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# An efficient regeneration protocol for *Agrobacterium*-mediated transformation of melon (*Cucumis melo* L.)

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**ABSTRACT.** An efficient selection and plant regeneration protocol for *Agrobacterium*-mediated transformation, using cotyledon node zone-stem connection region of melon, has been developed. The new *Agrobacterium*-mediated transformation methodology, independent of organ culture, used the entire germinated seed as explants. The transformation system was maximized to maintain the integrity of melon itself, thus avoiding the limitations of traditional tissue culture methods. The transformation was carried out under a non-sterile environment. The incorporation of a selectable marker (neomycin phosphotransferase II) into the genome of transgenic plants was confirmed by PCR and Southern blot analyses. The transformation frequency based on the PCR was 13%. Transgenic melon plants were usually detected by PCR in less than 1 month after *Agrobacterium* inoculation, and seeds could be harvested in 3 months. The growth characteristics and morphology of the transgenic plants were identical to the untransformed wild-type plants. This method would be beneficial for facilitating the characteristics of gene functions

 

and for boosting the manipulation of melon transformation for commercial purposes.

**Key words:** Transformation method; *Agrobacterium tumefaciens*; Melon; Adventitious shoots induction

## INTRODUCTION

Melon (*Cucumis melo* L.) is a group of high-value crops that include muskmelon, honeydew and casaba melon. Despite the development of genetic transformation protocols for melon reported in the last 20 years, transformation remains genotype-dependent and efficiencies are relatively low (0- to 12.5%) (Nuněz-Palenius et al., 2006, 2008). Moreover, the introduction of transgenes creates the possibility of gene escape and pollution. Adventitious shoots induction transformation technology offers several unique advantages, including a high level of transgene expression and the absence of epigenetic transgene instability effects. In addition, increased biosafety associated with transplants is of particular relevance to future applications of genetic engineering in agriculture. Because of these advantages, the transgenic technology could be useful for engineering agronomic traits, and for the metabolic engineering and production of biopharmaceuticals in plants.

Although melon is susceptible to *Agrobacterium* infection and responsive to regeneration from explants derived from the cotyledon (Dirks and van Buggenum, 1989) and callus (Moreno et al., 1985), the transformation regeneration frequency varies widely among cultivars (Galperin et al., 2003; Akasaka-Kennedy et al., 2004; Rhimi et al., 2006; Hao et al., 2011) and sources of explants (Fang and Grumet, 1990; Guis et al., 2000; Ren et al., 2012). Moreover, in melon transformation, a high frequency of tetraploids and false- positive regenerants are common phenomena.

Bordas et al. (1997) reported transformed melon plants that over-expressed the yeast *HAL1* gene, related to salt tolerance. Resistance to various plant diseases has also been achieved in transgenic melon through the incorporation of genes coding for the coat protein gene of various plant viruses, including cucumber mosaic virus, watermelon silver mottle virus (Huang et al., 2011), watermelon mosaic virus, and zucchini yellow mosaic virus (ZYMV) (Fuchs et al., 1998). Other researchers have reported the development of genetically engineered melon plants through transformation with the ACC oxidase antisense gene (Nora et al., 2001; Nuněz-Palenius et al., 2006). However, there is relatively little published data on the regeneration and/or transformation of oriental melon, with most of such information originating from the studies of Moon et al. (2000) on the regeneration of whole oriental melon plants using cotyledon explants, and Wu et al. (2009) on the regeneration of transgenic melon resistant to ZYMV.

In the present paper, we report the development of a stable adventitious shoots induction transformation system for melon. This transformation system may be useful for introducing agronomically and biotechnologically relevant traits into melon. The production of transplant melons with maternal inheritance of the transgene could solve problems related to out-crossing between the genetically modified crops and conventional crops or its wild relatives.

