Transformation of Recalcitrant Melon (*Cucumis melo* L.) Cultivars is Facilitated by Wounding with Carborundum

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Transformation of the recalcitrant melon (*Cucumis melo* L.) cultivars Kırkağaç 637 and Noi Yarok was accomplished by wounding cotyledon explants by vortexing with carborundum prior to inoculation with *Agrobacterium tumefaciens*. The addition of silver nitrate to the regeneration-selection medium reduced the transformation efficiency, as the percentage of the explants forming putative transgenic calli and bud-like protuberances was decreased and no transgenic shoots were produced. Chimeric transgenic plants were obtained after the regeneration of putatively transformed callus, bud-like protuberances, buds and shoots on selective medium with kanamycin. The treatments producing the most buds or shoots from explants after 30–40 days of cultivation were the most successful for the production of transgenic plants. Only treatments where explants were vortexed with carborundum produced transgenic melon shoots of either cultivar. Subculture every 18–20 days on fresh regeneration-selection medium containing 50 mg/L kanamycin after either a relatively high (100 mg/L) or low level (50 mg/L) of kanamycin in the first regeneration-selection medium was necessary for the successful transformation of cultivar Kırkağaç 637. These techniques are now being used in breeding programs for the production of melon lines bearing resistances to zucchini yellow mosaic virus and cucumber mosaic virus, important viruses limiting agricultural production.

1 Introduction

Turkey is a secondary center for the diversification of melon [1] and the second largest melon producing country in the world [2]. There are many conventional melon varieties widely grown in Turkey. However, the majority of these are susceptible to several viruses [3], fungal diseases [4], and other pests, although some imported melon cultivars have useful resistances and/or attributes (earliness, high yields, etc.).

Genetic engineering is a valuable biotechnological approach for breeding purposes [5], and can be used to introduce resistance genes not found in the gene pool of particular species. There have been several reports on the genetic transformation of melon with a variety of marker genes, as well as genes for viral resistance and fruit quality attributes [5–11]. In our laboratories, transformations of several cultivars (cvs.) of melon (including cvs. Kırkağaç 637 and Noi Yarok) employing the methods of Fang and Grumet [6] as well as Vallés and Lasa [10] were unsuccessful presumably due to non-defined methodological or environmental differences, as well as genotype and/or seed batch dependence. Cultivar (cv.) Kırkağaç 637 is the most common cv. in Turkey and is therefore of commercial importance, and Noi Yarok is an Israeli melon variety; both these cvs. have good regeneration ability [12, V. Gaba et al., unpublished results].

Many physical treatments that damage plant tissue to create sites for *Agrobacterium* infection have been reported to increase transformation frequency in different plant species. Fang and Grumet [6] suggested cutting all edges of melon cotyledons with a blunt scalpel blade to maximize wounding for *Agrobacterium* inoculation. Sonication-assisted *Agrobacterium*-mediated transformation opens micro-pores in the tissue surface for *Agrobacterium* colonization [13]. Crushing apple leaves before inoculation produces infection sites for *Agrobacterium tumefaciens* resulting in a higher transformation rate [14]. Shaking the plant materials mechanically with glass beads [15] and pectolytic treatment of the explants [16] also enhances transformation efficiency. Wounding tobacco leaf and sunflower meristematic explants by particle bombardment increases transformation frequency by subsequent inoculation with *Agrobacterium tumefaciens* [17, 18]. Transformation of melon of cv. Kırkağaç 637 cotyledon explants was attempted by particle wounding/*Agrobacterium* inoculation in preliminary experiments, but transgenic plants were not obtained (unpublished results).

The objective of this study was to develop an *Agrobacterium*-mediated transformation method for cvs. Kırkağaç 637 and Noi Yarok. A transformation method by vortexing melon explants with carborundum, prior to *Agrobacterium* inoculation, was developed while investigating several factors affecting transformation efficiency. Wounding of embryogenic tissues by vortexing with carborundum prior to *Agrobacterium* treatment is a reliable and efficient method for papaya transformation [19]. Wounding of cotyledon explants of melon, cvs. Kırkağaç 637 and Noi Yarok, by vortexing in water with carborundum enabled genetic transformation in otherwise recalcitrant material.

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2 Materials and Methods

2.1 Plant Material

Seeds of *Cucumis melo* L. cv. Kırkağaç 637 were obtained from Çağdaş Seeds Ltd. (Turkey), and seeds of cv. Noi Yarok were obtained from Hazera Seeds Ltd. (Israel).

2.2 Tissue Culture Conditions and Media

Coats of the melon seeds were removed, and the seeds were washed in 70 % ethanol for 5 min, rinsed three times with sterile distilled water, and sterilized in a solution of 1 % sodium hypochlorite with 2 drops of Tween 20 per 100 mL solution for 45 min. After rinsing with sterile distilled water three times, the seeds were further washed for 15 min in 500 mg/L carbenicillin and 500 mg/L cefotaxime. Seeds germinated on MS [20] medium, in a growth room at 28 ± 1 °C, with a 16-h photoperiod and 90–120 μmol/m^{2}s cool white fluorescent light. Cotyledons were excised from 4- and 5-day-old seeds for cv. Kırkağaç 637 and Noi Yarok, respectively, and cut across to make 3 explants per cotyledon, including trimming off the tip of the cotyledon but not all edges.

