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Transformation of NHX-1 Gene into Some Melon Varieties

Tolga İZGÜ[(1)] Başar SEVİNDİK[(2)] Özhan ŞİMŞEK[(2)] Yeşim YALÇIN MENDİ[(2)*]

Abstract

In the present study, an efficient method for shoot regeneration and transformation by *Agrobacterium tumefaciens* strains in two different melon cultivars (*Cucumis melo* var. *inodorus* cv. 'Kirkagac 637' and *Cucumis melo* L., *cv.* Védrantais) have been developed. *A. tumefaciens* AGL1 race provide pUBINHX1 plasmid, resistant to salinity. Expression of AtNHX1 (Na-H antiporter) gene driven by CaMV 35S control in pUBINHX1 plasmid. In the structure of the plasmid hygromisin phospotransferase gene located as plant selection marker gene in T DNA region. The best regeneration result was obtained from MS medium supplemented with 2.2 µM BAP and 0.5 µM NAA. DNA isolation, PCR and gel electrophoresis were done from putative transgenic plants, 200 bp band size was obtain. Regeneration of putative transgenic plants obtained from proximal and cotyledon explants showed differences in both cultivars.

Key Words: *Cucumis melo*, Transformation, Salinity, Gene transfer, PCR

Bazı Kavun Çeşitlerine NHX-1 Geninin Transformasyonu

Özet

Bu çalışmada, iki kavun çeşidinde (*Cucumis melo* var. *inodorus* cv. 'Kirkagac 637' ve *Cucumis melo* L. *cv.* Védrantais) *Agrobacterium tumefaciens* suşu kullanılarak sürgün rejenerasyonu ve transformasyonu için en uygun metod geliştirilmiştir. *A. tumefaciens* AGL1 ırkındaki pUBINHX1 plazmidinde tuzluluğa dayanıklılık geni bulunmaktadır. pUBINHX1 plazmidi içerisinde bulunan AtNHX1 (Na-H antiporter) geni, CaMV 35S promotoru kontolü altında ifade olmaktadır. T-DNA bölgesinde lokalize olan hygromisin phospotransferase geni bitki seleksiyon marker geni olarak görev almaktadır. En iyi rejenerasyon sonucu, 2.2 µM BAP ve 0.5 µM NAA içeren MS ortamından sağlanmıştır. Putatif transgenik bitkilerde, DNA izolasyonu, PCR ve jel elektroforez çalışmaları yapılmış, 200 bç bant büyüklüğü saptanmıştır. Proksimal ve kotiledon eksplantlarından elde edilmiş putative transgenik bitkilerin rejenerasyonu, her iki çeşit içerisinde farklılıklar göstermiştir.

Anahtar Kelimeler: *Cucumis melo*, Transformasyon, Tuzluluk, Gen transferi, PCR

Introduction

Melon belongs to *Cucurbitaceae* family and known to be one of the most important horticultural crop. Varieties of *Cucumis melo* species spread large area in Asia, especially Korea, China and Japan (Zhang et al. 2014). Melon originates from China and secondary diversification center is Turkey (Pitrat et al. 2000) Turkey is one of the most important country in the world for melon production as 1.7 million tonnes annually according to 2013 statistics after China (FAO, 2014). There are some reports on genetic transformation in different melon varieties for disease resistance, biotic and abiotic stress resistance (Mendi et al. 2012).

Recently, biotechnology has wide usage among plant breeding techniques and one of the most important technique is genetic transformation. It presents improvement of new plant varieties and new prospects for conventional melon breeding (Mendi et al.

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[1]Department of Horticulture, Faculty of Agriculture, University of Ege, 35100, Bornova, İzmir, Turkey
[2]Department of Horticulture, Faculty of Agriculture, University of Çukurova, 01330, Balcalı, Adana, Turkey
*ymendi@gmail.com

2012). The development of biotechnological tools have made it possible to overcome some of conventional problems. Transformation researches were developed for melon, watermelon, and other plants in the last decades and the first gene transformation to melon was performed successfully by Fang and Grumet using cotyledon explants (Bezirganoglu et al. 2014).

It was represented that *Cucumis melo* susceptible to *Agrobacterium* infection and it has high transformation frequency (Zhang et al. 2014). Especially, *Agrobacterium tumefaciens* mediated method has been most successful one for obtaining transgenic plant (Fang and Grumet 1990).

Salinity is an important abiotic stress factor for open-field to produce *Cucumis melo* varieties (Kuşvuran et al. 2007). Researchers have identified and isolated many genes connected salt stress (Serrano and Gaxiola 1994). NHX-1 gene is one of the novel antiport identified from *S. cerevicesae* and the role of this gene is Na+/H+ antiporters in salt tolerance and pH homeostasis.

