44 (±0.09)	41.53 (±0.09)	5.26 (±0.09)
UC82b	9.66 (±0.10)	48.79 (±0.10)	36.16 (±0.10)	5.38 (±0.10)
LP4	8.66 (±0.09)	53.67 (±0.09)	32.96 (±0.09)	3.02 (±0.09)
LP2	10.39 (±0.11)	60.44 (±0.11)	29.05 (±0.11)	1.83 (±0.11)

Table 5 Analysis of the ploidy level of the transgenic tomato plants

Tomato genotype	No. of plants tested	Ploidy level of tested plants		% of 2n plants[a]
		2n	4n	
p73	210	42	158	20.0 ± 2.8
UC82b	145	102	43	70.3 ± 3.8
LP0	72	46	26	63.9 ± 5.7
LP2	18	13	5	72.2 ± 10.5
LP4	7	4	3	57.1 ± 18.7

[a] Results presented in percentage (%) were calculated as the number of diploid transgenic plants per total number of transgenic plants assessed by flow cytometry analysis ± SE

Table 6 Fertility analysis of the transgenic tomato plants under greenhouse conditions

Transgenic cultivar	Date of culture	Diploid transgenic plants (2n)							Tetraploid transgenic plants (4n)						
		Plants in greenhouse	Plants with fruits		Seed weight (SW) per plant			% of plants with SW > 0.5 g	Plants in greenhouse	Plants with fruits		Seed weight (SW) per plant			% of plants with SW > 0.5 g
			No.	%	0	<0.5 g	>0.5 g			No.	%	0	<0.5 g	>0.5 g	
p73	Jun–Oct	16	16	100	0	2	14	87.5	8	8	100	8	0	0	0
	Jun–Oct	5	5	100	0	0	5	100	8	8	100	8	0	0	0
	Mar–Jul	12	12	100	0	0	12	100	16	16	100	11	5	0	0
UC 82	Jun–Oct	16	16	100	2	3	11	68.7	–	–	–	–	–	–	–
	Jun–Oct	10	10	100	3	2	5	50.0	6	6	100	4	2	0	0
LP0	Jul–Nov	17	16	94.1	1	–	16	94.1	2	2	100	0	2	0	0

transformation procedure. With method A, from a sample of 53 transgenic plants of cv UC82b, 40 were diploid (75.5%) and 13 tetraploid (24.5%). However, when the same protocol was applied to cultivar p73, from 71 transformants tested, 31 were diploid (43.7%) and 38 tetraploid (56.3%). These results suggest that the receptor genotype used in *Agrobacterium*-mediated transformation of tomato might influence the ploidy level of the primary transformants.

When the optimised protocol for transformation was applied (Method B), we observed (Table 5) a drastic increase of tetraploid plants with cultivar p73: from 210 primary transformants analysed for their DNA contents, only 42 plants were diploid (20.0%). Comparing this rate with the results obtained with Method A, it seems that the ploidy level in transgenic plants of p73 is affected by the regeneration and/or transformation protocol. With method B, cultivar UC82b and inbred lines LP2, LP0 and LP4 were much more stable than p73; the percentages of diploid transgenic plants reached 70.3%, 72.2%, 63.9% and 57.1% respectively with these four genotypes.

Fertility analysis

Some of the transgenic plants (117) confirmed by the rooting test and the PCR analysis for the *nptII* gene, were transferred to the greenhouse in six different experiments. All transgenic plants produced fruits, independently of the date of culture (Table 6). When fruits were harvested, 90% of the diploid plants (75 transformants) produced fruits with more than 0.5 g of normal seeds per plant. Inversely, 75% of the tetraploid tomato plants (41) were seedless and 25% exhibited a very low number (<0.5 g) of seeds per plant.

Discussion

The first objective of this work was to evaluate the relative distribution of 2C/4C nuclei in the cotyledon explants from five tomato cultivars and to compare it with the percentage of diploid/ tetraploid transgenic plants regenerated from them.

All cultivars and inbred lines were diploid and the frequency of 2C nuclei ranged from 39.6% (LP0) to 60.4% (LP4). These results confirm that cotyledonary tissue is a mixed population of polysomatic cells, as described by Smulders et al. (1994, 1995). In cv Moneymaker these authors determined that cotyledons exhibited more polyploid nuclei (39% of 4C, 9% of 8C and 1% of 16C) than leaves (16% of 4C, 2% of 8C and 0% of 16C). In our study, we also observed a polysomatic pattern in the tomato cotyledon, and the differences between cotyledons and leaves were also confirmed (data not shown). In addition, we reported that the polysomatic pattern in cotyledon explants is cultivar dependent.

In order to apply genetic transformation via *Agrobacterium*, cells of the explant source have to be competent for both regeneration and integrative transformation (Potrykus 1991). Control experiments (Table 2) showed

different competence for organogenesis in the five tomato cultivar candidates for the transformation experiments. In particular, cells of cotyledons proceeding from cultivars LP2 and LP4 exhibited a very different regeneration competence (26.3% and 95.5% of explants with adventitious shoots respectively). According to these results, in LP2 cotyledons a minority of cells are competent for regeneration while in LP4 these cells are predominantly present. Surprisingly, the transformation rate of LP2 (the less organogenic cultivar) was 3-fold higher compared with LP4, the more-organogenic cultivar. These results showed that for LP2, the few cells competent for regeneration are also competent for transformation. In contrast, for LP4 cotyledon tissue, most of the cells are able to differentiate adventitious shoots but are non-competent for genetic transformation. Our results agree with the Potrykus (1991) statements and confirm that plant tissues are mixed populations of cells with competence for many different responses. They also suggest that the relative composition of cell populations in tissues within the same species (*Lycopersicon esculentum* Mill.) may be genotype dependent. In addition, the similar mixoploid pattern of LP2 and LP4 suggest that the organogenic competence is not directly dependent on the polysomatic level of the tissue used as an explant source.