In transformation experiments, N medium (MS salts and vitamins, 30 g/L sucrose, 8 g/L agar (Sigma, A1296), 0.88 mg/L IAA, 1.13 mg/L BA, 0.26 mg/L ABA) [6], NB00101 medium (BM3 medium [MS salts, 100 mg/L myo-inositol, 1 mg/L thiamin-HCL, 30 g/L sucrose, 8 g/L agar] with 0.01 mg/L NAA, 0.1 mg/L BA) [10] and BM3 medium [10] were used as regeneration, elongation and rooting media, respectively. *In vitro* cultures were placed at 28 ± 1 °C with a 16 h photoperiod of a 90 μmol/m^{2}s photon fluence rate during regeneration-selection, elongation and rooting.

2.3 *Agrobacterium* Strain and Plasmid

The *A. tumefaciens* strain EHA 105 containing the binary plasmid pME504 [21] that encodes the NPTII, *bar* and *uid*A-intron c-DNA was used for transformation. A colony of this bacterium taken from an LB plate with 100 mg/L kanamycin kept at 4 °C was incubated overnight in 2 mL liquid LB medium with 100 mg/L kanamycin at 28 ± 1 °C and 125–140 rpm (final OD_{600} = 1). From the overnight culture 200 μL were added to 20 mL of liquid LB without antibiotics and shaken (as above) for 2–3 h prior to inoculation. Approximately 20 mL culture were used for the inoculation of the 100 cotyledon explants obtained from 16–23 seedlings.

2.4 Vortexing, Inoculation and Co-Cultivation

Half-gram lots of carborundum powder (Fisher Scientific C192–500; 320 grit, 0.045 mm diameter; or Prolabo (Italy), 0.037 mm diameter) were sterilized by autoclaving with 30 mL double distilled water in 150 × 25 mm diameter tubes. The water-carborundum slurry was poured into 50 mL sterile centrifuge tubes (Corning), and the 100 explants cut from 16–23 seedlings were gently added. Each tube was capped and vortexed at speed 5 or 7 for 0.5, 1, 1.5 or 2 min in a vertical position on a 220 V Heidolph REAX 2000 Vortex. After vortex treatment, the contents of the centrifuge tube were poured into an empty Petri dish, and explants were transferred to the *Agrobacterium* culture in a fresh Petri dish. The explants were inoculated with *Agrobacterium* for 10 min, with occasional agitation, then blotted dry with sterile Whatman No. 1 filter paper and approximately 25 explants were distributed per plate of N medium without antibiotics, and co-cultivated for 2 or 3 days in the growth room. After co-cultivation, the explants of both melon cultivars were washed 3 times with sterile double distilled water, blotted dry with Whatman paper, and placed in 90 × 15 mm Petri dishes (25 mL medium and 17–21 explants per dish) with the adaxial side on N medium with carbenicillin (500 mg/L) and kanamycin.

2.5 Sensitivity to Kanamycin

To determine the concentration of kanamycin that suppresses the regeneration of cv. Kırkağaç 637 in regeneration and elongation media, cotyledon explants were excised from 5- or 7-day-old seedlings and placed adaxial side down on N medium without ABA containing 0, 40, 80, or 120 mg/L kanamycin. The cultures were incubated under white fluorescent lamps at 60–75 μmol/m^{2}s with a 16-h photoperiod at 28 ± 1 °C. Explants with bud-like protuberances (green nodular structures with leaf primordia [22]) or shoots were scored after 22 and 50 days.

Explants of cv. Kırkağaç 637 with buds and/or shoots which had regenerated on MSBA1 medium (MS salts and vitamins, 30 g/L sucrose, 8 g/L agar, 1 mg/L BA), were transferred onto NB00101 elongation medium supplemented with 0, 50, 75, or 100 mg/L kanamycin. After 5 weeks, the clumps were sub-cultured to the same medium for a further 2 weeks, and a percentage of bleached explants was scored.

2.6 Sensitivity to Phosphinothricin (PPT)

The roots of 8 to 12-day-old *in vitro*-grown seedlings of cv. Kırkağaç 637 showing the first true leaf were cut and discarded. The hypocotyls of the plants were placed into BM3 media supplemented with 0, 1, 2, 4, or 8 mg/L PPT (glufosinate ammonium) the active ingredient of the herbicide Basta. Rooting percentage was scored after 19 days in the culture.

2.7 Selection, Elongation and Rooting of cv. Kırkağaç 637

Three selection regimes were tested on N medium for 18–20 days with:

(i) 100 mg/L kanamycin, and subculture with 50 mg/L kanamycin;
(ii) 75 mg/L kanamycin, and subculture with 75 mg/L kanamycin;
(iii) 50 mg/L kanamycin, and subculture with same concentration.

The duration of subcultivation was 18–20 days in each case. At the end of the regeneration-selection period, the explants were scored for the production of callus, bud-like protuberances (a type of protuberance known to become buds on regeneration or elongation medium), buds and/or shoots. The effect of adding 5 mg/L silver nitrate to the regeneration-selection medium with 50 mg/L kanamycin on regeneration was determined on cv. Kırkağaç 637. The regenerating parts of the explants were excised and transferred onto NB00101 elongation medium supplemented with 50 or 75 mg/L kanamycin and 500 mg/L carbenicillin for 2 weeks. Transfers were performed at 2–3-weekly intervals on this media for up to 6 repetitions. Shoots expressing GUS (β-glucuronidase) activity were rooted on BM3 with 0 or 50 mg/L kanamycin and 500 mg/L cefotaxime, which apparently stimulates root formation.