The aim of this study is to develop a reproducible *Agrobacterium tumefaciens* mediated genetic transformation technique followed by an efficient regeneration system which can together provide stable expression and transmission of the introduced ATNHX-1 gene by the use of cotyledon and proximal explants isolated from germinated seeds.

Material and Method

Plant Material

Cucumis melo var. *inodorus* cv. 'Kirkagac 637' and *Cucumis melo* L. *cv.* Védrantais were used as plant material. Seeds of genotypes were obtained from a commercial seed company.

Bacterial Strain and Plasmid

Agrobacterium tumefaciens EHA 105 recombinant bacteria was used for transformation assays. Bacteria was provided from USA California Davis University Plant Science Department. *A. tumefaciens* AGL1 race provide pUBINHX1 plasmid, resistant to salinity. Expression of AtNHX1 (Na-H antiporter) gene driven by CaMV 35S control in pUBINHX1 plasmid. In the structure of the plasmid Hygromisin phospotransferase gene located as plant selection marker gene in T DNA region. Antibiotic resistant gene located, except T-DNA region.

Method

In vitro Regeneration and Transformation Protocols

Coats of melon seeds were removed, and the seeds were sterilized by ETOH solution 70% and 20% NaOCl with 2 drops of Tween 20 per 100 ml solution for 15 min. After rinsing with sterile distilled water three times, the seeds were incubated in dark condition and hormone free MS medium (Murashige and Skoog, 1962) and the cotyledons excised. Totally 400 seeds were used 200 of them *Cucumis melo L. cv.* Védrantais cotyledones and 200 of them *Cucumis melo* var. *inodorus* cv. 'Kirkagac 637' (200 proximals and 200 cotyledones).

In order to identify the concentration of *A. tumefaciens* incubated in LB medium for overnight, spectrophotometric measurement was carried out. Incubated bacteria races, which has $O.D._{600}$ =(0.5-0.6) absorbance, were used in transformation experiments. The proximal and cotyledon part of the cotyledons without apical meristem was used as an explant. The explants were incubated in *Agrobacterium* culture for 15 min. After pouring of the bacteria, explants were blotted dry with sterile filter paper and placed on MS medium and incubated in dark for 3–4 days.

Inoculation and Co-cultivation

Totally 400 melon cotyledon explants from 200 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, 1 g l^{-1} MES, 30 g l^{-1} sucrose, 2.2 µM BAP, 0.5 µM NAA, 8 g l^{-1} agar, pH: 5.8) and M2 medium (MR with 750 mg l^{-1} Cefax) 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 (DM) (4.4 g l^{-1} MS (Murashige and Skoog, 1962) basal

medium, 30 g l^{-1} sucrose, 1 µM BAP, 0.2 µM GA_3, 1 g l^{-1} MES, 8 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, 0.5 µM NAA, 1 g l^{-1} MES, 8 g l^{-1} agar, pH: 5.8).

Molecular Analysis

DNA Isolation

Leaves from all samples coming from tissue culture were immediately frozen in liquid nitrogen and stored at -80 °C. High molecular weight genomic DNA was extracted from the leaf of each sample following the CTAB protocol for minipreps (Edwards et al., 1991). DNA concentration was measured using a NanoDrop (ND 100) spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and gel electrophoresis. DNA was diluted in water to a final concentration of 50 ng/µL and stored at -20 °C.

PCR Reactions

Primers for NHX-1 genes (F:ACCACGAAGCTGCTTTTCAT, R: TCAAGTCGAAAAGCTCAGCA) were designed using Primer 3, expected DNA size was optimized to be 200 bp. All plantlets were checked weather they have expected DNA size or not. Amplification reactions were performed in 20-µL volumes containing 2X PCR Mastermix (Fermentas K0171, Waltham, MA, USA), 1 U *Taq* DNA polymerase (Fermentas EP0402), 25 mM $MgCl_2$, 20 µM of the forward and reverse primer and 50 ng of melon DNA. Mixtures were assembled at 0 °C, transferred to a thermal cycler, then precooled to 4 °C. The amplification was carried out in a model Master Gradient thermal cycler (Eppendorf, Hauppauge, NY, USA) using an optimized in-house program consisting of an initial denaturation step of 2 min at 94 °C, and then 35 cycles of 2 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C, followed by a 10-min elongation step at 72 °C. PCR products were stored at 4 °C before analysis. Amplification products were separated by electrophoresis on 1.5% agarose gels and 0.5 g/mL ethidium bromide in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 3 h at 70 V. The fragment patterns were photographed under UV light for further analysis. A 100-bp DNA ladder (Fermentas) was used to determine fragment size.

