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Agrobacterium-Mediated Transformation of 'Kirkagac 637' a Recalcitrant Melon (*Cucumis melo* L.) Cultivar with ZYMV Coat Protein Encoding Gene

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Summary

A gene encoding for zucchini yellow mosaic virus (ZYMV) coat protein was transformed into 'Kirkagac 637', an important Turkish melon cultivar using *Agrobacterium tumefaciens,* and putatively resistant transgenic lines were obtained. Proximal cotyledonary explants were incubated with *Agrobacterium* carrying the binary plasmid PGA643 including the *nptii* and *zymv* coat protein genes under the control of the CaMV 35S promoter.

ELISA tests and PCR analyses were performed to determine which plants were transformants. Transformation efficiency was 1,6 %. Four transformants were self pollinated to obtain R_1 generation. The plants from the R_2 generation were tested by PCR to demonstrate the integration of the *zymv* coat protein gene (1000 bp) in to the 'Kirkagac 637' genome. Segregation of 4 transformants was tested by X^2 analysis and the results were consistent with predicted Mendelian ratios. In each case the P values were much greater than the rejection level of P=0.05.

Zusammenfassung

Transformation des Hüllprotein-Gens von ZYMV mittels *Agrobacterium* in die Melonensorte 'Kirkagac 637'. Das Gen für das Hüllprotein des Zucchinigelbmosaikvirus (ZYMV) wurde mittels *Agrobacterium tumefaciens* in 'Kirkağaç 637', eine sehr wichtige Melonensorte in der Türkei, transformiert, was zu vermutlich resistenten transgenen Linien führte. Kotyledonen ohne apikalmeristematischen Teil wurden als Explantate verwendet und mit *Agrobacterium tumefaciens*, welches das binäre Plasmid PGA643 mit dem *nptii*-Gen und dem *zymv*-Hüllproteingen kontrolliert vom CaMV 35S-Promotor aufweist, inkubiert.

Zur Erkennung transformierter Pflanzen wurden ELISA-Tests und PCR-Analysen durchgeführt. Dabei zeigte sich, dass die Transformationsrate 1,6 % betrug. Vier Transformanten wurden geselbstet, um eine R_1-Generation zu erhalten. Um die Integration des *zymv* Hüllproteingens (1000 bp) in das Genom von 'Kirkagac 637' in den R_2-Pflanzen nachzuweisen, wurden PCR-Analysen durchgeführt.

Die Aufspaltung der vier Transformanten wurde mit dem χ^2 –Test überprüft. Die Ergebnisse stimmten mit der erwarteten Mendel-Spaltung überein. In jedem Fall waren die P-Werte wesentlich größer als 0.05.

Key words. melon – *Agrobacterium tumefaciens* – ZYMV CP gene – regeneration – transformation

Introduction

Turkey is a secondary centre of diversification of melon (*Cucumis melo* L.) (PITRAT et al. 1999) and the second largest producing country, after China, of 1.9 million tons annually (FAO 2002). There are many conventional melon varieties widely grown in Turkey. However, the majority of these are susceptible to several viruses (YILMAZ et al. 1992), fungal diseases (SARI et al. 1994) and other pests, although some imported melon cultivars have useful resistances. The most serious disease-causing agents are viruses, which cannot be directly controlled. Two of the most severe viral diseases found in melon, zucchini yellow mosaic virus (ZYMV), and cucumber mosaic virus (CMV), are widespread throughout Turkey (YILMAZ et al. 1992).