2.8 Characterization of Transgenic Plants

GUS Assays

The X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) histochemical assay was used to detect GUS expression in roots, stems and leaves of putative transformants [23].

Parts of putatively transformed plants growing on kanamycin were immersed in 100–200 μL of substrate and incubated overnight at 37 °C. If necessary, green tissue was cleared with 70 % ethanol 2–3 times and fixed in FAA (5 % formalin, 5 % acetic acid, in 70 % ethanol).

DNA Preparation and PCR Analysis

Polymerase Chain Reaction detection of the NPTII gene was performed. DNA from 100 mg of plant leaves was extracted [24], DNA pellets were washed twice with 70–75 % ethanol and re-suspended in 500 μL of sterile double distilled water.

Five μL of suspended DNA were used for 45 μL of reaction mixture (5 μL buffer for Taq DNA polymerase [×10], and 3 μL of 25 mM $MgCl_2$, 2.5 μL of 2.5 mM dNTP, 28.5 μL of double distilled water, 2.5 μL [diluted: 1/10] primer 1 [5'-CACGCAGGTTCTCCGGCCGC-3'], 2.5 μL [diluted: 1/10] primer 2 [5'-TGCGCTGCGAATCGGGAGCG-3'], and 1 μL Taq DNA polymerase [Promega, Madison, WI, USA]) were employed for each sample. Each reaction cycle (36 cycles) consisted of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and polymerization at 72 °C for 1 min.

PPT Treatment of Putative Transgenic Buds or Shoots

Buds and plantlets of putative transgenic plants growing on kanamycin-containing medium, which scored positive for GUS and PCR assays for NPTII, were excised and subcultured twice on elongation or rooting medium supplemented with 0–8 mg/L PPT for 15–20 days.

At the end of the second round of cultivation, bud or shoot survival was recorded. Putative transgenic plantlets were micropropagated and acclimated, and then transplanted into pots in a greenhouse. The plants were sprayed with 0.5–2 % Basta® 20 SL (20 % a.i.) (30–40 L/1000 m^2). As a control, untransformed plants of cv. Kırkağaç 637 were sprayed with 1 % Basta.

Southern Blot Analysis

For Southern analysis, DNA was prepared from melon leaves according to Baudracco-Arnas [25]. For Southern blot, 5 μg of sample DNA were restriction-digested according to the manufacturer's instructions (Boehringer-Mannheim), run on 0.8 % agarose gels, and blotted onto charged nylon membranes (Genescreen Plus, Du Pont) using the alkaline blotting method. Hybridization was performed at 60 °C in 6 % PEG, 5 % SDS, 5× SSPE, and 5 μg/mL denatured salmon DNA.

The blots were hybridized with a purified 3.8 kbp *Bam*HI fragment from the binary vector pME504, encompassing the NPTII gene and 35S CaMV promoter. The probe was labeled using the random hexamer method with $^{[32]}$P-CTP (Boehringer-Mannheim protocol). After hybridization, blots were washed three times for 15 min at the hybridization temperature with 1 % sodium chloride and trisodium citrate buffer, 1 % SDS, and exposed to a phospho-imager for 4 days.

2.9 Ploidy Determination

The ploidy level of leaf samples (from 3 plants of each line) of transgenic shoots was analyzed by flow cytometry [26] by Plant Cytometry Services (Schijndel, Holland).

2.10 Data Analysis

The experiments were designed and analyzed in terms of the completely randomized factorial design [27] or completely randomized design (3–6 replicates and 6–21 explants in each replicate). Values expressed as percentage were transformed (arcsin (P = original percentage value)$^{0.5}$) ac-

cording to Bartlett [28], and were analyzed using the analysis of the variance (ANOVA). Tukey's Honestly Significant Difference (HSD value) test was used to compare the treatments at $p < 0.05$ level, unless otherwise noted. Data were converted back to a non-transformed value for presentation. When necessary, analysis using a χ^2-test under the hypothesis was performed [29].

3 Results and Discussion

Preliminary experiments were carried out to determine the effects of wounding by vortexing with carborundum on gene transfer to cv. Noi Yarok. According to the growth on the selection medium with kanamycin, the expression of GUS gene product in R_0 and R_1, PCR detection in R_0 and Southern blot detection in R_1 plants, the only treatment to provide transgenic shoots (5 %: 1/20 explants) were explants exposed to the process of "2 min vortex at speed 7, 10 min of incubation with *Agrobacterium*, 2 days of co-cultivation, and two rounds of selection on regeneration media containing 125 mg/L kanamycin for 20 and 15 days" (see Tab. 1). Treatment without vortexing produced intermediate levels of callus, but a low level of bud-like protuberances and no buds or shoots, and no transgenic plants (see Tab. 1).

Table 1. Effect of vortex duration on the transformation and regeneration from cotyledon explants of cv. Noi Yarok.
Regeneration responses of 5-day-old cotyledon explants inoculated with *A. tumefaciens* after wounding by vortexing at speed 7 in carborundum, and co-cultivated for 2 days. The explants were cultured for two rounds, 3 and 2 weeks, respectively, on N medium containing 125 mg/L kanamycin. The concentrations required to prevent bud or shoot regeneration of cv. Noi Yarok were 125 mg/L kanamycin in the regeneration medium and 50–75 mg/L kanamycin in the elongation medium (Gaba *et al.*, unpublished results).

