Genetic Stability in Melon Transformation System: Assessment by Flow Cytometry and ISSR Markers

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Research Article

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Abstract

Genetic instability in melon species sometimes occurs as a result of *in vitro* tissue culture and transformation systems. This study describes a new regeneration technique for agrobacterium-mediated co-culture of muskmelon explants (*Cucumis melo* L. c.v. 'Khatooni'). Here, no genetic instability was observed in positive PCR regenerants. 4-day-old cotyledonary explants had been infected with LBA4404 *Agrobacterium* suspensions. The co-cultivation occurred in the presence of 100mg/l rifampicin and 50mg/l kanamycin. The bacteria contained a binary vector pBI121 carrying nopaline synthase by the promoter-neomycin phosphotransferase gene. The regeneration succeeded 65% in selective MS medium containing N^6-benzylaminopurine (600 µg/l), β-naphthoxyacetic acid (25 µg/l) and 50mg/l kanamycin in inoculated 4-day-old cotyledonary explants. According to the polymerase chain reaction analysis of neomycin phosphotransferase II gene, transformation was merely successful (8.4%), indicating a substantial miss on a large number of regenerated plants in the selective medium, as a consequence of PGR and antibiotic imbalances. Inter Single Sequence Repeat markers and flow cytometry analyses were used for evaluating the genetic stability and ploidy level of transplants, respectively. The integrated approach underlined that *Agrobacterium* inoculation and plant growth regulators were successfully combined *in vitro* to enable muskmelon transformation.

Introduction

Muskmelon (*Cucumis melo* L.) is a common vegetable in several tropical and subtropical areas (Feyzian et al., 2009). Though belonging to the *Cucurbitaceae* family and the *Reticulatus* subspecies, muskmelon can sometimes be taken mistakenly for cantaloupes which are vegetables of the *Cantalupensis* subspecies (Malik et al., 2014). Pitrat (2008) reported that muskmelon originated in South Africa, but Schaefer et al. (2009) and Pitrat (2014) later claimed that muskmelon may have bio-historical roots in Asia. Muskmelon is increasingly becoming important in the drug and food industries because the fruits components (Kim et al., 2015). Melon seed oil can treat intestinal worms, reduce blood LDL levels (low-density lipoprotein cholesterol) and common blood clots. Also, it can prevent irregular heartbeats and reduce the likelihood of kidney and bladder stones (Paksoy and Aydin 2004; Sena et al. 2017; Eloka-Eboka et al., 2017; Mallek et al., 2018). Pectin in melon peels is an intricate component of polysaccharides which is abundantly utilized in the preparation of jellies, jam and useful sugars for diabetics.

The development of agronomic traits in melons is increasingly becoming a focus of attention as part of efforts to counter the damages of bio stresses. Using classic pathways to improve morphology traits is sometimes held back by time- and labor-consuming limitations (Ju et al., 2014). Furthermore, intra- and inter-specific incompatibilities can hamper success in traditional breeding methods. New breeding ways such as gene transferring for the improvement of new cultivars are effectively related to proliferation systems (Vengadesan et al., 2005; Bezirganoglu et al., 2013). Despite good levels of performances in handling muskmelon transformation and tissue culture *in vitro* (Ren et al., 2012), morphogenesis

variation is frequently observed in regeneration and transformation systems which can be attributed to genetic and somaclonal variation (Guis et al., 1998; Xing et al., 2010).

The culture conditions may affect genetic instability (Orzechowska et al. 2016).

Wei et al. (2006) discovered three factors that can assist in increasing organogenesis: the kind of explant, combination and concentration of PGRs, and culture situation. *Agrobacterium* mediated transformation is the preferable procedure by which DNA fragments are inserted into herbaceous plants and genetic instability is altered. Exposing explants to co-cultivated *Agrobacterium* can arrest plant tissue growth for an amount of time, thereby reducing proliferation. Some antibiotics such as ampicillin, neomycin and carbenicillin are often employed in suspensions to alleviate this problem. Ren et al. (2013) observed irregular phenotypic traits such as slow plant growth and small plant size, no flowering, fruits or seed set in tetraeuploid transgenic melon lines, as compared to diploid plants. DNA content and changes in chromosome number have been reported in several transgenic melon subspecies through flow cytometric analysis. In most reports concerning melon transformation, the ploidy levels of the transgenic lines were not specified (Switzenberg et al., 2014; Bezirganoglu et al., 2014) and in some cases a high percentage of instability was observed. In one of the latest melon transformation expriments (GarciaAlmodovar et al., 2017), a large number of transgenic plants became tetraploid, whereas the diploid level decreased significantly in tranformed melons when the infected explants grew from seedling.