## MATERIAL AND METHODS

### Vector construction and plant transformation

We first isolated the melon *CmACS-7* (EU791279.1) coding sequence by RT-PCR with the following primers: 5'-GAAAACAAGGATTTCTTTTTCTTTTTCCTCAG-3' and 5'-TTCAACAAATCTTCAGTTCAATTTCTCTC-3'. Finally, the *CmACS-7* CDS construct preceded by a GUS leader as a *Sac*I-*Hid*III fragment, was transferred to the binary vector pBI121 to generate pBI-ACS-73'UTR. *Agrobacterium tumefaciens* EHA105 was transformed with pBI121-*CmACS-7* by electroporation. The binary vector pBI121-cm harbored hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase II (*npt*II), whereas pBIcdS contained the ACS (*CmACS-7*) deaminase gene (Figure 1).

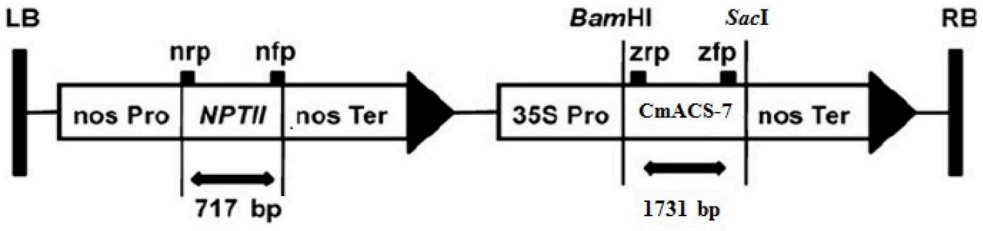


**Figure 1.** T-DNA construct of the binary vector plasmids used for genetic transformation in "melon". EHA105 (pBI121-cm); RB = right border; LB = left border; nptII = neomycin phosphotransferase gene; *NPTII* = neomycin phosphotransferase resistance gene.

### Transformation of melon by adventitious shoots induction

For the transformation of melon seeds, mature embryos were derived from viable seeds of melon andromonoecious inbred lines (M-15). The andromonoecious lines have only male flowers in the main stem. The seeds were supplied by the Watermelon & Melon Genetic Breeding Laboratory of the Horticulture College at Northeast Agricultural University (Figure 1A). Seeds were soaked in 55 to 60°C water and mixed constantly with a glass rod; when the water temperature had reached room temperature, the seeds were soaked for a further 12 h, and then placed in a training dish for the germination. After 2 days, pending that the cotyledon had not expanded, two cotyledons between apical buds were carefully removed with a blade. The wound was zoned for treatment with drops plus induction liquid. The induction liquid contained concentration for 1/3MS (Murashige and Skoog), 2.0 mg/L 6-benzyladenine (6-BA), and 0.5‰ SilWet L-77. The receptor material was prepared, and then drops plus liquid containing 1/3MS, 1.5 mg/L 6-BA, 100 µM AS(3,5-methoxy-4-hydroxyacetophenone), and 0.5‰ SilWet L-77 were disseminated; daily for 2 days dark culture, and then in light culture. To determine the antibiotic concentration useful for selecting transgenic buds, kanamycin at nine different concentrations, 0, 1000, 2000, 3000, 4000, 5000, 6000, and 8000 mg/L was used. A Smudge Brush was impregnated with the solution for the corresponding leaf surface, and each concentration of antibiotic solution was smearing on 40 strains, applying a total of

three times a day for three consecutive days. A smudge on the 3rd, 6th, and 9th days of observation was recorded for the degree of yellowing of leaves.

A 20 µL suspension of EHA105 (pBI121-cm) was cultured 24 h in a reciprocal shaker (120 rpm) at 28°C in 50 mL liquid LB (Luria-Bertani) medium containing 50 mg/L kanamycin, 50 mg/L hygromycin (Wako Pure Chemical Industries, Osaka, Japan) and 50 mg/L rifampicin, The bacterial suspension was centrifuged and then resuspended to a final density of $OD_{600}$ = 0.6 in inoculation medium containing 1/3 MS, 1.5 mg/L 6-BA, 100 µM AS, and 0.5‰ SilWet-77; plants were then dipped in the bacterial suspension. After inoculation, moisturizing cultivation was carry out, and the drops were repeated on the 2nd day plus one additional times.