Vortex duration [min]	Explants [%] forming			
	callus	bud-like protuberances	buds or shoots	transgenic shoots[b)]
0	85[a)]	3[a)]	0[a)]	0[a)]
0.5	82	35	18	0
1	94	22	6	0
1.5	90	10	14	0
2	95	15	10	5

a) Since there were not enough replicates, the data within the column were not analyzed statistically. Sample size was 17–21 explants per treatment.
b) Transgenic shoots were assessed based on the growth of the shoots on selection medium with kanamycin, the expression of the GUS gene product in R_0 and R_1, the PCR detection in R_0 and the Southern blot detection in R_1 plants.

3.1 Transformation of cv. Kırkağaç 637

Kanamycin at a concentration of 80 mg/L in the regeneration medium significantly inhibited the production of bud-like protuberances or shoots on explants of cv. Kırkağaç 637 ($P < 0.05$) (data not shown). The appearance of explants with bud-like protuberances or shoots was decreased from 97 % in the regeneration medium without kanamycin, to 6 % in the medium supplemented with 80 mg/L kanamycin (data not shown). The low level of escapees was further reduced to near zero by further selection on the elongation medium. The addition of 50 mg/L kanamycin to the elongation medium caused 53 % of the explants to bleach and die, and 47 % of the explants to partially bleach (at least 50 % of each explant, especially the parts of the explants in contact with the medium) of cv. Kırkağaç 637 by the end of the first subculture, and this concentration was used for selection, unless otherwise noted (data not shown).

The duration of vortex treatment (0.5, 1 or 1.5 min at a setting of 7) was examined as a factor in transformation parameters and production of transgenic plants (see Tab. 2). On the basis of the growth on selection medium with kanamycin, the expression of the GUS gene product in R_0 and R_1, and the PCR detection in R_0 plants, two putative transgenic lines of cv. Kırkağaç 637 were obtained from 0.5 or 1.5 min vortex treatments, each with a transformation efficiency of 1 % (1/94 explants). However, the influence of vortex treatment was not significant ($P > 0.05$) in the formation of buds or shoots, bud-like protuberances or calli.

Table 2. Effect of vortex duration on the transformation and regeneration from 4-day old cotyledon explants of cv. Kırkağaç 637.
Regeneration responses of cotyledon explants inoculated with *A. tumefaciens* after wounding by vortexing at speed 7 in carborundum, and co-cultivated for 2 days. The explants were cultured for 18 days on N medium containing 100 mg/L kanamycin followed by 18 days on N medium supplemented with 50 mg/L kanamycin.

Vortex duration [min]	Explants [%] forming			
	callus	bud-like protuberances	buds or shoots	transgenic shoots
0	97	9	1	0
0.5	79	15	4	1[a)]
1	76	9	2	0
1.5	78	7	3	1[b)]
HSD 5 %	NS	NS	NS	NS

The means within the columns are not significantly different (NS) when employing Tukey's Honestly Significant Difference (HSD) test; sample size was 94–100 explants per treatment.
a) Transgenic shoots were assessed according to the growth of the shoots on the selection medium with kanamycin, the expression of the GUS gene product in R_0 and R_1, and the PCR detection in R_0 plants.
b) Transgenic shoots were assessed based on the growth of the shoots on the selection medium with kanamycin.

The effects of a combination of treatments during the co-cultivation period, the addition of $AgNO_3$ and kanamycin concentration on the production of putative transgenic callus, bud-like protuberance, buds or shoots were examined following vortex wounding with carborundum at a lower speed (see Tab. 3). No significant differences amongst the

treatments were found in the formation of buds or shoots, or callus. However, the highest frequency of bud-like protuberance formation was obtained from the explants co-cultivated with *Agrobacterium* for 2 days and regenerated on medium with 50 mg/L kanamycin and without $AgNO_3$, the only treatment which allowed in this case to produce a transgenic plant (1 %: 1/102 explants) ($P < 0.05$). The treatments producing the most buds or shoots at this stage were also most successful in the production of transgenic plants (see $r = 0.89$ in Tab. 2 and $r = 0.94$ in Tab. 3).

Table 3. Effect of treatments of combination of co-cultivation duration, $AgNO_3$ and kanamycin concentration on the regeneration of 4-day-old cotyledon explants of cv. Kırkağaç 637. Cotyledon explants were inoculated with *A. tumefaciens* after wounding by vortexing at speed 5 for 1 min in carborundum, and co-cultivated for 2 or 3 days. Treatments noted with kanamycin are the initial concentration of the antibiotic in the selection medium following co-cultivation, followed by the same concentration of kanamycin in the next subculture medium for 20 days each.

Treatments	Explants [%] forming			
	callus	bud-like protuberances	buds or shoots	transgenic shoots
Two-day co-cultivation, 50 mg/L Kn, 5 mg/L $AgNO_3$	69	5ab	1	0
Two-day co-cultivation, 50 mg/L Kn, without $AgNO_3$	77	12a	3	1*
Three-day co-cultivation, 50 mg/L Kn, 5 mg/L $AgNO_3$	63	1b	0	0
Three-day co-cultivation, 50 mg/L Kn, without $AgNO_3$	77	11ab	0	0
Three-day co-cultivation, 75 mg/L Kn, 5 mg/L $AgNO_3$	54	3ab	0	0
Three-day co-cultivation, 75 mg/L Kn, without $AgNO_3$	63	4ab	0	0
HSD 5 %	NS	15.53	NS	NS

The means within the columns with the same letter are not significantly different (NS) when employing Tukey's Honestly Significant Difference (HSD) test; sample size was 99–105 explants per treatment.
* Transgenic shoots were assessed based on the growth of the shoots on selection medium with kanamycin and PPT, the expression of the GUS gene product in R_0 and R_1, and the resistance to PPT (Basta) in the greenhouse in R_0.