Biotechnology is a potential tool for improving new plant cultivars. Genetic transformation presents new prospects to conventional melon breeding, by allowing the introduction of traits such as virus resistance that could not be provided by conventional breeding methods. There have been several reports of the genetic transformation of melon with a variety of marker genes, as well as genes for viral resistance, abiotic stress resistance and fruit quality attributes (FANG and GRUMET 1990, 1993; DONG et al. 1991; YOSHIKA et al. 1992;

GONSALVES et al. 1994; VALLES and LASA 1994; GABA et al. 1995; AYUB et al. 1996; CURUK 1999; BORDAS et al. 1997; GUIS et al. 2000; GALPERIN et al. 2003). GABA et al. (1995) transformed cv. 'Galia' a recalcitrant cultivar by particle bombardment with 1 % transformation efficiency. The highest transformation efficiencies from cotyledon and leaf explants of melon were reported by FANG and GRUMET 1990, GUIS et al. 2000 and GALPERIN et al. 2003 with 7 %, 2.4 % and 100 % (1.5 transgenic shots per explant), respectively. However, it has been reported that the Turkish melon cv. 'Kirkagac 637' is a recalcitrant melon cultivar with the transformation methods used by FANG and GRUMET (1990) and VALLES and LASA (1994), presumably due to non-defined methodological or environmental differences, as well as genotype and/or seed batch dependence (CURUK 1999). Therefore, a different transformation method was used by CURUK (1999) to transform cv. 'Kirkagac 637' by vortexing melon cotyledon explants in water with carborundum causing wounding, which enabled genetic transformation with 1 % frequency.

The objective of this study was to transfer the *zymv* resistance gene into 'Kirkagac 637', a commercially important Turkish melon cv. using the *Agrobacterium*-mediated transformation method described by FANG and GRUMET (1990) with several modifications, as the transformation method reported by CURUK (1999) was tedious, time consuming and labour-intensive.

Materials and Methods

Plant Material

Seeds of *Cucumis melo* var. *inodorus* cv. 'Kirkagac 637' were obtained from Ege Agricultural Research Center (Turkey). This cultivar is resistant to *Fusarium oxysporum* f.sp. *melonis* 0 and 1 strains (SARI et al. 2000).

Agrobacterium *and Binary Plasmid*

Agrobacterium tumefaciens strain C58 containing the binary plasmid PGA643 that harbor the *nptii* and *zymv* coat protein genes both under the control of the CaMV 35S promoter (FANG and GRUMET 1990) was used for transformation (obtained from Prof. Rebecca Grumet, Horticulture Department, Michigan State University, USA).

Tissue Culture Conditions During Transformation

Melon seed coats were removed, and the seeds were sterilized in a solution of 15 % laundry bleach with 2 drops of Tween 20 per 100 ml solution with occasional shaking for 10–12 min. After rinsing with sterile distilled water three times, the seeds were incubated in darkness in sterile distilled water for 16–18 hours, and the cotyledons excised. Approximately 3600 explants from 1800 melon seeds (2 proximal cotyledonary explants without shoot apex per seed) were used for transformation. MR medium (4.4 g l^{-1} MS basal medium, 30 g l^{-1} sucrose, 0.88 mg l^{-1} IAA, 1.12 mg l^{-1} BAP, 0.26 mg l^{-1} ABA, 8.0 g l^{-1} agar, pH 5.8.), M2 medium (MR with 400 mg l^{-1} timentin) and M3 medium (MR with 400 mg l^{-1} timentin and 200 mg l^{-1} kanamycin) were used as selective regeneration media. *In vitro* cultures were incubated at 25–26 °C in a 16 h photoperiod (30 µmol m^{-2} s^{-1} fluorescent white light) for all regeneration selection treatments. Following regeneration selection, a shoot elongation medium was used (MSG) (4.4 g l^{-1} MS basal medium, 30 g l^{-1} sucrose, 0.67 mg l^{-1} BAP, 8.0 g l^{-1} agar, pH 5.8), and shoots were rooted on a MSR medium (4.4 g l^{-1} MS basal medium, 30 g l^{-1} sucrose, 0.01 mg l^{-1} NAA, 8.0 g l^{-1} agar, pH 5.8).

