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Agrobacterium tumefaciens mediated transformation and regeneration of muskmelon plants

Guowei Fang and Rebecca Grumet

Department of Horticulture, Michigan State University, East Lansing, MI 48824, USA

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Abstract. Transgenic muskmelon (*Cucumis melo* L.) plants were produced efficiently by inoculating cotyledon explants with *Agrobacterium tumefaciens* strain LBA4404 bearing a Ti plasmid with the NPT II gene for kanaymcin resistance. After co-cultivation for three days, explants were transferred to melon regeneration medium with kanamycin to select for transformed tissue. Shoot regeneration occurred within 3-5 weeks; excised shoots were rooted on medium containing kanamycin before transferring to soil. Morphologically normal plants were produced in three months. Southern blot analysis confirmed that ca. 85% of the regenerated plants contained the NPT gene. Dot blot analysis and leaf callus assay of progeny of transgenic plants verified transmission of the introduced gene(s) to the next generation. Factors affecting transformation efficiency are discussed.

Abbreviations: ABA - abscisic acid; BAP - 6-benzylaminopurine; IAA - indole 3-acetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; NPT II - neomycin phosphotransferase II.

Introduction

One of the most effective means of gene transfer into dicotyledonous plants is to utilize the natural transformation mechanism of *Agrobacterium tumefaciens* (Gasser and Fraley 1989). Genes located between the border sequences of the *Agrobacterium*'s Ti plasmid are inserted into the genome of the host. Utilization of this mechanism for gene transfer requires both susceptibility to infection by *A. tumefaciens*, and the ability to regenerate plants from individual transformed cells via tissue culture. Although many dicots are suitable hosts for *Agrobacteria*, only a limited number of species have been transformed and regenerated successfully (Gasser and Fraley 1989).

The cucurbit family includes many high value vegetable and fruit crops (cucumbers, melons, squashes). The only published report of successful transformation and regeneration of a cucurbit species is for cucumber (*Cucumis sativus*) using the vector *A. rhizogenes* (Trulson et al. 1986). Several groups have recently reported successful regeneration from muskmelon explants (e.g. Neidz et al. 1989). In this paper, we describe an efficient procedure for the production of transgenic muskmelon (*Cucumis melo*) plants using the vector *A. tumefaciens*.

Materials and methods

Plant material and Agrobacteria. Peeled muskmelon seeds (Hale's Best Jumbo) were sterilized in 15% (v/v) Chlorox (5.25% sodium hypochlorite) with a drop of Tween 20 for ten min. then rinsed three times with sterile distilled water and placed on hormone free MS medium (Murashige and Skoog 1962) with 0.8% agar. The tissue culture growth room conditions were 25 - 26°C with a 16 h photoperiod provided by cool white fluorescent lamps (ca. 2500 lux). The *Agrobacterium* strain was disarmed *A. tumefaciens* LBA4404 containing the CIBA-GEIGY binary vector pCIB10 (Rothstein et al. 1987) with a transferable selectable marker for kanamycin resistance (the neomycin phosphotransferase gene, NPT II). *Agrobacterium* cultures were grown and maintained on AB medium (Chilton et al. 1984) with 50 $mg \cdot l^{-1}$ kanamycin.

Inoculation and co-cultivation. Excised 4 - 5 day old cotyledons were cut on all edges with a dull scalpel blade (to maximize wounding), soaked in a fresh overnight culture (10^7-10^8 bacteria $\cdot ml^{-1}$) of LBA4404 + pCIB10 for 10 - 60 min, blotted dry with sterile filter paper to remove excess bacteria, and then transferred to melon regeneration (MR) medium [MR medium = MS medium with 5 uM IAA, 5 uM BAP, 1 uM ABA and 3% sucrose; Neidz et al. 1989] with or without tobacco (*Nicotiana tabacum*) nurse cultures for a co-cultivation period of 1 - 7 days. In the experiments where tobacco nurse cultures were used, three ml of log stage tobacco

Offprint requests to: R. Grumet

suspension cultures grown in MS medium with 0.2 mg·l^{-1} 2,4-D and 180 mg·l^{-1} monobasic potassium phosphate were pipetted onto MR plates three days prior to transformation and kept at 25 - 26°C with 16 h photoperiod. Just before co-cultivation two pieces of sterile #3 filter paper were laid over the tobacco cells, explants were put on the paper.