The only treatments observed to produce transgenic melon shoots were those where the plant material was vortexed with carborundum. In each case, vortexing with carborundum had to be accompanied by the correct selection procedure for that cultivar to obtain transgenic plants. Transformation of cv. Noi Yarok required subculture every 2–3 weeks on fresh regeneration-selection medium with a high concentration of kanamycin (125 mg/L). However, successful transformation of cv. Kırkağaç 637 necessitated a regeneration-selection for 18–20 days on a high (100 mg/L) (similar to [30]) or low (50 mg/L) kanamycin level, followed by subcultivation every 18–20 days on fresh regeneration-selection medium with a low concentration of kanamycin.

The co-cultivation period (2 or 3 days) during our investigation had no significant effect on the formation of bud-like protuberance or callus, and silver nitrate had a negative effect on these processes ($P < 0.05$) (see Tab. 4), although it had a positive influence on the formation of bud-like protuberance, buds and shoots when uninoculated explants were used ($P < 0.05$) (see Tab. 5). Stimulation of regeneration responses has previously been noted when using silver nitrate in melon [31] and potato [32]. An advantage in the use of silver nitrate for the transformation has become apparent in melon [11]. The difference in transformation response observed here could result from different physiological stages between the works, the bactericidal effect of silver nitrate [33], or a transformation stage-specific silver toxicity.

Table 4. Effect of the co-cultivation period and silver nitrate on the frequency of bud-like protuberance and callus formation. Cotyledon explants of cv. Kırkağaç 637 were vortexed at speed 5 for 1 min, inoculated with *A. tumefaciens* and cultured on N medium containing 50 mg/L kanamycin with or without 5 mg/L $AgNO_3$. The responses were scored after 5 weeks on the regeneration-selection medium.

Co-cultivation period [days]	Explants forming					
	callus [%]			bud-like protuberances [%]		
	+$AgNO_3$	–$AgNO_3$	Means	+$AgNO_3$	–$AgNO_3$	Means
2	69	77	73	5	12	8
3	63	77	70	1	11	6
Means	66 x	77 y		3 b	12 a	
HSD 5 %	6.14		NS	7.68		NS

HSD 5 % (co-cultivation period X $AgNO_3$ on bud-like protuberances formation): NS (not significant); HSD 5 % (co-cultivation period X $AgNO_3$ on callus formation): NS. The means with same letter within the column or row are not significantly different when employing Tukey's Honestly Significant Difference (HSD) test. Sample size was 100–105 explants per treatment.

Table 5. Effect of vortex duration and silver nitrate on the frequency of bud-like protuberance and bud or shoot formation of un-inoculated explants.
Cotyledon explants of cv. Kırkağaç 637 were vortexed at speed 7 for 0, 0.5 or 1 min, cultured on N medium with or without 5 mg/L $AgNO_3$. The responses were scored after 5 weeks on the regeneration medium.

Vortex duration [min]	Explants forming					
	bud-like protuberances [%]			buds or shoots [%]		
	+$AgNO_3$	–$AgNO_3$	Means	+$AgNO_3$	–$AgNO_3$	Means
0	94	78	86 a	78	38	58 a
0.5	83	61	72 ab	44	22	33 ab
1	67	22	44 b	39	0	19 b
Means	82 x	54 y		54 x	20 y	
HSD 5 %	18.35		27.51	13.46		20.17

HSD 5 % (vortex duration X $AgNO_3$ on bud-like protuberances formation): not significant. HSD 5 % (vortex duration X $AgNO_3$ on buds or shoots formation): not significant. The means with same letter within the column or row are not significantly different when employing Tukey's Honestly Significant Difference (HSD) test. Sample size was 18 explants per treatment.

3.2 Characterization of R_0 Transformed Plants

Each transformation event resulted in a group of buds derived from bud-like protuberances or callus; line 5/V/C was from cv. Noi Yarok, and lines 3/V/B, 1/V/A, and 2/II/A from cv. Kırkağaç 637. After the initial selection procedures (above), the products of each transformation event were subcultured several times at 2–3-weekly intervals, during which the number of bud groups from each transformation event was greatly multiplied. Putative transgenic buds or shoots of line 3/V/B were unable to root and their growth was very poor on medium containing 50 mg/L kanamycin. Many of the bud groups produced from putative transformed lines 1/V/A and 2/II/A bleached during the subculture process, suggesting that these transgenic lines were chimeric. Continuous selection was effective in breaking chimeras on elongation medium with 50 mg/L kanamycin for up to six cycles of subculture, and by increasing the kanamycin concentration up to 75 mg/L for a few cycles (data not shown). As a result of this selection, putative transgenic plants of lines 1/V/A, 2/II/A, and 5/V/C rooted on rooting medium with 50 mg/L kanamycin, while the control plants could not root. Reiterative selection has previously been shown to be successful in sunflower [34].

