Efficiency of SSR markers for determining the origin of melon plantlets derived through unfertilized ovary culture

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Abstract

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The effects of temperature pre-treatment, thidiazuron, naphthaleneacetic acid, and 6-benzylaminopurine on *in vitro* gynogenic plant production from un-pollinated melon (*Cucumis melo* L.) ovaries were investigated. Simple sequence repeat (SSR) marker analysis was conducted to identify the homozygous diploid individuals. The temperature pre-treatment (4°C) for 4 days increased embryo formation frequency (63.3%) significantly. Addition of thidiazuron (0.04 and 0.02 mg/l) in the induction medium significantly increased the number of responding ovaries (46.6%, 65.83%), respectively. The maximum number of plantlet regeneration (22.5%) was achieved by culturing the ovary derived embryos on Murashigue and Skoog medium (MS medium) supplement with 0.6 mg/l 6-benzylaminopurine. Spontaneous doubled haploids originated directly through embryogenesis were subjected to genetic analysis using SSR molecular marker with 23 primers pair for homozygosity. SSR markers with microsatellite CMGA172, confirmed that the alleles in the parental material were also present in the gynogenic plantlets, but amplified only two alleles as compared to four alleles of the heterozygous parent material at same locus. Therefore these regenerated plantlets were consider homozygous and produced through a process of gametophytic embryogenesis.

Keywords: *Cucumis melo* L.; *in vitro* gynogenesis; embryo formation; microsatellites markers

Melon (*Cucumis melo* L.) is an open-pollinated crop with both dioecious and monoecious flowering characteristics (Rashid, Singh 2000). The production of pure lines in different crops is a lengthy process and generated inbreds may not be 100% homozygous after successive selfing (Bajaj 1990). However, haplo-diploidization through gametic embryogenesis allows for single step development of complete homozygous lines from heterozygous parents (Zhao et al. 2006). Early attempts to produce haploids of melon by anther or ovule culture were not successful (Dryanovska, Ilieva 1983). The first successful recovery of gynogenic plant in melon was achieved by stimulating haploid parthenogenesis through pollination with irradiated pollen. (Sauton 1989; Cuny et al. 1992; Ficcadenti

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et al. 1995). Production of haploid plants using the irradiated pollen technique is not a practical approach in some research labs, due to the unavailability of gamma radiation facilities. Thus unfertilized ovary culture in melon is more useful method. However, *in vitro* culture of unfertilized ovaries of melon has been reported only once, but due to low gynogenic efficiency and preliminary results with only one plant growth regulator combination for the induction and regeneration medium limited its role for the formulation of reliable melon gynogenesis protocol (Ficcadenti et al. 1999). Gynogenesis is highly genotypic dependent, requiring temperature pre-treatment specific growth medium and culture conditions for induction and development of intact plants (Mukhambetzhanov 1997). Therefore, it is pertinent to study other suitable and efficient protocol for *in vitro* gynogenesis in melon.