Plant regeneration and selection. Control melon tissue was tested for sensitivity to kanamycin using MR medium containing 0 - 200 mg kanamycin·l^{-1}. Based on those results 75 mg·l^{-1} was used in transformation experiments. After co-cultivation with *Agrobacteria*, explants were washed with sterile distilled water to remove excess bacteria, blotted dry, and transferred onto MR medium containing 75 mg·l^{-1} kanamycin (to select for transformed tissue) and 100 mg·l^{-1} cefotoxime (to eliminate bacterial carry over). Developing shoots were transferred to hormone free, root inducing medium (MS salts with 3% sucrose, 100 mg·l^{-1} cefotoxime) with or without 50 mg·l^{-1} kanamycin. Regenerated plantlets were transplanted to sterile Bacto soil mix as soon as roots appeared and transferred to the greenhouse.

Genetic analyses. Genomic DNA was extracted from young leaf tissue of putative transformed plants using the procedure of Dellaporta et al. (1985) and either digested with *Bam* HI that cuts on one side of the NPT II gene, or with *Bam* HI and *Bcl* I to drop out the NPT II gene from the T-DNA. Southern blot analysis was performed according to Maniatis et al. 1982. To prepare the hybridization probe, the NPT II gene was isolated from pCIB10 as a 2.2 kb *Bgl* II-*Bam* HI fragment and random primer labeled with ^{32}P (Feinberg and Vogelstein 1983). To test for inheritance of the introduced NPT gene, transformed melon plants were either selfed, or crossed to control plants, in the greenhouse. The progeny were examined for the NPT gene by dot blot analysis; genomic DNA was extracted from leaves of three week old plants, spotted onto nitrocellulose and probed with labeled NPT fragment. Explants from cotyledons of progeny were also tested for expression of the NPT II gene by culturing on MR medium with kanamycin.

Results and discussion

Transformation conditions

Several factors including kanamycin level, *Agrobacterium* concentration, inoculation time, length of co-cultivation period, and the use of tobacco nurse cultures were tested to optimize the melon transformation system. The sensitivity of non-transformed melon tissue to kanamycin was examined using MR medium containing 0, 10, 25, 50, 75, 100, or 200 mg kanamycin·l^{-1}. Normal callus and shoots only developed on medium without kanamycin, some callus and shoots were formed on medium with 10 mg·l^{-1}. At 25 mg·l^{-1} a few explants produced slowly growing callus and buds, but no shoots were formed. Kanamycin levels at or above 75 mg·l^{-1} completely inhibited growth of control explants, and so this level was chosen for routine selection of transformed tissue.

Bacterial concentration for inoculation is a critical variable for lettuce transformation (Michelmore et al. 1987), but has little influence on petunia transformation (Horsch et al. 1985). To examine the effect of bacterial titer on gene transfer efficiency for melon, explants were inoculated with concentrations ranging from 10^5-10^{10} bacteria·ml^{-1}. Although there was little influence of bacterial titer on transformation and regeneration (data not shown), the condition of the bacterial culture was very important. Fresh bacteria grown under optimal conditions (overnight from a fresh inoculum culture, 30°C, well-aerated) gave the highest transformation efficiency. We routinely used a 15 - 24 h culture at a concentration of 10^7 - 10^8 bacteria·ml^{-1}.

To study the influence of exposure time to bacteria, explants were soaked in bacteria for either 10, 20, 30, or 60 min (Table 1). There was little difference in transformation efficiency up to 30 min. A 60 minute exposure reduced efficiency by 40% relative to a 10 min exposure, probably due to subsequent bacterial contamination that inhibited explant growth. Ten minute soaks were routinely used. Co-cultivation periods of 1, 3, 5 and 7 days were tested (Table 2).

Table 1. Effect of *A. tumefaciens* inoculation time on callus and shoot production by melon explants cultured on MR medium with 50 mg·l^{-1} kanamycin.

Inoculation time (min)	Total explants	# Producing callus (%)	Shoots produced (%)
10	79	24 (30.4)	8 (10.1)
20	81	21 (25.9)	9 (11.1)
30	77	19 (24.7)	8 (10.3)
60	73	13 (17.8)	4 (5.5)

Explants were co-cultivated for three days, then transferred to medium with kanamycin, and counted five weeks after inoculation. The data are compiled from two experiments.

Table 2. Effect of co-cultivation period on callus and shoot production by melon explants cultured on MR medium with 75 mg·l^{-1} kanamycin.

Co-cultivation (days without kanamycin)	Total explants	# Producing callus (%)	Shoots produced (%)
0	117	14 (11.9)	3 (2.6)
1	121	29 (23.9)	11 (9.1)
3	119	48 (40.3)	15 (12.6)
5	113	37 (32.7)	12 (10.6)
7	114	17 (14.9)	5 (4.4)

Explants for all treatments were inoculated with *Agrobacterium* for 10 min; counting was done 40 days after inoculation. The data are compiled from three experiments.