Results and Discussion

Transformation of ATNHX-1 gene through *Agrobacterium tumefactions* is mainly aimed in the present study. On the other hand efficient regeneration protocol was applied to transformed explants. Following the transformation and regeneration assays plants were confirmed by PCR analysis. Totally 400 seeds belonging to *Cucumis melo* L. *cv.* Védrantais and Kırkağaç 637 were germinated in hormone free MS medium and germination rate was calculated as 100% (Table 1). In the transformation studies, proximal and cotyledon parts coming from seed germination were used. Transformation was subjected to total 400 cotyledons, 400 proximal explants to transfer ATNHX-1 gene for each genotype.

Totally 400 melon seeds were used as 200 *Cucumis melo L., cv.* Védrantais and 200 Kırkağaç 637 for transformation of AtNHX-1 gene. It was used 800 explants in total, which consisting of 4 explants (2 proximal and 2 cotyledons) from each seeds and *in vitro* cultured.

In this *Cucumis melo L. cv.* Védrantais genotype, 12 putative transgenic were observed as 5 proximal and 7 cotyledon part of the seeds, 1 putative transgenic was observed for *Cucumis melo* var. *inodorus* cv. Kırkağaç 637 genotype. Transformation efficiency of NHX-1 gene was determined as 6.87% from 200 *Cucumis melo L. cv.* Védrantais seeds. Instead, transformation efficiency was obtained as 0.625% for Kırkağaç 637 genotype. In some of the proximal and cotyledon explants, which were transferred to regeneration mediums, as well as the regeneration, callus formation was also observed (Figure 1, 2 and 3). Although, callus formation was observed from all of these explants, shooting were limited. A part of these regenerants, which maturated in regeneration mediums, turned into unorganised formation while some others transformed to the shoot forms (Figure 4, 5).

DNA extraction were done from putative transgenic plant which was observed after transformation of NHX-1 gene and DNA

quantity and quality were checked by spectrofotmeter. Quantity of extracted DNA were not too much but it was enough for PCR.

It was used 800 explants in total, which consisting of 4 explants (2 proximal and 2 cotyledon) from each seeds, different part of the seeds were *in vitro* cultured. Transformation efficiency were determined 3.25% as 13 transgenic plant for 400 seeds material. This study shows that explant is one of the most important factor for transformation studies.

AtNHX-1 gene amplification (200 bp) was observed lane 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 for *Cucumis melo* L. *cv.* Védrantais genotype in (Figure 6). PCR was also performed with the same samples using AtNHX-1 gene spesific primers. There was DNA band (200 bp) only in the lanes 1 for *Cucumis melo* var. *inodorus* cv. 'Kirkagac 637' genotype (Figure 6). No band was shown in negative control group. This showed that there was no contamination caused by materials used and chemicals in PCR solution (Figure 6). Bordas et al. (1997) studied on Amarillo canario and Pharo. They used real leaves in Amarillo canaraio genotype, cotyledon and real leaves for Pharo genotypes. They reported that explants taken from Amarillo canario were most effective than Pharo cotyledons and real leaves. In this study, efficient regeneration were observed from cotyledon explants of *Cucumis melo L. cv.* Védrantais seeds.