## Genomic DNA extraction and PCR

Genomic DNA was isolated from 0.2 g young leaves of the putative transgenic plantlets and non-transgenic melon plants. The CTAB method (Luan et al., 2008) was employed. The presence of the *CmACS-7* gene in the genome of the transformed lines was confirmed by polymerase chain reaction (PCR) amplification on total DNA samples. Specific primers 5'-CAGAAAACAAGGATTTCTTTTTCTTTTTCCTCAG-3' and 5'-TTCAACAAATCTTCA GTTCAATTTCTCTC-3' were used for the amplification of the *CmACS-7* gene, respectively. The PCRs were carried out in a 50 µL reaction mixture containing 100 ng DNA, 1 µL 10 mM each primer, 0.5 µL 10 mM dNTPs, 2.5 µL 10 X Taq buffer (Takara, Dalian, China), 1.5 µL 25 mM $MgCl_2$, 1 µL Taq DNA polymerase (Takara,) and $ddH_2O$, using the following conditions: 94°C for 5 min, then 40 cycles of 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The amplification products were electrophoresed on 1% agarose gel and detected by ethidium bromide staining.

## Southern hybridization analysis

Genomic DNA of some PCR-positive plantlets was digested with *Eco*RI (with only one recognition site in the T-DNA region), transferred from the gel to a nylon membrane using the vacuum method, and hybridized with a DIG-labeled beta (900-bp) probe. Hybridization was per-Results, formed according to the manufacturer protocols for the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer). After digestion, all 260 mL of the digestion reaction was loaded into deep wells of a 1% agarose gel and separated by running at 30 V for 16 to18 h. Washing and detection were performed according to the instruction manual of the DIG labeling and detection system (Roche Diagnostics Mannheim, Germany). For detection of hybridization signals, the membrane was exposed to a detection film (Lumi-Film Chemilum inescent Detection Film; Roche Diagnostics) for 1 h.

# RESULTS

## Regeneration of transgenic melon

The experimental study on melon leaf showed that melon had a certain resistance to kanamycin, but with the increase of kanamycin concentration, the differentiation of ad-

ventitious buds was inhibited to a certain degree. Specifically, the kanamycin concentration of 6000 mg/L showed a marked increase in this trend. Processing of the different cards of kanamycin concentrations showed that the antibiotic could inhibit the growth of new leaves, and the changes in the response of blades to different concentrations occurred in a faster response time. Shoot survival decreased at kanamycin concentrations of 4000 to 5000 mg/L, and growth was totally inhibited when kanamycin exceeded 6000 mg/L. Therefore, we chose to add 6000 mg/L kanamycin to the regeneration medium in order to select transformed cells (Figure 2).