Depending upon the regenerative pathways, *in vitro* regenerated gynogenic plants differ in their ploidy level (Diao et al. 2009). In melon (*Cucumis melo* L., $2n = 2x = 24$) gynogenesis, Ficcadenti et al. (1999) indicated that a sporophytic haploid condition is unstable in melon and that the regenerated diploid plants could be the results of a spontaneous diploidization occurred precociously, may be during embryo formation in the ovule. The spontaneous chromosome doubling is a random process without any relationship to the genotype and it is impossible to evaluate the exact time of chromosome doubling event (Bouvier et al. 2002). Therefore, these diploid gynogenic plantlets should be investigated for their homozygosity and source of origin before using in breeding programs. Occurrence of spontaneous diploidization is also a common phenomena in other plant species (*Cocos nucifera* L.: Perera et al. 2008; *Pyrus communis* L.: Bouvier 2002; *Cucumis sativus* L.: Diao et al. 2009; Hui et al. 2007). Among these regenerated diploid gynogenic plants, those originating from megaspore mother cell are equivalent to haploids (homozygous) and can be used directly in breeding programs, while those originating from normal somatic cell are heterozygous and would require further inbreeding prior to use as parents (Perera et al. 2008). Thus, it is very important to identify the origin of putative spontaneous double haploid regenerated plants obtain through gynogenesis. However, it is very difficult to distinguish spontaneous doubled haploids (DH) from normal somatic diploid using flow cytometry and cytogenetic observation, because they contain the same amount of nuclei and chromosomes numbers (Diao et al. 2009). Identification of homozygous dihaploid plantlets by morphological characterization has been reported (Kernan, Ferrie 2005; Hui et al. 2007), but this procedure is less effective due the effect of environmental variation on morphological traits (Chen et al. 1998). To identify large numbers of spontaneous double haploids or homozygous plants at an early stage of development using molecular markers would provide a fast, efficient, and cost-effective method for breeding programs. Simple sequence repeats (SSRs) are microsatellites are short sequences of nuclear DNA, consisting of tandemly repeated nucleotide units (1–5 nucleotides long). They are highly polymorphic, somatically stable, and inherited co-dominantly (Morgante, Olivieri 1993), making them an ideal markers for conforming origin and homozygosity (Bouvier et al. 2002). SSRs has been successfully used for identification of homozygous, spontaneous double haploids in *Cucumis sativus* L. (Diao et al. 2009), *Cocos nucifera* L. (Perera et al. 2008), *Solanum tuberosum* L. (Chani et al. 2000), and *Pyrus communis* L. (Bouvier et al. 2002).

Our objectives were aimed at improving the procedure for recovery of double haploid melons and demonstrate the use of molecular markers (SSRs) in identifying such spontaneous double haploids. To this end, we investigated the influences of low temperature pre-treatment and thidiazuron (TDZ) on embryo formation frequency, the effect of plant growth regulators on plant regeneration and the use of SSR marker to distinguish homozygous regenerants among ovary derived diploids.

METHODS AND MATERIALS

Plant material and sterilization

Commercial F_1 hybrid Jin Man Di (*Cucumis melo* L., $2n = 2x = 24$) was used as donor parent in this study. This genotype found to be responsive for gynogenesis out of $n = 10$ genotypes and inbred lines tested in a preliminary experiment (data not presented). The plants were grown in walk through plastic tunnels using standard cultural practices (Rashid, Singh 2000). Un-pollinted ovaries were harvested at anthesis, when the anthers contained binucleate pollen and emasculated before placing in Petri dishes on moist filter paper and stored in an ice box (Gémesné Juhász et al. 1997). For temperature pre-treatment studies the ovaries were kept at 4°C before surface sterilization for 0, 4, and 8 days. The surface disinfection of peeled ovaries were undertaken by rinsing

the explants in 70% (v/v) ethanol for 30 s followed by 0.1% (w/v) aqueous mercuric chloride for 5 min, then several rinses with sterile distilled water under aseptic conditions and finally blotted to remove excessive moisture. Each ovary (10–12 mm in length) was cut horizontally into 1–2 mm thick slices and then cultured onto the induction medium.

Media preparation and culture requirements

Ovary slices were cultured on the induction medium for one week, which comprised Murashigue and Skoog (MS) basal medium (Murashigue, Skoog 1962) supplemented with 30 g/l sucrose, 7 g/l agar, and various concentrations (0.0, 0.02, 0.04, 0.08 mg/l) of TDZ were used. TDZ is a thermo liable compound; therefore it was added to the medium after autoclaving through filter sterilization. An initial screening experiment with various plant growth regulators previously conducted indicated that several combinations of naphthaleneacetic acid (NAA), and 6-benzylaminopurine (BAP) could induce shoots directly from the ovary explants (data not shown). Therefore, in the present experiment, responding ovary slices were then transferred onto the differentiation medium consisting of MS basal medium, supplemented with 30 g/l sucrose, 7 g/l agar, 50 mg/l L-proline and different combinations of BAP (0.0, 0.3, 0.6, 0.9 mg/l) and NAA (0.0, 0.05, 0.1 mg/l), (Table 1). The pH of all the media was adjusted to 5.8 before being autoclaved at 121°C (1.2 kg/cm) for 18 min. All the cultures were transferred to growth chamber at 25°C with a 16 h light and 8 h dark photoperiod (~ 50 mmol/m/s).

Table 1. Effect of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) on plantlet regeneration from unfertilized ovary culture of melon (*Cucumis melo* L.)