There was no GUS activity in plants of line 3/V/B. GUS activity was observed in only some buds and plants of line 1/V/A in the first round on selection-elongation media. The majority of GUS negative buds and shoots were eliminated by continuous selection on kanamycin-containing medium. GUS expression could not be detected in all organs of GUS positive plants of line 1/V/A; the stem and leaves were negative, while the roots were positive (see Fig. 1A). The organ-specific pattern of GUS expression observed in the first or second subcultures (see Fig. 1A) disappeared during later subcultures. These results were consistent with the report by Christou [35] of the possibility of interconversion of shoot apex of layers (L1, L2, and L3) to each other. Nevertheless, there was very weak GUS expression in the stems of some *in vitro* grown plants. Different levels of GUS activity were detected in leaf tips and ribs (see Fig. 1B), leaf blades and leaf hairs (see Fig. 1C) of line 1/V/A.

GUS gene expression was observed in line 5/V/C: roots and vascular bundles of stem bases (see Fig. 1D), petioles and midribs (see Fig. 1E) gave a strong GUS response, while ribs and interribs of an *in vitro* grown leaf produced a weaker GUS reaction (see Fig. 1E). However, strong GUS activity was found in *in vitro* grown buds or shoots of line 2/II/A, localized especially in the vascular bundles and pith of a cross section of the stems, while the epidermis and other tissues usually displayed a weaker GUS response (see Fig. 1F).

There was strong GUS expression in the male flowers (see Fig. 1G), and ovary of female greenhouse-grown flowers (see Fig. 1H), and weaker GUS expression in the ovary hairs of female flowers of line 5/V/C (see Fig. 1H). It is notable that control (non-transgenic) male and female flowers of cv. Noi Yarok expressed low levels of endogenous GUS activity (see Fig. 1I). The flesh of the fruit exhibited a strong GUS response, while there was no GUS response in the flesh of the non-transgenic control fruit (see Fig. 1J).

The rooting of control plants of cv. Kırkağaç 637 was not affected by 1 mg/L PPT, but plants died on medium supplemented with 2 mg/L PPT (data not shown). All shoots of lines 3/V/B and 5/V/C died on medium supplemented with 4 mg/L PPT. Buds and shoots of line 2/II/A grew, developed and rooted on medium containing 4 or 8 mg/L PPT. Putative transgenic plants of lines 1/V/A and 5/V/C died after spraying with 1 % Basta, but putative transgenic plants of line 2/II/A were not affected (see Fig. 1K).

PCR results clearly demonstrated that lines 5/V/C and 1/V/A (characterized as possessing GUS activity) contained the NPTII gene (see Fig. 2).

3.3 Southern Blot Analysis

Three transgenic R_1 sibling plants of line 5/V/C of cv. Noi Yarok were analyzed by Southern blot (see Fig. 3). Two restriction enzymes, XbaI and SacI, cut only once in the T-DNA, each giving two major bands suggesting that the probe had labeled two fragments (containing respectively the NPTII gene and a CaMV 35S promoter) which had been separated by the enzyme. The restriction enzyme *Bam*HI cuts twice in the T-DNA, releasing an internal fragment of 3.8 kbp (identical to the probe used) observed in Fig. 3. Other lesser bands of about 5 kpb and 1.5 kbp also became visible. One of these bands (probably the 5 kbp) was due to the probe also marking the 35S promoter adjacent to the *bar* c-DNA, still attached to the genomic DNA. The three plants analyzed here are the R_1 progeny of a single transformant, and therefore had the same SacI, XbaI, and *Bam*HI patterns. The control of untransformed plants of cv. Noi Yarok did not react to the probes (see Fig. 3).

3.4 Ploidy Analysis of R_0 Plants

Two transgenic lines of cv. Kırkağaç 637 (1/V/A and 2/II/A) were tetraploid and 1 line (3/V/B) was mixoploid (diploid/tetraploid), and line 5/V/C of cv. Noi Yarok was diploid. The morphology of R_0 plants of 1/V/A and 2/II/A confirmed the result of the flow cytometry, with the characteristically enlarged flowers, and oblately flattened fruit of tetraploid melon. The occurrence of polyploid melon transformants is not confined to our transformation protocol. Polyploid regenerants have often been noted from melon cotyledons, occurring in both transgenic and non-transgenic plants [9, 11].