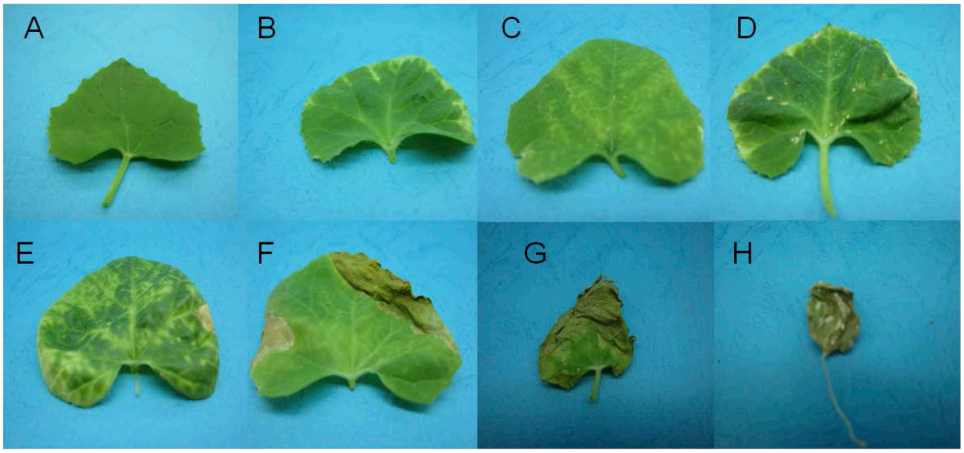


**Figure 2.** Leaves of different concentrations of kanamycin apply reaction. **A.** 0 mg/L. **B.** 1000 mg/L. **C.** 2000 mg/L. **D.** 3000 mg/L. **E.** 4000 mg/L. **F.** 5000 mg/L. **G.** 6000 mg/L. **H.** 8000 mg/L.

Transformation of melon with *A.tumefaciens* EAH105 containing pBI121-cm was performed by adopting the proposed improved method. The results, after removal of latera buds and apical buds of melon to the germination of 3 days processing, are shown in Figure 2. After the *Agrobacterium* infection, the induction of adventitious shoot differentiation and growth with liquid every 2 days in the wound, plus the adventitious bud induction by liquid drops, plus the 2 drops at all times, caused differentiation after 7 to 10 days at the growing point of expansion, along with adventitious buds differentiation. With the continued growth of adventitious buds, the vitality of the buds started growth elongation. When the new leaves of bud elongation had reached the two-leaf stage kanamycin was applied. After 9 days of observation, large areas of leaves that did not have kanamycin resistance started turning from green to yellow. We used scissors to discard the negatives from the base of the plant, until the filter on the same receptor plant leaves showed no obvious change of resistance. Smudge-resistant strains going through the filter for the first time in turn produced different parts of three filters, recorded as kanamycin plants. The results are shown in Figure 3. The kanamycin-resistant plants acquired were transplanted to soil and cultivated in a greenhouse. For the $T_0$ generation plants, we avoided the traditional group culture method of following generation and root regeneration. For the original growth of roots, plants that were closer to real healthy seedlings were obtained, in soil in which the normal training strength rate higher. After cotyledons shed-

ding and sections compartmentalizing, we observed an obvious differentiation to the expansion state. As the growth variable was rough, after kanamycin section removal of negative buds of the base department, it was clearly visible that most the plant retained a 1 positive bud, where less appears part 2 a and 3 a positive bud (Figure 3). In the greenhouse test. Following a hardening process, all plantlets of these 30 lines were quite stable with a normal shoot and root system, and were used for further studies. The plantlets from individual shoots that were PCR-confirmed to be regenerated from explants were defined as plantlets of individuals for further assays. The established $T_1$ transgenic melon lines, which appeared morphologically normal, were analyzed by PCR and southern blot assay.