NAA (mg/l)	BAP (mg/l)	No. of cultured ovaries	No. of regenerated plants	Response (%)
0.00	0.0	35	0	0.00
0.00	0.3	40	5[b]	12.50
0.00	0.6	40	9[a]	22.50
0.00	0.9	40	0	0.00
0.05	0.0	35	0	0.00
0.05	0.3	35	2[c]	5.70
0.05	0.6	35	1[c]	2.85
0.05	0.9	35	0	0.00

Values in each column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range tests

Cytological observation

The young shoot tips of gynogenic plants were used for ploidy determination (Cao, Chen 2003). The young shoot tips of gynogenic plants were used for ploidy determination, rinsed in tap water to remove the media and treated in 0.02% 8-hydroxyquinoline at 4°C for 3 h. The roots were fixed in a Carnoy's fluid for 24 h. Root tips from the fixed roots were hydrolyzed in 1N HCl for 10 min at 60°C and then stained with acetocarmine. Stained root tips were squashed in 45% acetic acid and observed under light microscope.

SSR analysis

Putative spontaneous doubled haploid plantlets derived from gynogenesis were analyzed with SSR markers to determine their source of origin and homozygosity. A set of 23 pairs of microsatellite melon specific primers (Table 2) were used on melon parental material. Genomic DNA was extracted from young leaves tissue according to a modified protocol Doyle and Doyle (1990). Twenty-three melon SSR primer pairs were synthesized according to the sequences previously reported by Danin-Poleg et al. (2001). The DNA amplifications were carried out in each 20 µl of the reaction mixtures (30 ng template DNA, 1.0µM each of the SSR primer pairs, 1 U Taq DNA polymerase, 3mM dNTP, 3mM $MgCl_2$, 10 × buffer), using a thermal cycler (PTC-200, MJ Research, Waltham, MA, USA). PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and finally extension at 72°C for 5 min. The PCR products were separated on 7.2% polyacrylamide gels (PAGE) followed by silver staining (Bassam et al. 1991).

Statistical analysis

The frequencies of embryo formation were recorded on quotient of the number of ovaries responding and the total number of treated ovaries. Data

Table 2. The simple sequence repeat (SSR) designation and sequences of the primers used on melon (*Cucumis melo* L.) parental material

SSR designation	Sense primer 5' to 3'	Anti-sense primer 5' to 3'
CMTC163-1	CTAAAACCTAACTCTTTTCC	TTTGTGGGACTCGTTGAATGA
CMTC158-2	CCCCCATATTCATCAAAACT	TCAGCTCACTTTTCCATTCA
CSLHCPA-3	TTCTCCATGTTTGGATTCTTT	ACCACAAATAATAATTCAACA
CSHPRAG-4	GTTTAACTCAATCCAACTCAA	CGAAACATTCATAACTCTACT
CMGA15-5	CGGCAAGACGATTGGCAGC	ATCACCGTAGCGAAGCACC
CMCT44-6	TCAACTGTCCATTTCTCGCTG	CCGTAAAGACGAAAACCCTTC
CMACC146-7	CAACCACCGACTACTAAGTC	CGACCAAACCCATCCGATAA
CMCTT144-8	CAAAAGGTTTCGATTGGTGGG	AAATGGTGGGGGTTGAATAGG
CMAT141-9	AAGCACACCACCACCCGTAA	GTGAATGGTATGTTATCCTTG
CMCCA145-10	GAGGGAAGGCAGAAACCAAAG	GCTACTTTTGTGGTGGTGG
CMGA172-11	CAATCGCAGATACTTCCACG	TGCTTGTCCCAACGGTGTCAT
CMCT123-12	CGGATTGTACTTATTGCCAAG	CATGTGCATGTGTGCATGTAC
CMGT108-13	CTCCTTCAAACATTGTGTGTG	GAGATAGGTATAGTATAGGGG
CMTAA166-14	GGAACAGACACCTCTTCTGAG	TCCGTCTACAAGCGTGACTGT
CMGA165-15	CTTGTTTCGAGACTATGGTG	TTCAACTACAGCAAGGTCAGC
CMCT160a-16	GTCTCTCTCCCTTATCTTCCA	ACGGTGTTTGGTGTGAGAAG
CMCT505-17	GACAGTAATCACCTCATCAAC	GGGAATGTAAATTGGATATG
CSCTTT15a-18	GTTTGATAATGGCGGATTGT	GTAGAAATGAAGGTATGGTGG
CSGTT15b-19	ACCTTGTTGATTCGGTTCTCC	AGTTCGGTTTAACTACCCACG
CSTCC813-20	GTTGTGCTCCCCAATAGTTG	CACCACTTCTTCCACCGAA
CSCT335-21	CCTTCACTTCCATCTTCATC	CGGTCCTTCATTTCATAGAC
CSATT214-22	TTGAGTACCATTGTCATAGAT	TTAGTTTAATTTCATCTCTGT
CSAT425a-23	TAGGGCAGGTATTATTTCAG	ACGGACTGATTTAGTATAGGC