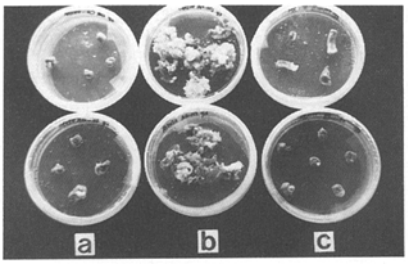


Fig. 1. Cotyledon explants of melon cultured on medium with 75 $mg \cdot l^{-1}$ kanamycin. (a) uninoculated control explants; (b) explants inoculated with *A. tumefaciens* LBA4404 + pCIB10; (c) control explants inoculated with *A. tumefaciens* LBA4404 without plasmid. The cultures were photographed three weeks after inoculation.

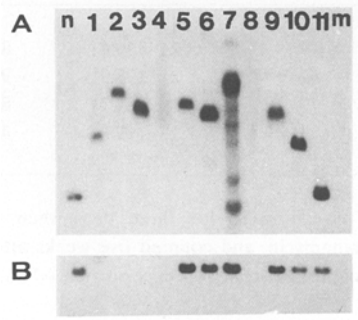


Fig. 2. Southern blot hybridization analysis of genomic DNA from putative transgenic melon plants. (A) The DNA was digested with *Bam* HI and probed with isolated, ^{32}P-labeled NPT fragment. Lane n: one copy reconstruction (50 pg) of 1.9 kb NPT gene isolated from pCIB10; Lanes 1 - 11: genomic DNA extracted from young leaves of individual putative transgenic plants (12 $\mu g \cdot lane^{-1}$); Lane m: 12 μg genomic DNA from untreated control melon plant. (B) A subset of the above samples (#5, 6, 7, 9, 10, 11) were doubly digested with *Bam* HI and *Bcl* I to release the NPT II gene. Other aspects of DNA preparation and probing were as for A.

Three days of co-cultivation resulted in the highest percentage of shoot regeneration on kanamycin. With longer periods it was not possible to completely eliminate the *Agrobacteria.*

Tobacco nurse cultures are sometimes used to increase transformation efficiency (Rogers et al. 1986). The effect of nurse cultures on melon transformation was tested after the optimal inoculation and co-cultivation times had been determined. In two of four experiments the presence of tobacco nurse cultures resulted in a 10 - 15% higher rate of shoot regeneration, in the other two experiments there was no obvious difference. Since the nurse cultures did not make a large difference in transformation efficiency they were not routinely included.

Transgenic plants

Explants were either inoculated with LBA4404 + pCIB10, with LBA4404 without plasmid (no NPT gene), or were not exposed to *Agrobacteria*. Although explants from all treatments readily formed callus and shoots on medium without kanamycin, only explants inoculated with LBA4404 + pCIB10 produced callus and regenerated shoots on MR medium with 75 $mg \cdot l^{-1}$ kanamycin (Fig 1). Buds were produced along the cut edges of the explants 2 - 3 weeks after inoculation. In 2 - 3 more weeks approximately 20 - 30% of the buds developed into shoots that were large enough to be transferred to rooting medium (this is an ca. 40% reduction relative to the number of shoots that regenerate from non-inoculated explants on MR medium without kanamycin). To further select for transformation, 50 $mg \cdot l^{-1}$ kanamycin was included in the rooting medium. In another 3 - 5 weeks, about 30% of the shoots produced roots (vs. 80% of shoots from non-inoculated explants when rooted on medium without kanamycin). Plantlets were transferred to soil mix as soon as possible after root initiation, longer growth on medium reduced the survival rate in soil. Although there was some variation in growth rate of the regenerated plants, they appeared to be morphologically normal and were fertile.

In other plant transformation systems, antibiotics were reported to affect development of the regenerated plant (Michelmore et al. 1987) and inhibit rooting of the transformed shoot (James et al. 1989). Although the reduced rooting observed for melon may be the effect of the antibiotic, the use of kanamycin in the rooting medium was helpful in reducing the number of putative transgenic, regenerated plants that did not test positive by Southern blot assay (data not shown).

The only cultivar tested in our experiments was Hale's Best Jumbo. Of the four genotypes tested by Neidz et al. (1989) this cultivar gave the highest percent regeneration. Although cultivar specific differences in regeneration efficiency of melon have been reported by several investigators (e.g. Orts et al. 1987; Dirks and Van Buggenum 1989) we do not have reason to anticipate that the transformation steps will be cultivar dependent.

Southern blot analysis was performed to determine if the regenerated plants were transgenic. DNA was isolated from several control melon plants, and from 25 of the ca. 60 regenerated plants that were rooted in the presence of kanamycin. The NPT probe did not hybridize with DNA from the control plants but did hybridize with DNA from 22 of the 25 kanamycin-resistant regenerated plants.