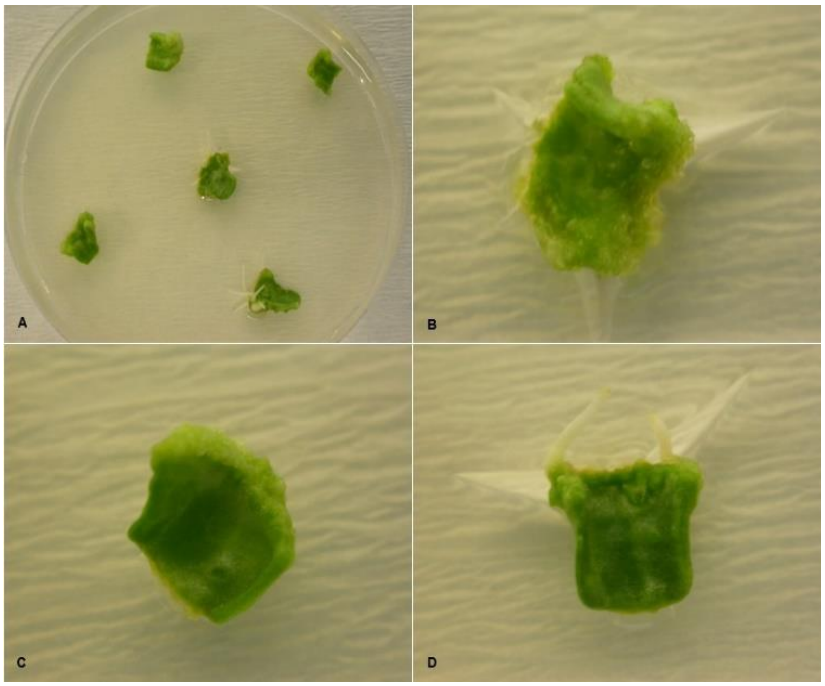

Figure 1. *Cucumis melo* var. *inodorus* cv. Kırkagaç 637 genotype in regeneration medium A-B) regeneration of cotyledon explants (7^{th} day), C-D) Cotyledon explants in regeneration medium

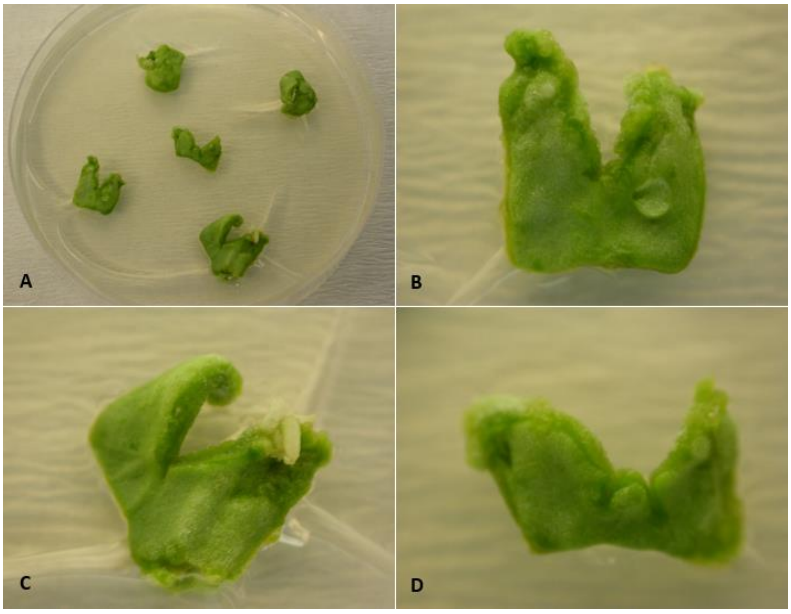

Figure 2. *Cucumis melo* var. *inodorus* cv. Kırkağaç 637 genotype in regeneration medium regeneration of proximal explants (7th day), C-D) Proximal explants in regeneration medium

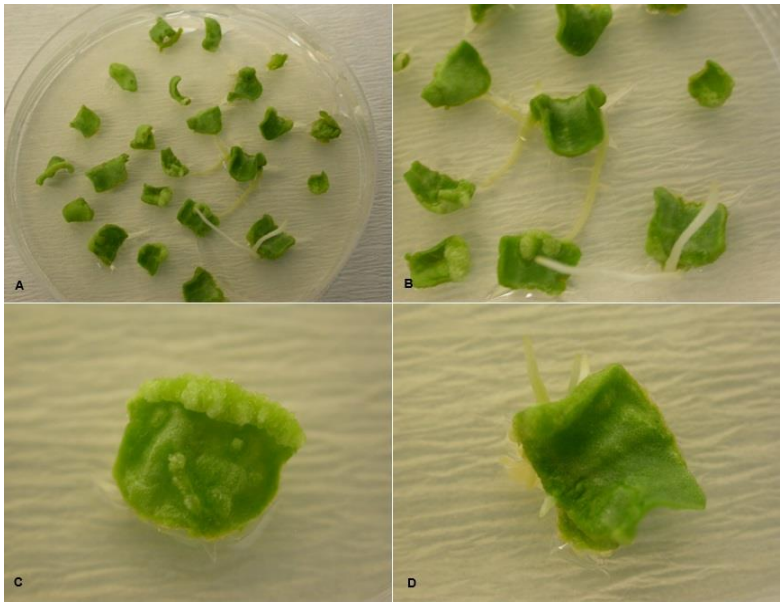

Figure 3. *Cucumis melo L, cv.* Védrantais genotype in regeneration medium A-B) regeneration of cotyledon explants (7th day), C-D) Cotyledon explants in regeneration medium

Figure 4. A) Shoot development in *Cucumis melo* L. *cv.* Védrantais genotype B) Shoot development in *Cucumis melo* L. *cv.* Védrantais genotype