were subjected to arcsine transformation in order to normalize the distribution. Analysis of variance was performed according to RCB design with three replication and the Duncan's multiple range test was used for comparing the mean ($P < 0.05$).

RESULTS AND DISCUSSION

Effect of low temperature pre-treatment on embryo formation frequency

The temperature pre-treatment (4°C) for different intervals (0, 4, and 8 days) resulted in different regeneration efficiencies (Table 3). Ovaries exposed to 4°C for 4 days produced significantly highest number of gynogenic ovules (63.3%), followed by ovaries at control treatment with embryogenic response of 49.16%. However, no gynogenic response was noted in the ovaries pre-treated for 8 days at 4°C. A switch from gametophytic to sporophytic pathway requires some specific pre-treatment conditions applied to explants prior to culture (Wang et al. 2000). Therefore low temperature pre-treatments (4°C) have a strong influence on embryo induction frequencies in the present study. Our results are also in agreement with Lux et al. (1990) and Gürel et al. (2000) who reported that cold pre-treatment of (*Beta vulgaris* L.) flower buds at 4°C for 4–5 days led to an increase in embryo yield.

Table 3. Effect of temperature pre-treatment and thidiazuron (TDZ) on embryo formation frequency from unfertilized ovary culture of melon (*Cucumis melo* L.)

Induction medium TDZ (mg/l)	Temperature pre-treatment (0 days)			Temperature pre-treatment (4°C) 4 days			Total (%)
	No. of cultured ovaries	No. of responding ovaries	Embryo formation frequency (%)	No. of cultured ovaries	No. of responding ovaries	Embryo formation frequency (%)	
0.00	120	0	0.00	120	0	0.00	0.00
0.02	120	52	43.30[d]	120	60	50.00[c]	46.60
0.04	120	66	55.00[b]	120	92	76.60[a]	65.83
0.08	120	0	0.00	120	0	0.00	0.00
Total	–	118	49.16	–	152	63.33	–

Values followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range tests

Effect of TDZ on embryo formation frequency

Culture medium is a principle factor controlling the induction and development of intact plants (Mukhambetzhanov 1997). In many crop species, TDZ stimulated embryogenesis with a higher efficiency and frequency compared with other cytokinins or the combination treatments of auxins and cytokinins (Visser et al. 1992; Zhang et al. 2001). Correspondingly, in the present investigation significant variations in the response of ovary slices was observed on media containing different concentration of TDZ (Table 3). In the control treatments, ovaries became swollen but no sign of ovule growth or enlargement were observed and later turned to brown. Whereas the ovary slices on the induction medium containing 0.02 and 0.04 mg/l TDZ showed minor swelling and ovules started to grow and appeared on the surface of ovary slices. The maximum number of the responding ovaries 65.83% and 46.6% were noted on induction medium supplemented with 0.04 and 0.02 mg/l TDZ, respectively. Embryo formation frequencies obtained using the optimized TDZ procedure was considerably higher than those reported previously by Ficcadenti et al. (1999). The induction medium having maximum concentration of TDZ (0.08 mg/l) found to be lethal for young ovaries, ovaries became yellow or brownish without showing any visible growth of unfertilized ovule in the ovary slices after 4–5 days. In the present study, TDZ proved to be effective for embryogenesis in melon unfertilized ovary culture when included in the induction medium in lower concentrations of 0.02 and 0.04 mg/l, similar to earlier reports in *Cucumis sativus* L. gynogenesis (Diao et al. 2009; Gémesné Juhász et al. 1997).