Thus in almost every case there was successful incorporation of the NPT gene into the melon genome. A *Bam* HI digest of the DNA from eleven of these plants is shown in Fig. 2a. Nine were positive for the presence of the NPT gene; there was no hybridization in the control lane. Since *Bam* HI only cuts on one side of the NPT gene, the other *Bam* HI site must come from the melon genome. Variable hybridization patterns were observed among the NPT-positive plants indicating the expected random integration of the NPT gene into the melon genome. Most transformed plants appeared to contain a single copy of insert DNA (single band), plant #7 may have two or more integrations into its genome (multiple bands). The DNA from several plants was also double-digested with *Bam* H1 and *Bcl* I to release an internal fragment from the transferred DNA. In each case, the expected ca. 1.9 kb band was observed (Fig. 2b).

Four transformants (plant #'s 1, 5, 6, 7) were either self-pollinated, or backcrossed to non-transformed control plants, to produce the next generation. The progeny were examined for the NPT gene by dot blot analysis; the results are summarized in Table 3. The segregation ratios of the progeny of all six families was consistent with predicted Mendelian ratios as tested by X^2 analysis. In each case the P values were much greater than the rejection level of P=0.05. The progeny from self-crosses of plants 1, 5, and 6, gave the expected 3:1 ratio (NPT positive: NPT negative) for the incorporation of a single gene.

Similarly, when plants 1 and 6 were backcrossed withthe parental genotype, the progeny gave the expected 1:1 ratio for single gene incorporation. The 15:1 ratio observed for the progeny of selfed plant #7 implies that two genes were incorporated. In each case, the number of genes determined by progeny analysis was consistent with the observed Southern blot pattern for the original regenerated plants (Fig. 2).

A leaf callus assay was conducted to determine expression of the kanamycin resistance trait (Fig. 3). Young leaves from NPT positive (dot blot) progeny were cultured on MR medium with 75 $mg \cdot l^{-1}$ kanamycin. Explants from NPT-positive progeny produced callus within two weeks; no callus was produced by non-transformed (dot blot negative) progeny or by control explants. Thus kanamycin resistance behaved as a dominant trait as reported for other plants (e.g. Chyi et al. 1986; Catlin et al. 1988).

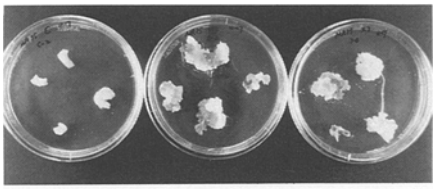
Table 3. Summary of dot blot data of progeny of transgenic regenerants.

Family	NPT[a] +	-	# Plants tested	Probable # of integrations[b]	Expected ratios	X^{2c}	P
1 - self	13	5	18	1	3:1	.073	1.00
1 - cross	9	10	19	1	1:1	.053	1.00
5 - self	13	3	16	1	3:1	.330	0.80
6 - self	15	3	18	1	3:1	.667	0.65
6 - cross	9	11	20	1	1:1	.200	0.90
7 - self	17	1	18	2	15:1	.015	0.75

[a] Genomic DNA was extracted from leaves of three week old plants and probed with ^{32}P-labeled NPT fragment. + hybridized to the NPT probe; - did not hybridize.

[b] Based on number of NPT-positive progeny and Southern blot hybridization patterns.

[c] Calculated as $X^2 = \Sigma [(|o - e| - ½)^2/e]$ using the Yate's correction factor.



a b c

Fig. 3. Sample leaf callus assay for expression of the NPT-II gene. Young leaves from control plants (a) or NPT positive, F_1 progeny (b,c) were cultured on MR medium with 75 $mg \cdot l^{-1}$ kanamycin.

Conclusions

We have successfully transformed and regenerated muskmelon plants using *Agrobacterium tumefaciens* and a modified leaf disk procedure. The best conditions in our experiments included the use of 4-5 day old cotyledons soaked for ten min in a fresh overnight culture of *A. tumefaciens*, a three day co-cultivation period, regeneration on MR medium containing 75 $mg \cdot l^{-1}$ kanamycin, rooting in the presence of 50 $mg \cdot l^{-1}$ kanamycin and immediate transfer to soil. Efficiency of transformation and regeneration is 3 - 7% (calculated from initial explants to independently derived transgenic plants). Time from initiation of the experiment to plants in the greenhouse is approximately three months.

Southern blot data and progeny analysis verify integration of the NPT gene into the melon genome and transmission of the NPT gene to subsequent generations.

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