Chovelon et al. (2011) illustrated that 85% of transgenic melons, being derived from cotyledon explants, were tetraploid. Histological reports indicate that no study so far has addressed the identification of complete genetic fidelity in transgenic melon clones derived from *Agrobacterium*-mediated samples. Inter Simple Sequence Repeat (ISSR) is a useful molecular marker to evaluat somaclonal variation in regenerants (Bhowmik et al., 2016).

Nonetheless, no study has applied this marker for the evaluation of transgenic melons. Since high levels of genetic instability are common in regeneration and transformation systems when studying melons, the purpose of this study was to introduce a new method for achieving transformation with high genetic sustainability in muskmelon. The genetic fidelity of transgenic melon was examined by flow cytometry and for the first time by molecular markers.

Materials And Methods

Seed sterilization

Mature seeds were obtained from the muskmelon cultivar ‘Khatooni’. The seeds were uncoated and surface sterilized according to Raji et al. (2018). So, the seeds were dried under laminar flow and on sterile paper.

Explant preparation

In following seeds were placed in jars filled with half-MS media (Murashige and Skoog 1962) and after germination, explants were prepared from 4 day-old seedlings. Then those were used to co-cultivation and shoot regeneration.

Transformation and co-cultivation

Further to transformation, 4-day-old cotyledons were cut into small pieces (2 by 5 mm) and then infected by the LBA4404 *Agrobacterium tumefaciens* strain containing a binary vector pBI121 that harbored the NoS promoter-*nptII* gene (Fig. 1).

The *Agrobacterium* suspension was grown in 10 ml Luria–Bertani liquid media including 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin for 12 h at 28°C in an incubator shaker operating at 200 rpm. The *Agrobacterium* was sedimented by centrifugation at 10000 rpm for 5 min.

The sediment was resolved in the MS medium containing 25 mg/l acetosyringone. Cells were adjusted at OD600 (5·108 cells/ml). The explants were suspended in the *Agrobacterium* dilution for 2–3 min and were relocated to be dried on filter paper. They were cultured on MS medium without plant growth regulators (PGRs) in an incubator at 28°C for two days of co-cultivation. The incubation occurred in a dark environment.

Organogenesis and acclimation of co-cultivated explants

The co-cultivated explants in free MS, organogenesis in selective medium (SM), including MS media containing BAP (600 µg/l) mixed with NOA (25 µg/l) and adaptation in greenhouse were done based on our previous report ???? et al (2020).

The SM also containing kanamycin (50 mg l^{-1}) and cefotaxime (300 mg l^{-1}). After two weeks of explant culture, proliferation record was started. The irrigation and fertilization occurred in hydroponic systems.

DNA extraction

DNA was extracted from mother and transgenic plants according to bahmankar et al () study by the modified CTAB method (Doyle, 1987). The quality (260/230 and 260/280 ratios) and concentrations of DNA were assessed by a NanoDrop Spectrophotometer (Thermo Scientific - Waltham, Massachusetts).

Polymerase chain reaction

The presence of the *NptII* gene in regenerated lines was analysed by PCR using specific forward (5′-GAACAAGATGGATTGCACGC-3′) and reverse primers (5′-GAAGAACTCGTCAAGAAGGC-3′, melting temperature 58°C) amplifying a fragment of 786 bp.

The fragments in the extracted DNA were amplified under the following conditions: 35 cycles with 5 min pre-incubation at 94 °C, 45 s at 94°C, 45 s at 58°C and then 45 s at 72°C. The amplified DNA fragments

were exposed to 1% agarose gel electrophoresis and were observed under ultraviolet light in a Gel Documentation System.

SSR fingerprinting

DNA fragments were amplified using ISSR primers (Siragusa et al., 2007) for positive PCR plants. PCRs were performed in a 25 µl reaction mixture as described by Meziane et al. (2016). Then, the PCR results were observed on electrophoresis for 4 h at 110 V. For increased accuracy, PCR test was repeated twice more.