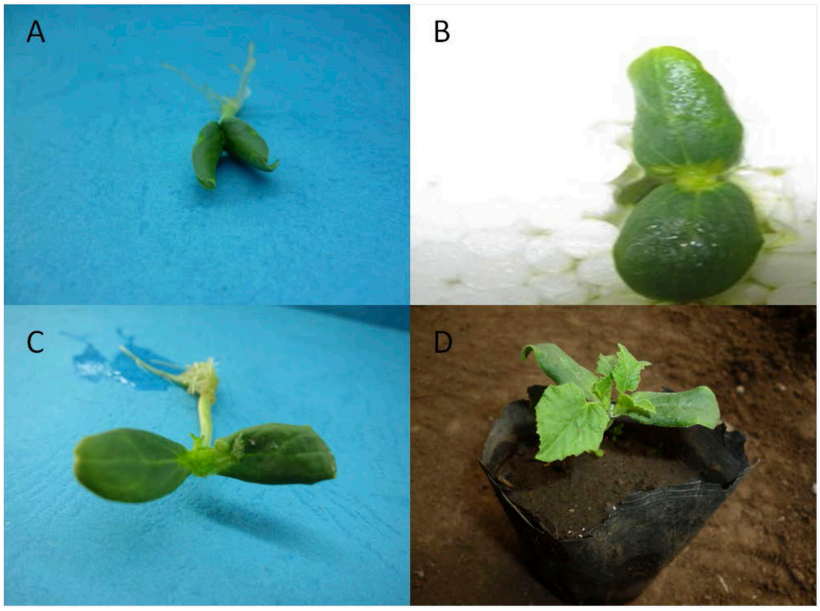


**Figure 3.** Culture and shoot inducing. **A.** Culture for 2 days. **B.** Shoot inducing. **C.** Shoot elongation. **D.** Transplant.

## Analysis of transgenic plants

PCR detection of the genomic DNA from four putative transgenic melon four lines with the transgene specific primer pair resulted in an expected 1.7 kb product (Figure 4). The 0.8 kb product PCR-amplified with the nptt specific primer pair also corroborated this is inference (data not shown). Southern blot analysis was carried out using probes on two transplants to verify the homoplasmic condition and transgene integration. Southern blot hybridization revealed the 1.7 kb fragments in transplants to plants, respectively, when a DNA blot was probed with the probe targeting sequence. Only one a fragment in the four transplants lines and no hybridization signal in non-transformed plants were detected when a DNA blot was probed (Figure 5). In order to test that the expression of the *CmACS -7* gene must come from the plant genome, after several subcultures on rooting medium, 10 transgenic plants, two from each transgene, were analyzed by Southern hybridization as described above. Variable

hybridization patterns were observed among the transgenic plants, indicating the expected random integration of the *CmACS-7* gene into the ‘‘Melon’’ genome. Among the tested lines, line 2-6 infected with EHA105 (pBI121-cm) had a single copy of T-DNA integrated in the plant genome.

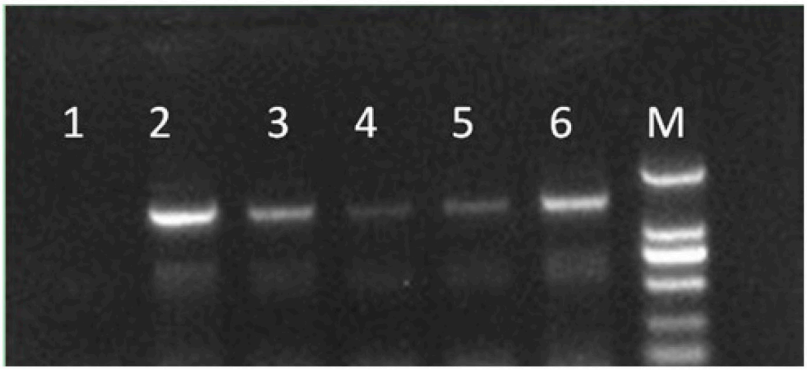


**Figure 4.** Molecular analysis of transgenic plants. Ethidium bromide-stained agarose gel showing amplification by PCR of the *CmACS-7* genes in transgenic “melon” plants. *Lane M* = 2-kb molecular size marker; *lane 1* = non-transformed control; *lane 2* = positive control (plasmid DNA); *lanes 3-6* = transgenic lines transformed with EHA105 (pBI121-cm).