In tomato, the effect of the *Agrobacterium* concentration for co-cultivating cotyledon explants was previously described by Fillatti et al. (1987). When the concentration of the bacteria increased or decreased from 5.10^8 bacteria per ml, the rate of transformation was reduced by at least 20%. In our work, by reducing the optical density we achieved a 5-fold enhancement of the transformation rate (from 2.5% to 11.2%) with the p73 cultivar. In addition, two other inbred lines (LP0 and LP2) have been efficiently transformed and some transgenic plants have been regenerated from LP4.

Ploidy variations have been reported in both regenerated (Smulders et al. 1995) and transgenic tomato plants (Van Roekel et al. 1993; Ultzen et al. 1995). With the p73 cultivar, the rate of tetraploid plants (Table 5) was much higher than that expected according to the distribution of nuclei with different DNA contents in the explant source (Table 4). In the case of cv UC82b and inbred lines, the percentage of tetraploid primary transformants was similar (LP0) or lower than expected (UC82b, LP2 and LP4) on the basis of the polysomatic pattern of the cotyledon (Table 4). This result showed that these four genotypes are more stable than the p73 cultivar and confirmed a genotype effect on the regeneration/transformation process of diploid plants. Such differences also suggests that the original polysomatic pattern of the explant source may influence, but does not determine, the tetraploid percentage of the tomato transformants (LP0 and LP2 have a quite different polysomatic pattern; Table 4).

In various cultivars of tomato, several examples of recessive and some dominant monogenic mutations have been obtained through plant regeneration from cells or tissues (Van der Bulk et al. 1990). The same authors clearly demonstrate the influence of the source of the explants on the percentage of tomato regenerated plants with a different ploidy level, showing values of 1.5%, 11.9% and 58.0% for leaf, cotyledon and hypocotyl respectively. Similar results were also observed in our group by regenerating tomato plants from cotyledon and leaf explants of p73, UC 82b, LP0, LP2 and LP4 (unpublished data).

In several species, changes in the ploidy level generally enhanced the characters of certain vegetative or reproductive organs. Although a more-complete analysis would be suitable, it was impossible for us to distinguish between diploid and tetraploid transformants during the period of in vitro culture. In these conditions, tomato transgenic tetraploids have the same morphology, colour and rooting capacity as the diploid transformants.

In our work, over 100 transgenic plants (confirmed by the rooting test, PCR and Southern analysis for the nptII gene), were transferred to the greenhouse in six different experiments. All transgenic plants produced fruits independent of the period of culture (Table 6). In greenhouse conditions it was very difficult to identify tetraploid plants on the basis of morphological traits. When fruits were harvested, 90% of the diploid plants (75 transformants) produced tomatoes with more than 0.5 g of normal and viable seeds per plant. In contrast, 75% of the tetraploid tomato plants (41) were seedless and 25% exhibited a very low number (<0.5 g) of seeds per plant. The decrease of fertility has already been reported in several tetraploid species but, as far as we know, the loss of fertility in the tetraploid primary transformants has not been reported in transgenic tomato.

Regeneration of polyploid plants is a usual phenomenon in plant tissue-culture. In genetic transformation experiments the emphasis is generally put on the molecular characterization of the primary transformants, but the ploidy state of the transgenic material is only checked in relatively few cases. However, results obtained in our laboratory with experiments of genetic transformation with several crops (i.e. tomato, melon and watermelon), as well as those previously published by other authors in tomato transformation experiments (Van Roekel et al. 1993; Ultzen et al. 1995), suggest that it is necessary to take into account this kind of analysis in order to adequately perform a selection from the initial population of primary transformants. The confirmation of the ploidy level in transgenic material is particularly important when a polysomatic tissue (e.g. cotyledons in the previously mentioned species) is to be used as an explant source.

Indirect parameters (i.e. guard cell chloroplast number) have been used to identify polyploid plants in tomato (Koorneef et al. 1989). However, with the exception of the lack of fertility, we were unable to distinguish between diploid and tetraploid plants by visual study of the plant material in both in vitro and greenhouse conditions. This indicates that in transgenic tomato it is necessary to perform either a direct analysis (i.e. by flow cytometry or chromosome counting) or an indirect estimation (i.e.

guard cell chloroplast numbers in leaf epidermis) of the ploidy level. In the absence of this kind of analysis, the evaluation of transgenic material performed in primary tranformants and/or tissue cultures derived from these plants could be confusing. Recently, Galitsky et al. (1999) have provided some evidence for a ploidy driven mechanism of gene regulation in a lower eukaryote. The authors identified genes showing a ploidy dependent expression in isogenic *Saccharomyces cerevisiae* strains that varied from haploid to tetraploid. These genes were induced or repressed depending on the number of the chromosome set, regardless of the mating type. It is still unknown if a similar mechanism exists in higher plants. Irrespective of that, the foreseeable change of expression in endogenous and/or foreign genes in diploid and polyploid plants reinforces the convenience of checking the ploidy level before performing an evaluation of transgenic plant material.

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