Flow cytometric analysis (FCM)

FCM was done to determine the poloidy levels in leaf cells of transformants and mother plants (standard plant) by Partec PAS flow cytometer (Partec, http://www.partec.de/) described with Chovelon et al. (2011).

Data analysis

To confirm the transformed lines, 786 bp amplified fragments were considered for *nptII* genes, while no amplification of the bands in them should include a *vir* gene primer.

To consider genetic fidelity as per ISSR markers, the evaluations were focused only on the bands showing consistent amplification between 200 and 2,000 bp. PCR products were scored for the presence (1) or absence (0) of amplified bands. The polymorphism was measured base on allele frequencies and number of polymorphic loci/number of total loci.

Results

1.1 Shoot formation on selective media

At the beginning, the frequency of transformation was assessed by shoot formation on 4-day-old explants in SM (Fig. 2). After co-culturing and during 5 to 6 weeks of processing, DO occurred efficiently (65%) from 4-day-old cotyledon explants of ‘Khatooni’.

1.2 PCR analysis

Kanamycin selection was not completely efficient because PCR analysis showed several “escapes” and no amplified DNA fragment for the *nptII* gene in regenerants. The PCR system amplified the selective marker in 8.4% of the regenerants and was established in the presence of 786 bp fragments of nptII in 11 transgenic lines regenerated from SM (Fig. 3-a). There was no *Agrobacterium* contamination in nptII PCR since the PCR of the *Vir* gene did not indicate any amplification in nptII positive lines (Fig. 3-b).

1.3 Genetic fidelity

Genetic stability of the positive PCR plantlets were analyzed by ISSR markers. Ten ISSR primers were used in this study, and all ten are common in research applications (Haddad et al., 2017). A total 112 well-resolved bands were observed in electrophoresis gel (ranging between 7 to 13 and an average of 11 bands per primer). The size of amplified fragments ranged between 230 bp to 2.0 kb. All were same among the analyzed regenerants. These results (Fig. 4) indicated that all amplified profiles the transplants are true-to-type plants mother.

1.4 Flow cytometry

The FMC pattern of all transgenic and mother plants was homogeneous in channel 200 nuclei (Fig. 5), which resulting no differences between the transgenic lines and standard plants (Yang et al., 2008; Carra et al., 2012). These observations indicating that polyploidization did not occur in transformation system.

2. Discussion

No study has so far assessed somaclonal variation in muskmelon transgenic lines by molecular markers. For the first time, it was assessed in this study where no somaclonal differnces was observed among the transformed lines and standard line, according to ISSR markers. The recent results show that the chromosomal composition here is very stable compared to other reports. Ren et al. (2012) asserted that the type of bacterial strain, infection period and density of the *Agrobacterium* growth media affect regeneration, transformation rate and genetic inconsistency in melon. Nuñez-Palenius et al. (2006) and Ren et al. (2013) found that 80% to 100% of the transgenic plants were tetraploid. Chovelon et al. (2011) proved that genetic instability in transformed plants produced from cotyledons (53%) was higher than those obtained from melon leaf explants (12.2%). This could be due to a greater effect of Agrobacterium on younger cells of the cytodenic exudates.

Guis et al. (2000) had observed more tetraploidy in younger cotyledons. In this research the ISSR markers and FMC were utilized to compare chromosomal composition and genetic differences between transformed muskmelons and their mother plants. The transformation efficiency and genetic instability of melon tissues are generally influenced by several agents. Different results have been reported from *in vitro* differentiation following *Agrobacterium* transformation.

Our experiment suggests that shoot formation of co-cultivated explants decreases as compared to non-inoculated explants. The meristematic tissues of recalcitrant species like muskmelon destruct after the infection and co-cultivation of explants with *Agrobacterium* suspension which could be due to the production of ethylene in the culture medium. Khanna et al. (2007) showed how ethylene affects many growth processes and reduces the frequency of melon regeneration and transformation.

Ntui et al. (2010) reduced ethylene production by explants after *Agrobacterium* infection, even as the ACC gene expression reduced and increased the transformation rate. A cytological study by Dan et al. (2010)

on melon epidermal and sub-epidermal cells revealed a lower regeneration rate of the infected explants which can be related to the disability of cells in causing regeneration. Also, this may be due to the genetic instability of the infected meristematic parts.