Effect of low temperature pre-treatment and TDZ interaction

In interaction studies between temperature pre-treatment and different concentration of TDZ (Table 3), ovaries exposed to temperature pre-treatment (4°C) for 4 days and cultured on the induction medium (0.04 mg/l TDZ) showed significantly higher gynogenic response (76.6%) compared to the control (55%). Similarly, ovary slices cultured on the induction medium supplemented with 0.02 mg/l TDZ have better gynogenic response at 4°C for 4 days, than without temperature pre-treatment.

Effect of BAP and NAA on plantlet regeneration

After transferring from the induction to the regeneration media, ovary slices reacted by forming clusters of globular embryo like structures from ovules after 3–4 weeks (Fig. 1). The MS media supplemented with 0.6 and 0.3 mg/l BAP alone gave significantly ($P < 0.05$) higher regeneration rate of 22.5% and 12.5%, respectively (Table 1). The minimum regeneration (2.85% and 5.7%) was noted in the medium containing NAA (0.05 mg/l) and BAP (0.3 and 0.6 mg/l), respectively. Surprisingly, ovules in some of the ovary slices did not show further growth on regeneration medium containing different concentration of NAA and started normal organogen-

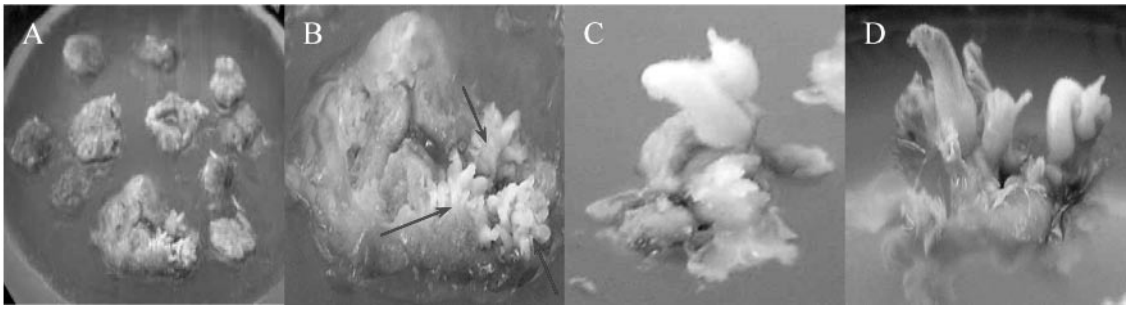

Fig. 1. Embryogenesis and plantlet regeneration from unfertilized ovary culture of melon (*Cucumis melo* L.) (A) ovary slices on induction medium; (B) clusters of sprouting embryos; (C) shoot organogenesis; (D) plantlet regeneration

esis when transferred onto the medium containing only BAP (0.6 mg/l). This sensitivity of embryos to NAA is contrary to the results of Ficcadenti et al. (1999), who obtained higher regeneration efficiency in melon on medium supplemented with 0.05 mg/l NAA. However, Kumlehn and Nitzsche (1995) reported that the sensitivity to 2,4-D in *Lolium multiflorum* gynogenesis is genotypic-dependent and dedifferentiating effect of 2,4-D seems to be weak enough to ensure a comparatively high quality of embryo morphogenesis and a satisfactory maintenance of regeneration potential. Ovary slices on MS medium supplemented with 0.1 mg/l NAA with different concentration of BA developed white callus but no sign of ovule growth was observed. Whereas the control treatment suppressed the ovule growth and ultimately ovary slices turned brown after 2 weeks of culture on the regeneration medium (data not shown). Cytokinins (BAP) are usually incorporated in tissue culture media for cell division and differentiation of adventitious shoots from callus and organs (Panaia et al. 2004). The incorporation of BAP at low levels was successfully applied in the present study for embryo maturation and ultimately plantlet regeneration. Our results are in agreement with Sharma et al. (2005), who reported that low level of BAP in the medium significantly effected embryogenesis and the maintenance of long term morphogenic capacity in *Hordeum vulgare* L. Likewise, application of higher concentration of BAP (0.9 mg/l) in regeneration media in present studies also failed to regenerate gynogenic plantlets, some callus was observed around the edges of melon ovary slices and remain green for 5–6 weeks without showing any organogenesis. Mukhambetzhanov (1997) reported that higher concentrations of plant growth regulators stimulate callus formation from somatic tissues and inhibit embryo formation from haploid elements of the embryo sac. The composition of nutrient media plays an important role in culture of non-fertilized female gametophytes.