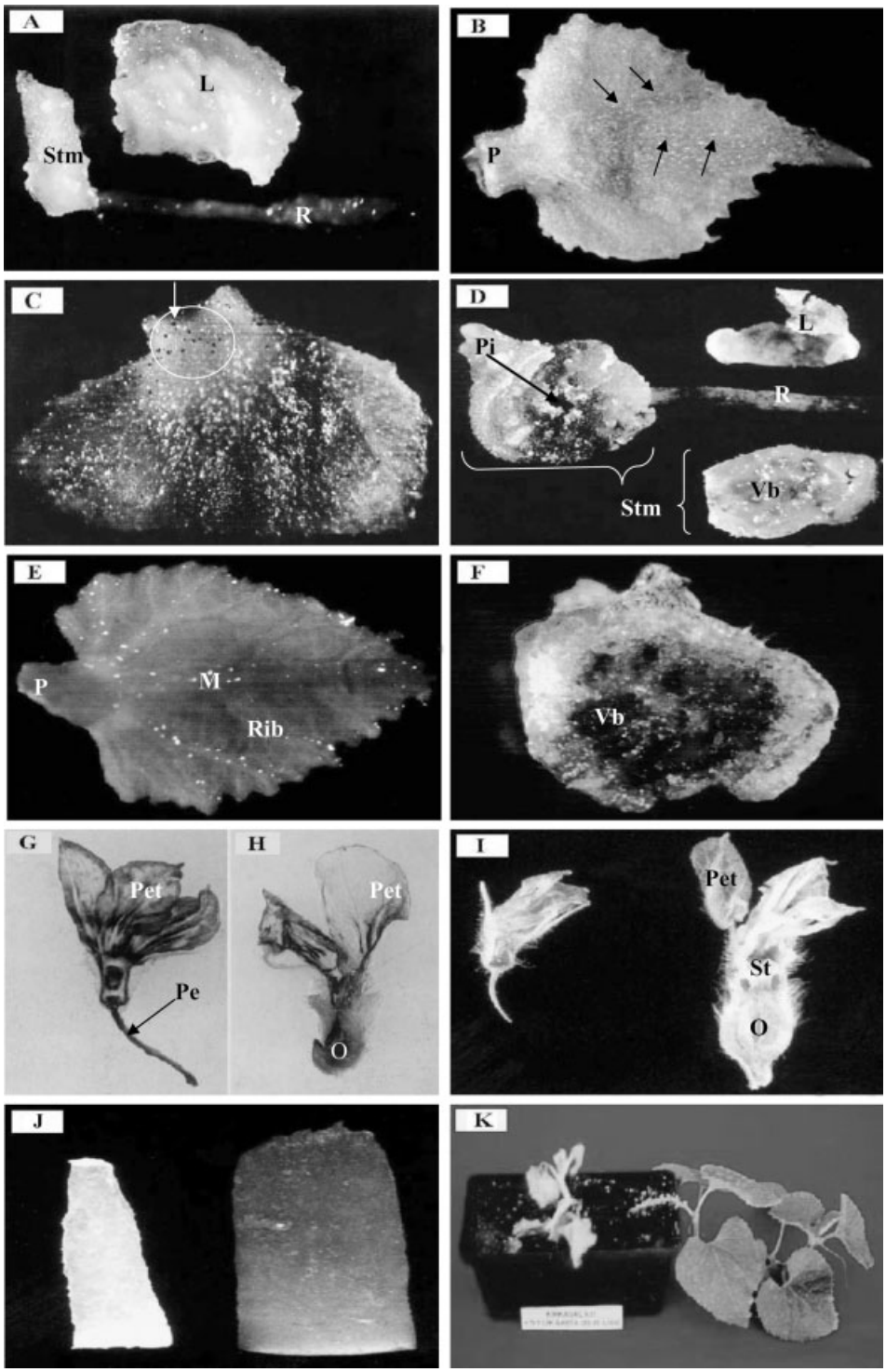

Figure 1. A) GUS activity in different organs of line 1/V/A, after one selection-elongation cycle on medium containing 50 mg/L kanamycin; (Note GUS activity in the root (R), but not in stem (Stm) or leaf (L) tissues (×10)). **B)** GUS activity in the leaf tip and ribs (arrows mark the ribs) of 1/V/A, after two selection-elongation cycles on medium containing 50 mg/L kanamycin (×10). **C)** GUS expression in the ribs, blade and hairs (the arrow marks stained hairs) of the leaf of 1/V/A, after two selection-elongation cycles on medium containing 50 mg/L kanamycin (×15). **D)** GUS expression in the root (R), base of the stem (Stm; stem, "Pi; pith), leaf (L) and in the vascular bundles of stem (Vb) of 5/V/C, after 1–2 selection-elongation cycles (×10). **E)** GUS expression in the petiole (P), midrib (M), ribs and interribs of the leaf of 5/V/C, after 1–2 selection-elongation cycles (x×10). **F)** Strong GUS activity in the vascular bundles (Vb) and pith, and weaker GUS activity in epidermal cells of the stem of 2/II/A, after 1–2 selection-elongation cycles (×20). **G)** GUS activity in the male flowers of 5/V/C (Pet; Petal, Pe; Peduncle) (×1.2). **H)** GUS assay in the female flowers of R_1 generation of 5/V/C (O; Ovary); (Note weaker GUS activity in the ovary hairs of the female flowers (×1.2)). **I)** Weak endogenous GUS activity in the flowers of non-transgenic plants of cv. Noi Yarok (St; Stigma). Left – male; right – female (×1.2). **J)** GUS expression in the flesh of the transgenic fruit (right) of R_1 generation of 5/V/C and the control (non-transformed) (left) fruit of Noi Yarok; (The rind is at the foot of the picture (×1)). **K)** Response to the application of the herbicide Basta (1 %) to melon, 10 days after spraying, right – transgenic melon plant of line 2/II/A, left – non-transformed melon control plant (×0.1).