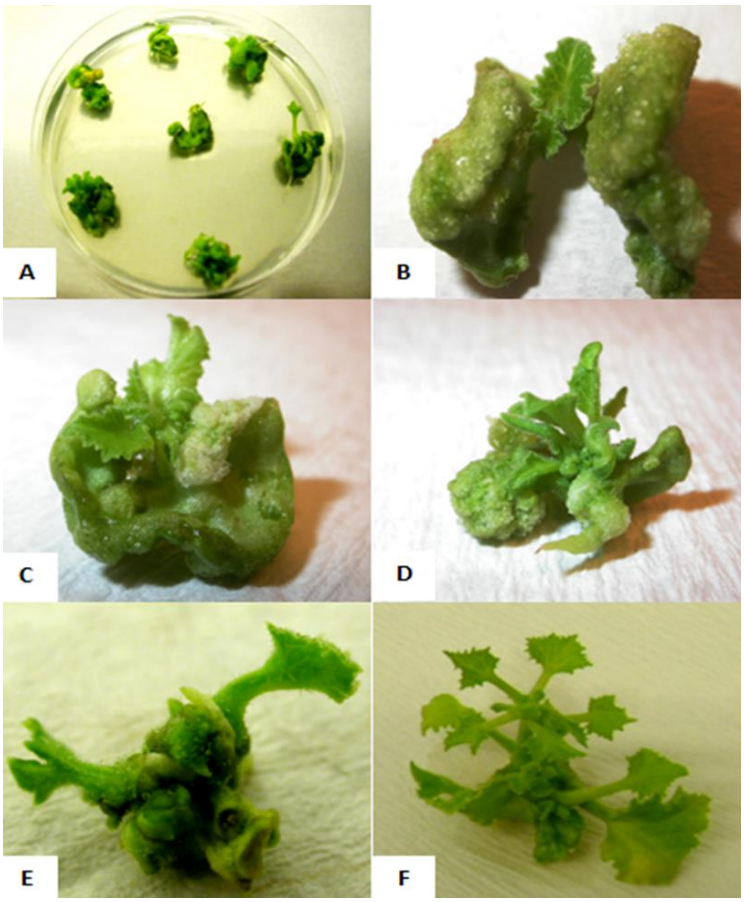

Figure 5. Shoot development of *Cucumis melo* L. *cv.* Védrantais, B) Regeneration of proximal explants, C) Developed shoots from proximal explants, D-E) Shoots from proximal explants at 15th day, F) Shoots from proximal explants at 21th Day.

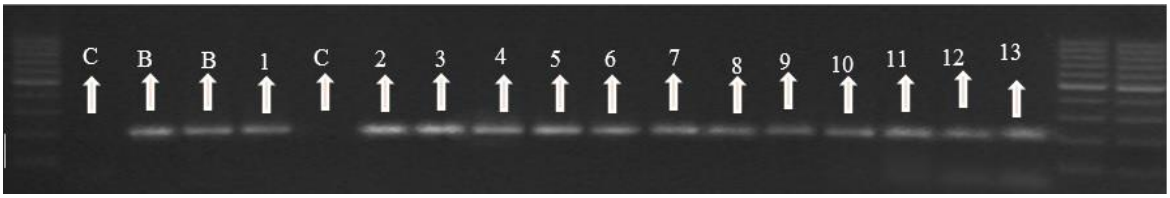

Figure 6. Ethidium bromide- stained agarose gel showing the amplification by PCR of the NHX-1 gene present in transgenic plant, C) Control, B) Bacteria strain, 1)Transgenic *Cucumis melo* var. *inodorus* cv. Kırkağaç 637 2-13) Transgenic *Cucumis melo* L. *cv.* Védrantais plants.

Table 1. Influence of an Agrobacterium-mediated transformation step on the regeneration frequency and transformation efficiency of melon cultivars recovered from different explants

Cultivars	Type of explant	Total no. explants tested	Explants with calluses (%)	Explants with shoots (%)	Total transformation efficiency (%)
Védrantais	Cotyledon	200	100	18	6.87
Védrantais	Proximal	200	100	14	5.53
Kırkağaç 637	Cotyledon	200	100	13	5.51
Kırkağaç 637	Proximal	200	100	7	2.625

Conclusions

We have established a transformation system for melons cv, "Kırkağaç 637" and "Védrantais" using *A. tumefaciens* as a vector. Although Kırkağaç 637 and Védrantais were the two cultivars tested, as the transformation of two different genotypes of muskmelon have been reported (Fang and Grumet 1990; Dong et al. 1991) and other Turkish commercial lines of melon have been tested for regeneration efficiency (Yalcin-Mendi et al. 2004), the results are considered to be significant steps in the development of commercial transgenic melon cultivars.

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