Identification of homozygous double haploid plants by SSR analysis

Cytological studies revealed that plantlets originating from embryos were all diploid. Therefore, to confirm weather the plantlets originated from megaspore mother cells (homozygous) and spontaneous doubling of chromosome occurred dur-

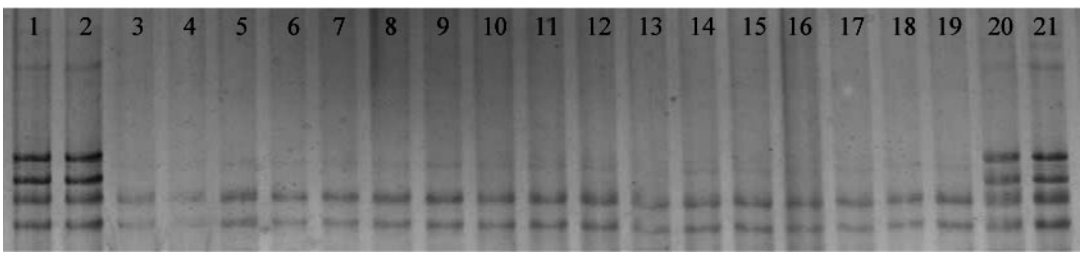

Fig. 2. Amplification pattern of microsatellite CMGA172 in polyacrylamide gel (7.2%); Lane 1, 2, 20, and 21 – Heterozygous ovary donor Parent; Lanes 3–19 – Homozygous/spontaneous dihaploid plantlets derived through melon unfertilized ovary culture

ing the plantlet development or embryogenesis initiated from diploid somatic tissue (nucellus and integuments) of ovary. Thus SSR analysis was carried out to identify the nature of plantlets. Five primer pairs (CSLHCPA, CSHPRAG, CMTAA166, CSATT214, and CSAT425a) did not amplify any band and hence were found to be non reactive at the annealing temperature (55°C). Six primer pairs (CMCT158, CMAT141, CMCCA145, CMGA172, CMCT505, and CSGTT15b) were found to be heterozygous for the parental material. These were used for genetic fingerprinting of the gynogenic plantlets for the detection of homozygosity.

One primer pair (CMGA172) generated banding patterns which distinguished gynogenic plantlets from the parental material. Thus, SSR markers with microsatellite CMGA172, showed 100% homozygosity and confirmed that the alleles in the parental material was also present in the gynogenic plantlets, but amplified only 2/4 alleles of the parent material at the same locus (Fig. 2). Thus, there were no partially heterozygous plants that were homozygous at this locus. By selecting this locus first to analyze unknown diploids, we could efficiently eliminate heterozygotes in a single amplification. Perera et al. (2008) also concluded that a single primer pair with the segregating allele in the donor parent is sufficient for distinguishing the population since all the analyzed structures were derived from the same donor parent. SSR markers were also successfully employed in other crops to identify spontaneous dihaploid plants and reveal homozygosity in very early "embryogenesis" (Diao et al. 2009; Perera et al. 2008; Chani et al. 2000; Bouvier et al. 2002). Gynogenic plantlets were genetically homozygous, derived from megaspore mother cells and considered spontaneous double haploid melon plants.

In this study, an efficient procedure was developed for induction of somatic embryogenesis from unfertilized ovary culture in melon using TDZ, and low temperature pre-treatment. Further development of somatic embryos, maturation and plantlet regeneration was also achieved on MS medium supplemented with BAP. The present results also illustrated the possibility of using SSR molecular markers with particular primers combination to distinguish melon plants which originated from maternal tissue in single amplification. This procedure would greatly increase the efficiency and reduce the time needed to screen large number of regenerants. Induced gynogenic development of the embryo sac is a possible approach to produce doubled haploid lines in melon.

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