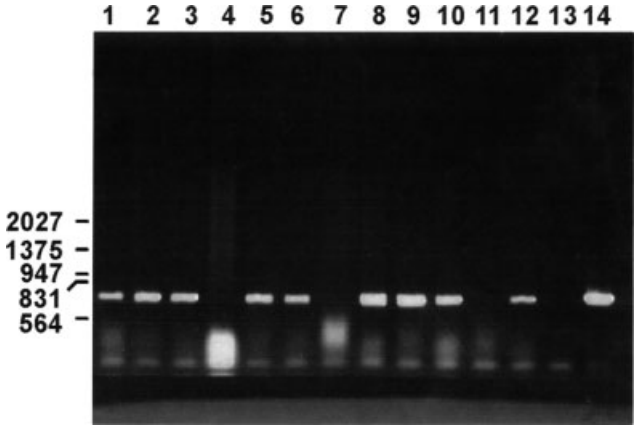

Figure 2. Ethidium bromide-stained agarose gel showing the amplification by PCR of the NPTII gene present in transgenic plants.
Samples from transgenic plants (lanes 1–3 and 5–6 [line 1/V/A], 8 [line 5/V/C], 9, and 10); non-transgenic control plant (lane 4), escapee plants (lanes 7 and 11), positive control (transgenic tobacco) (lane 12), negative control (lane 13) and positive control of pME 504 plasmid (lane 14), are indicated. Molecular weight markers (bp) are indicated on the side of the gel. Lines 2/II/A and 3/V/B were omitted from this test.

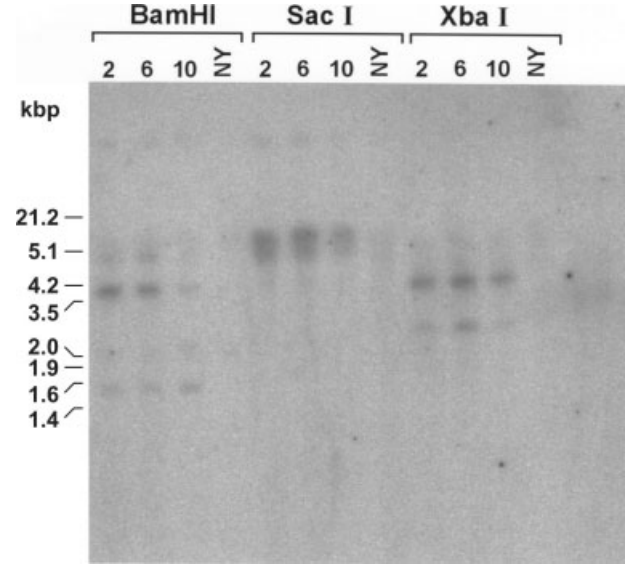

Figure 3. Southern blot gel of transgenic R_1 plants of line 5/V/C of cv. Noi Yarok.
DNA were restriction-digested, run on 0.8 % agarose gels, and blotted onto charged nylon membranes. The blots were hybridized with a purified labeled 3.8 kbp *Bam*HI fragment from the binary vector pME504, encompassing the NPTII gene and 35S promoter. After hybridization, the blots were washed and exposed to a phospho-imager. The DNA from three sibling plants was cut with three different restriction enzymes, as indicated. Molecular weight markers are indicated on the side of the figure. Lanes marked NY are non-transgenic controls of the same cultivar.

3.5 Genetic Segregation in the R_1 Generation

Several separate examinations of the segregation of the phenotype of GUS activity in the R_1 population of the diploid line 5/V/C generally gave a 3 : 1 segregation (i.e., 27/36 seedlings in a population were GUS positive). However, the distribution of the NPTII gene in this population was also examined by PCR, and it was established that 35/36 (97 %) of seedlings carried the NPTII gene. These results suggest that in line 5/V/C there were two or more separate insertions of T-DNA into the genome but also that in a certain proportion of the R_1 generation, GUS enzymatic activity cannot be detected. On the other hand, the NPTII gene was not detected in 30–40 % of R_1 plants of the tetraploid line 1/V/A by PCR, suggesting a single integration of T-DNA into the plant genome.

4 Conclusions

The transfer of three genes (*bar*, NPTII, GUS) to the melon genome was confirmed by the growth on selection medium supplemented with kanamycin and/or PPT, the expression of the GUS gene product, the PCR and Southern blot detection of the transgenes, the genetic transmission to the next generation, and Basta resistance tests of lines 2/II/A, 1/V/A, and 5/V/C. However, the *bar* gene may not always be expressed. Possible causes of this inactivation could be methylation, incomplete T-DNA transfer during the integration into the plant genome, or gene silencing [5, 36, 37].

Here we have expanded on the original observation of Cheng *et al.* [19] that wounding plant material by vortexing with carborundum prior to inoculation with *Agrobacterium* is a method that can enable genetic transformation of recalcitrant plant material. Our findings were consistent with reports that wounding of explants increases transformation [13–18]. We have used this method to transform melon cultivars recalcitrant in our hands by the usual techniques with this crop (e.g., [6, 10]). We are now using these techniques in breeding programs for the production of melon lines bearing resistances to zucchini yellow mosaic virus and cucumber mosaic virus, important viruses limiting agricultural production [38].

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Symbols used

ABA	abscisic acid
BA	benzyladenine
IAA	indole-3-acetic acid

NAA	naphthaleneacetic acid
GUS	β-glucuronidase
NPTII	neomycin phosphotransferase II gene
MS	Murashige and Skoog medium
bar	phosphinothricin acetyltransferase gene
PPT	phosphinothricin
*uid*A	GUS gene
X-gluc	5-bromo-4-chloro-3-indolyl-β-glucuronide
T-DNA	transferred DNA
HSD	Tukey's Honestly Significant Difference
NS	not significant

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