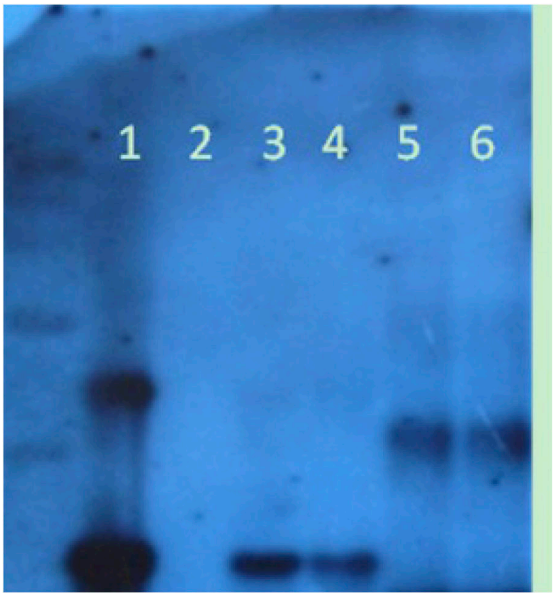


**Figure 5.** Southern hybridization were performed as described in the text. Genomic DNA from each line was digested with *Eco*RI and probed with *CmACS-7* gene fragment. *Lane 1* = plasmid PBI121; *lane 2* = non-trasformed plants; *lanes 3* and *4* = same plant tests transformed; *lanes 5* and *6* = same plant test. The bands represent the transgene insertions.

In general, the first bisexual flowers occurred earlier in the field (nodes 5-8) than in the no-transformation plant, which was likely due to more favorable environmental conditions, including a higher light intensity, which can be a limiting factor for bisexual bud production in the greenhouse. Although there was no significant difference in the time of appearance of the first bisexual bud for the *CmACS-7* transgenic lines (Figure 6), there was a significantly earlier appearance of the first female flowers in the main stem than for the transformation plants. The transgenic *CmACS-7* lines also produced significantly more mature bisexual flowers in the first 5-7 nodes of the main stem (Figure 6A, C) and a higher percentage of female flowers reached maturity. Although the transgenic melon line also showed an increased number of mature bisexual flowers and female flowers, the effect was more pronounced in the *CmACS-7* transgenic plants. There was also a higher frequency of adjacent female flowers on the main stem of transformation plants (Figure 6).

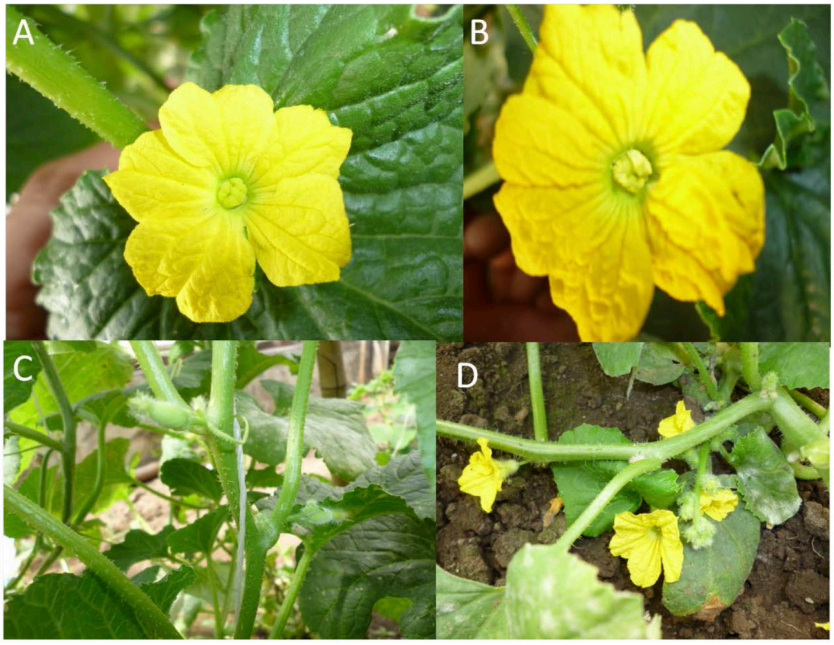


**Figure 6.** Evaluation of transgenic oriental melon lines carrying the ACS-7 transgene. **A.** Transgenic melon CM-15. **B.** No transgenic melon CM-15. **C.** Transgenic melon CM-15 Andromonoecious lines main stem have female flowers. **D.** Untransgenic melon CM-15 Andromonoecious line main stem have male flower.