Inoculation and Co-cultivation

The proximal part of the cotyledons without apical meristem was used as an explant. The explants were incubated in an overnight *Agrobacterium* culture for 15 min. After pouring off the bacteria, explants were blotted dry with sterile filter paper and placed on MR medium and incubated in dark for 3–4 days. Explants were then rinsed three times with sterile distilled water and blotted dry with sterile filter paper, placed on M2 medium and incubated under light in the growth room for 7 days. Afterwards, they were transferred to M3 medium (18 explants/plate).

After 3–5 weeks, shoots were excised and placed in Magenta G7 boxes containing MSG medium supplemented with 200 mg l^{-1} kanamycin and 400 mg l^{-1} timentin for 2–3 weeks. Elongated shoots were transferred to Magenta G7 boxes containing MSR medium with 400 mg l^{-1} timentin for 2–3 weeks.

ELISA Test for NPTII protein

Samples of leaf tissue (20–30 mg) from regenerated plants growing on medium with kanamycin were screened for expression of NPTII protein using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The NPTII assay kit was purchased from 5 Prime–3 prime, Inc. (Boulder, CO) and the assay was performed according to the manufacturer's instructions.

DNA Preparation and PCR Analysis

Genomic DNA was extracted from young leaves using a modified protocol of DELLAPORTA et al. (1985). DNA concentration was assessed by spectrophotometer. DNA was diluted in water to a final concentration of 50 ng μl^{-1} and stored at –20 °C.

The primers for the PCR reaction to detect the *zymv* coat protein gene were 5' AGATC TAAAT AACAA ATCTC AACAC 3' and 5'AGATCTCTGC AGCCC TTTTT TTTT 3', as per FANG and GRUMET (1990). PCR was performed in 25 µl reaction volumes containing 50 ng DNA, 1 x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.02 mM dNTP mixture, 2.5 µmol of each primers, 1 unit of Taq DNA polymerase and distilled water, overlaid by a drop of mineral oil. The mixtures were assembled at 0 °C, and then transferred to a thermal cycler, precooled to 4 °C. The amplification was carried out in a thermocycler (Perkin Elmer Cetus DNA Thermal Cycler 480) with an initial denaturation step of 2 min at 94 °C, 40 sec at 95 °C, 40 sec at 55 °C and 5 min at 72 °C. The amplification products (approximately 1.1 kb) were separated by electrophoresis in 2 % agarose gels in 1 x TAE buffer (40 mM Tris-Ac-

etate, 1 mM EDTA, pH 8.0) for 3 h at 70 V. The gel was stained for 20 min with ethidium bromide and the fragment patterns were photographed (Polaroid 667film) under UV.

Acclimatization and propagation

After the root system was formed, the putative transgenic plants were transferred to soil in multipots and covered with a plastic bag for 1 week. Two plants of line 20 and three plants of line 4 were grown in the greenhouse and selfed. About 20 seeds from each fruit were germinated on MS medium. Genomic DNA from these plants was isolated and PCR reactions conducted to observe the *zymv* coat protein gene in genomic DNA, and to examine the segregation of the transgenes in the R_1 generation.

Results and Discussion

Transformed shoots elongated and developed quickly. Non-transformed shoots remained small and bleached and/or yellowed due to the high kanamycin concentration (200 mg l^{-1}). Some of the non-transformed elongated shoots grew as well as transformed shoots. CURUK (1999) used different amounts of kanamycin (50, 75, 100 mg l^{-1}) in her transformation studies. She mentioned that successful transformation of cv. 'Kirkagac 637' required regeneration selection for 15–20 days on a high (100 mg l^{-1}) kanamycin concentration.

The shoots regenerated on M3 medium were placed on rooting media without kanamycin. After another 3–4 weeks 60 % of the shoots produced roots (Fig. 1).