Different results were also published about groundnut regeneration after *Agrobacterium* infection (Venkatachalam et al., 1998) whereas Guis et al. (2000) observed no difference in the regeneration frequency between infected and uninfected melon explants. Some studies have shown negative effects of *Agrobacterium* inoculation on regeneration. Deterrence or improvement of the proliferation in SM is possibly a manifestation of the role of some antibiotics in media culture (Bordas et al., 1997; Choi et al., 2012; Zhang et al., 2014). Antibiotics that were used in this study include cefotaxime, rifampicin and kanamycin which can be influential in regeneration. Valles and Lasa (1994) demonstrated that the frequency of shoot regeneration on an antibiotic-free medium was more than regeneration on media containing antibiotics. The frequency of regeneration has been affected positively by cefotaxime when specific concentrations are used in the culture media for some plant species such as durum wheat (Borrelli et al., 1992) and barley (Mathias and Mukasa, 1987). Cefotaxime is often supplemented in the organogenesis media after co-culture to prevent *Agrobacterium* growth (Yu et al., 2001).

This antibiotic is placed in the b-lactam class and is similar to plant PGRs. It has the least toxicity on *in vitro* plant culture, whereas some b-lactam antibiotics such as carbenicillin have shown inhibitory effects at higher concentrations and have reduced regeneration frequency (Grzebelus and Skop 2014). Phillips et al. (1981) affirmed that Rifampicin can effectively eliminate bacterial contamination but stated negligible effects on explant growth and regeneration.

Kanamycin has negative effects on the frequency of shoot and root formation *in vitro* regarding some melon subspecies (Bordas et al., 1997; Ren et al., 2012; Choi et al., 2012). Accordingly, we expected an increase in the regeneration of ploidy levels, whereas the ploidy level did not change after inoculation. In this study, a high genetic constancy (100%) of transformed muskmelon ('Khatooni') was successfully achieved using NOA+BAP PGRs.

Declarations

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Contribution

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Ethics declarations

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors

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Figures

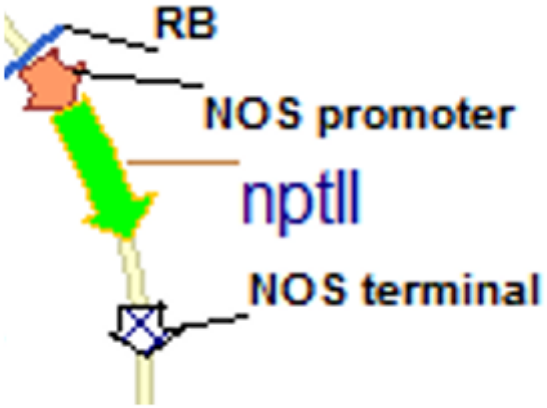

Figure 1

Binary vector pBI121 harboring CaMV 35S promoter-neomycin phosphotransferase (nptll) gene