## DISCUSSION

Melon tissue culture for transgenic methods, for explants of high processing requirements, require a specific bacteria-free environment, tissue culture, and complicated operations that involve a heavy workload. Most methods on the integrity of melon explants cause a lot of damage, such as cutting of the leaf and hypocotyls. Damage to the air can result from the artificially created plant tissue culture environment that is required to meet the high culture condi-

tions and needs for the normal growth of traumatized explants, as well as the full demands for differentiation of adventitious buds of explants. Thus, the culture conditions for the efficient genetic modification of traumatized explants are an important factor. Owing to the diversity of melon genotypes, however, the suitability of a variety of transformation culture conditions for each genotype is difficult to find making transformation of melon genotypes severely restricted. The various aspects of the transformation process also affect the final transformation efficiency, such as subculture and removal of bacterial contamination.

Compared with the traditional method, this study has established a transgenic method to maximized to maintain the integrity of melon, relying on its own commission to grow normally in the environment created by the induction of adventitious buds. The transgenic plants created were stable, the whole process took place in the open non-sterile environment, and groups woven training constraints were negated. Owing to the design of the path from the traditional way of tissue culturing and a bacteria-free environment, we created a method for melon genetic transformation with an environment suitable for growth and facilitating the transformation with the *Agrobacterium*-mediated method, as well as taking into account the light and humidity needs for transformed cells differentiation. The cultivation method used guarantee optimal humidity conditions, and hydroponic liquid nutrient control. While maintaining the integrity of the melon, the method also considered sensitive issues such as hormones, liquid induction by using the spotting (including hormone solutions) method of infection and induction of adventitious buds, and targeted screening of adventitious buds, to achieve transgenic plants.

This study on the method of establishing small subject to melon genotype, from infection to the PCR test, required 35-40 days, and access to the seeds for the entire cycle required 3 to 4 months. For $T_0$ generation seeds, the seed number was much higher, because the buds do not regenerate in the same way as a multiple-cell origin (Bordas et al., 1994; Choi et al., 2012). Thus, future generations would require several herbicide plant filters, eliminating chimeric plants and reducing late pure plants and the workload.

For melon, the first successful *Agrobacterum*-mediated gene transfer was published in 1990 (Fang and Grumet, 1990). Subsequently, many transgenic melon plants have been produced (Rhimi et al., 2006; Ren et al., 2012). However, the transformation efficiency in melon is not high enough. Moreover, regeneration and transformation in melon are genotype-dependent and still difficult, as well as being labor intensive with very low regeneration frequencies (Fang and Grumet, 1990). To produce a large number of transgenic plants, plant genetic transformation deals with the ability of a manipulated plant cell to differentiate into a complete organism. A robust differentiation protocol is one of the essential requirements to achieve this goal. So far, for several plant species, regeneration procedures have not been found and those species are labeled as recalcitrant.

Genetic improvement of melon (*Cucumis melo* L.) via the transformation method depends mainly on developing a favorable receptor system. Establishment of an efficient *in vitro* regeneration system plays an important role in the transgenic transformation. However, some problems have persisted in the genetic transformation of melon, such as the difficulty in promoting plant regeneration, the high frequency of somatic variance, the intense genotype-dependence, and the long transformation period required, all of which hinder the genetic improvement of melon.

In conclusion, we demonstrated the feasibility of a transformation method, by inducing melon adventitious shoots proliferation using 6-BA and selecting transformed shoots in the presence of kanamycin. The germinated entire seedlings of melon were used as explants.

The transformation system was maximized to maintain the integrity of melon itself, this overcome the limitations of traditional tissue culturing methods. The transformation was carried out under a non-sterile environment. With this procedure, fertile transgenic plants generating transgenic progenies were also obtained.

## ACKNOWLEDGMENTS

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