3.4 % of the explants formed putative transgenic shoots (122/3600 explants). ELISA tests for the *nptII* gene product was performed on 122 rooted shoots, of which 56 were positive and 66 rooted shoots were negative for NPTII protein. Thus, the ELISA test showed that non-transformed elongated shoots also grew on shoot proliferation and rooting media. FANG and GRUMET (1993) reported that 45 % of the muskmelon plants, regenerated in the presence of kanamycin, were positively tested for *nptII* expression by ELISA. DNA extracted from the plants found positive for the presence of the NPTII protein was then tested by PCR reaction. An agarose gel revealed that all these plants carried the *Zymv* coat protein gene.

The transformation efficiency of cv. 'Kirkagac 637' was 1.6 % (56/3600 explants) similar to GABA et al. (1995) and CURUK (1999), but lower than the transformation efficiency reported by FANG and GRUMET (1990), GUIS et al. (2000) and GALPERIN et al. (2003). Our results confirm that 'Kirkagac 637' is a recalcitrant melon variety, as described by CURUK (1999).

In our transformation system, the incubation of *Agrobacterium*-inoculated explants for 3–4 days in dark was the main modification of method of FANG and GRUMET (1990). The exact mechanism of how dark pretreatments stimulate subsequent light-dependent organogenesis is not completely understood but it is likely that incubation of plant tissues in darkness preserves light sensitive endogeneous plant growth regulators and other compounds (EVANS et al. 1981; HARTMANN et al. 1997; COMPTON 1999). Numerous reports exist in plant tissue culture literature where pretreatment of plant tissues in darkness improves adventitious shoot regeneration from cotyledons, leaf cells, petioles, nodal tissues (PUNJA et al. 1990; LEBLAY et al. 1991; MOHAMMED et al. 1992) and protoplasts cultured from cotyledons and leaves (SHAHIN 1984; CHENG and VEILLEUX 1991).

Four transformants (2 from line 20 and 2 from line 4) were self-pollinated to produce the next generation (R_1) and their fruits were obtained. The progeny were examined for the presence of the *zymv* CP gene by PCR. About 20 seeds from each fruit were germinated and seedling DNAs were isolated using a modified protocol of DELLAPORTA et al. (1985). A PCR reaction was performed to determine if the selfed plants were transgenic. The result of the PCR reactions is shown in Table 1.

The segregation ratios of the progeny of four fruits were consistent with predicted Mendelian ratios as tested by χ^2 analysis. P values were greater than the rejection level of P=0.05. The progeny from self crosses of plants 4.1, 20.1 and 20.2 gave the expected 3:1 and 1:3 ratios, respectively (*zymv* positive : *zymv* negative) for the incorporation of a single gene. The 15:1 ratio observed for the progeny of selfed plant 4.2 implies that two transgenes were incorporated. FANG and GRUMET (1993) analysed the progeny of self-fertilized transgenic tobacco plants by ELISA for the presence of the *nptii* gene. They showed that progeny of most of the nine tested lines segregated with a ratio of 3:1 (*npt*+ : *npt*–), indicating that the *npt* gene was expressed from a single locus.



Fig. 1. (A) Inoculated explants on M3 medium, (B) explants on shoot proliferation medium, (C) explants on rooting medium.

Table 1. PCR analysis of R_1 progeny of transgenic regenerants showing a PCR product from the *zymv* CP gene in the parents

Transgenic melone line	+*zymv* CP gene	–*zymv* CP gene	Plants tested	Probable integrations	Expected ratio	χ^2	P
20.1	13	7	20	1	3:1	1.06	30 %
20.2	7	13	20	1	1:3	1.06	30 %
4.1	16	4	20	1	3:1	0.26	50–70 %
4.2	18	2	20	2	15:1	0.48	50 %

We have demonstrated here the original observations of FANG and GRUMET (1993) and CURUK (1999) with a recalcitrant melon cultivar. The advantages of our method compared to method reported by CURUK (1999) are the slightly higher transformation efficiency and easier application with the same recalcitrant cultivar. However, more work is needed to improve the transformation efficiency for such recalcitrant cultivars.

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