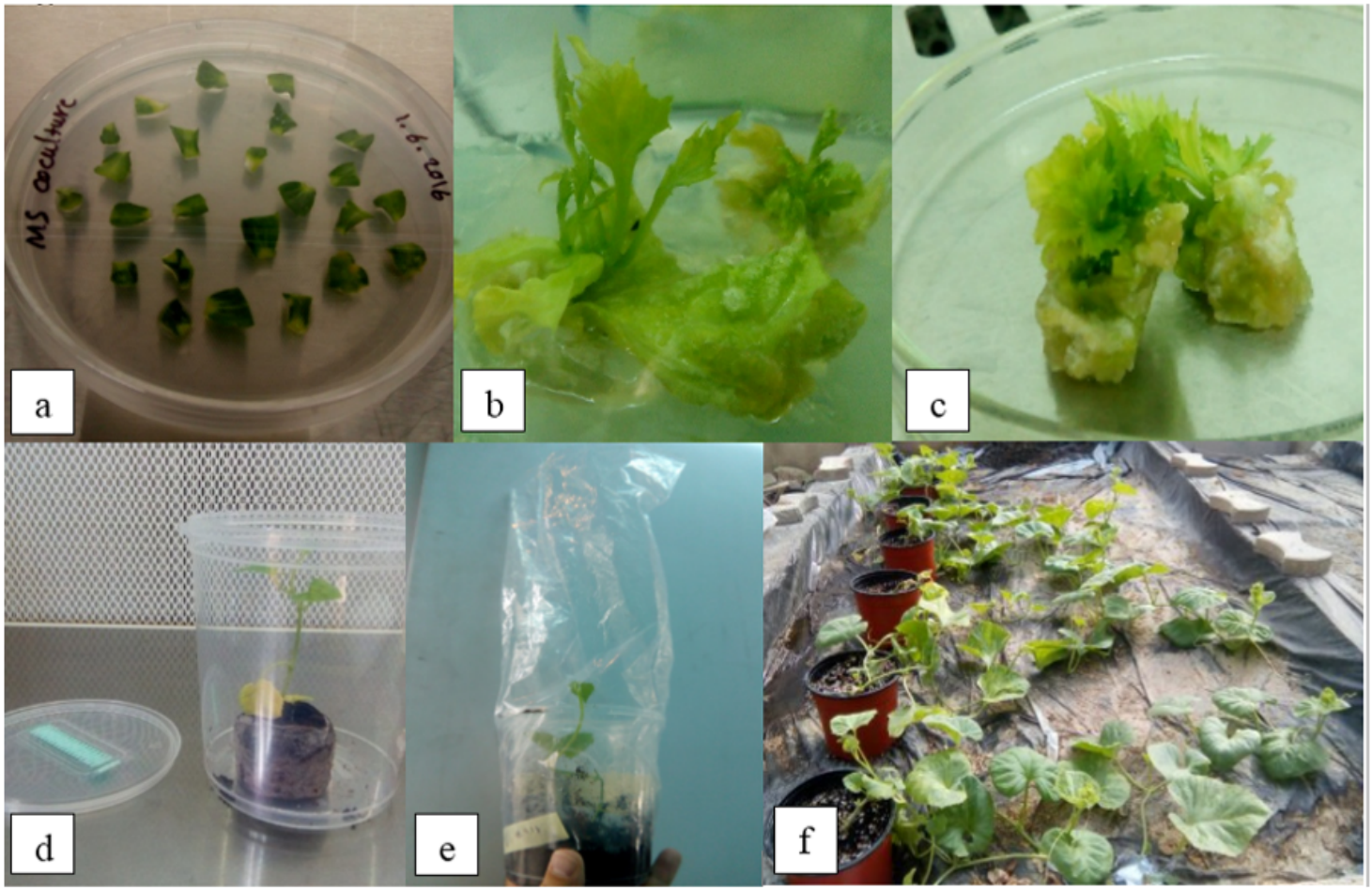

Figure 2

The steps of shoot formation on infected and co-cultivated cotyledon explants of muskmelon in selected media. a: Shoot primordia. b and c: Advantageous shoots formation. d: Transferring of plantlets to jiffy. e

and f: Acclimatized and rooted transplants.

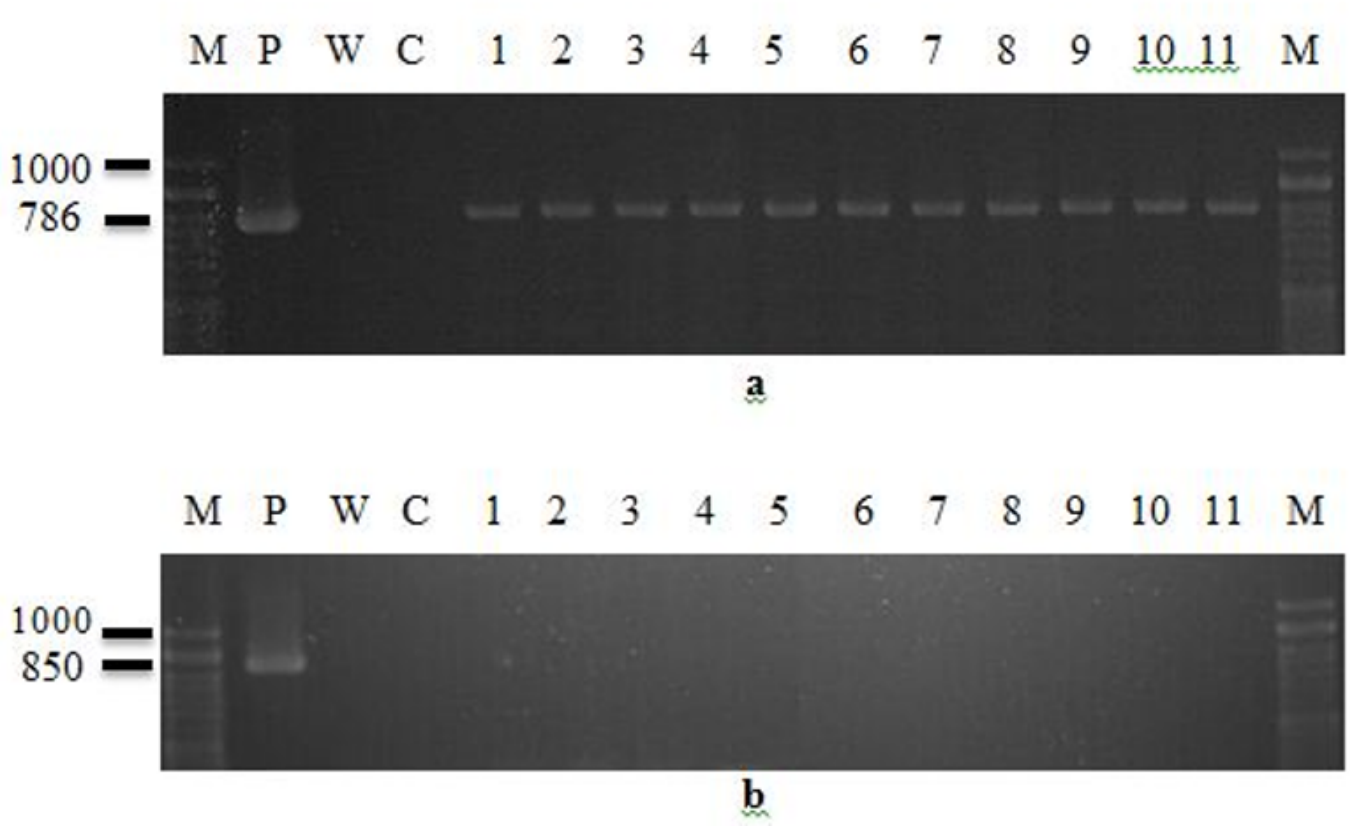

Figure 3

PCR analysis of neomycin phosphotransferaseII (nptII) gene (a) and Vir gene (b) in Genomic DNAs of putative transgenic plants. (a) Lanes L: 1 kb ladder, Lane P: plasmid, Lane W: water, Lane C, non-transgenic plants, lanes 1–11: putative transgenic plants.

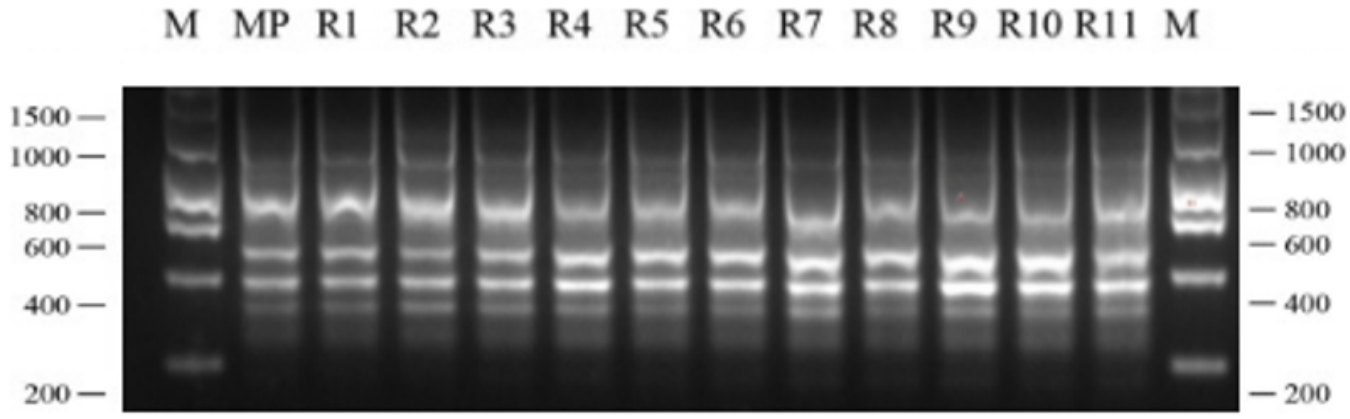

Figure 4

Genetic fidelity assessment of transformed lines L. (Khatooni cultivar) using ISSR markers, where in profiles were obtained with primer ISSR11+11b. The amplification bands of transgenic plants were similar to mother. Lane M: marker; lane MP: mother plant; lanes R1-11: transgenic plants.

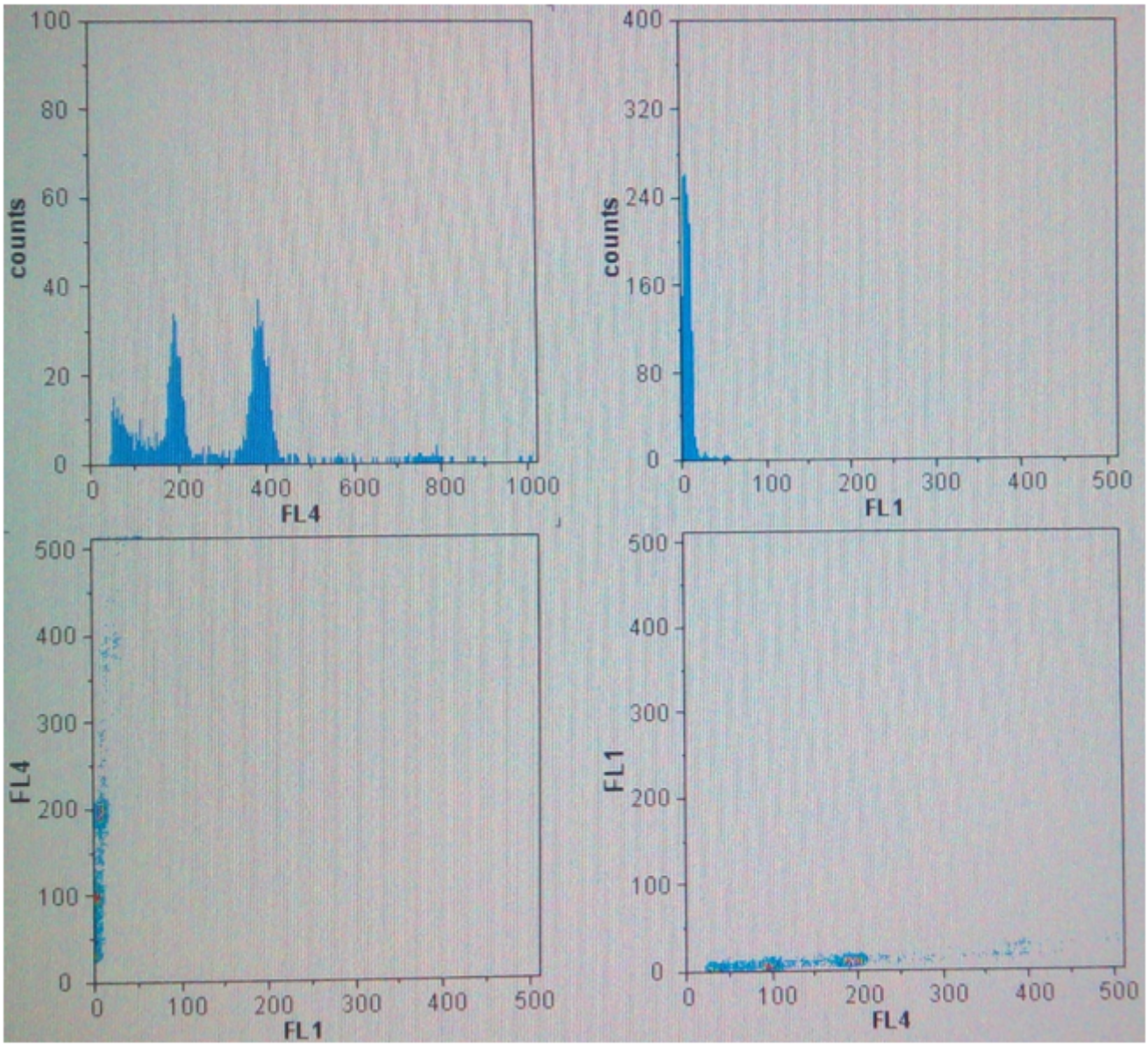

Figure 5

Measurement of chromosomal contents of nuclei in transgenic melon by FMC. Pattern similar of ploidy levels of transgenic and mother plant (standard plant).