



# Transformation of the *CmACS-7* gene into melon (*Cucumis melo* L.) using the pollen-tube pathway

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**ABSTRACT.** The aim of the present study was to develop a transformation system that may be useful for introducing agronomically and biotechnologically relevant traits into melon. The production of transplanted melon with maternal inheritance of the transgene could solve problems related to outcrossing between genetically modified crops and conventional crops or their wild relatives. By analyzing the main influencing factors systematically, the pollination time was ascertained and the pollen-tube pathway genetic transformation system was optimized. A screening system for resistant seeds from the T<sub>1</sub> generation was established. The transformed seedlings were grown under standard field conditions and selected using a polymerase chain reaction-based analysis. The resistant plants were detected at a rate of 5%. These results indicate that enhanced production hastens the initiation of bisexual flowers, development of mature bisexual flowers,

and fruit set in melon. We have established a melon transformation system based on the pollen-tube method.

**Key words:** Melon; Pollen-tube pathway; *CmACS-7*

## INTRODUCTION

Muskmelon (*Cucumis melo* L.) is one of the most economically important species within the family Cucurbitaceae. Other species in the family include cucumber, watermelon, and squashes. Melon is a complex plant that includes seven different sex combinations. Most of the thin-skinned, muskmelon cultivars have male identical strains (andromonoecious); these plants have both male bisexual and unisexual flowers, which results in flowers with different gender. Due to the presence of bisexual flowers, the pistil of the flower itself has to be used for artificial emasculation for preparing hybrids. The most commonly used methods for producing hybrid seeds are artificial emasculation, chemical emasculation, and male sterile lines. For producing hybrid melon seeds, artificial emasculation is the most commonly used method. However, during the process of artificial emasculation, the flowers may be completely emasculated, damaging the pistil. This results in poor pollination and a reduction in fruit set, further resulting in an increasing cost of seed production, difficulty in ensuring seed purity, and great inconvenience in hybrid production. According to previously published data, the cost of producing F<sub>1</sub> melon hybrid seeds is generally 12-30 times that of conventional varieties. The use of female inbred lines or the use of the same strains of dioecious flowers pollinated without emasculation greatly reduces the cost of producing hybrid seeds. Chemical hybridization affects the rate of fruit set but lacks the advantages of artificial selection. However, the production of hybrids using the methods described above has disadvantages such as lengthy cycles and heavy workload; hence, the use of exogenous genes that target genetic improvement is an effective way to solve the artificial emasculation, chemical emasculation, and male sterile lines.

Melon is susceptible to *Agrobacterium* infection and responds to regeneration from explants derived from both cotyledon (Dirks and van Buggenum, 1989) and callus (Moreno et al., 1985), although the frequency of transformation regeneration varies widely among cultivars (Galperin et al., 2003; Akasaka-Kennedy et al., 2004; Rhimi et al., 2006; Hao et al., 2011; Akbari et al., 2013) and sources of explants (Fang and Grumet, 1990; Gray et al., 1993; Ren et al., 2012, 2013; Bezirganoglu et al., 2013; Zhang et al., 2014; Zhang and Luan, 2015). However, the genes responsible for regeneration of melon continue to be poorly understood. Hence, developing simple and routine procedures to regenerate and transform all melon genotypes is still challenging.

The method of transformation through the pollen-tube pathway introduces exogenous DNA into plants through pollination, as first reported by Zhou et al. (1983). This approach has been successfully used in several crops (Yang et al., 2009). Although the underlying mechanism of transformation is still unclear, this tissue culture-independent procedure, which is more suitable for crops with low rates of regeneration, is simple, rapid, and efficient. In this study, a linear *CmACS-7* antisense gene cassette flanked by a T-DNA border was transformed into melon via the pollen-tube pathway method. The transformed seedlings were selected using a polymerase chain reaction (PCR)-based analysis, and were grown under standard field conditions. The melon fruits produced by these plants were tested for various traits.

## MATERIAL AND METHODS

### Plant material and transformation

Mature embryos were transformed from viable melon seeds from andromonoecious inbred lines (*Cm-23*). Andromonoecious lines only have male flowers on the main stem. Seeds were supplied by the Watermelon & Melon Genetic Breeding Laboratory of Horticulture College at Northeast Agricultural University, Harbin, China. Seeds were soaked in water kept at 55°-60°C and stirred constantly using a glass rod. Subsequently, the seeds were soaked for 12 h at room temperature and placed on a training dish for germination. After 2 days, if the cotyledon had not expanded, engraftment was performed in greenhouses.

### Genetic manipulation

First, the *CmACS-7* (EU791279.1) coding sequence of melon was isolated (Figure 1) using reverse transcription PCR with the following primers: 5'-GAAAACAAGGATTTCTTTTCTTTTTCTCAG-3' and 5'-TTCAACAAATCTTCAGTTCAATTTCTCTC-3'. The *CmACS-7* CDS construct was preceded by a glucuronidase leader as a *SacI-HindIII* fragment and was transferred to the binary vector pBI121 to generate the pBI-*ACS-7* 3'-untranslated region. *Agrobacterium tumefaciens* EHA105 was transformed with pBI121-*CmACS-7* using electroporation. The binary vector pBI121-Cm harbored hygromycin phosphotransferase and neomycin phosphotransferase II, whereas pBIcdS contained the 1-aminocyclopropane-1-carboxylate (*CmACS-7*) deaminase gene.

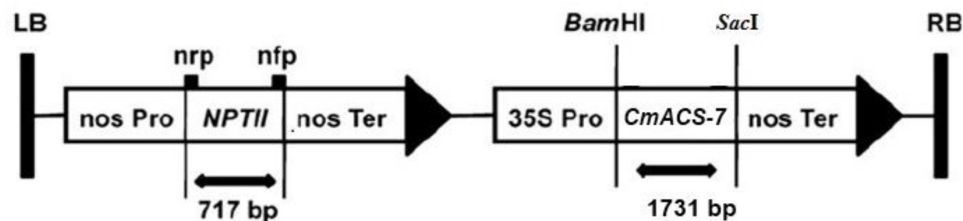


Figure 1. Plasmid map.

### Determination of pollen-tube time

In the afternoon, flowering melon plants were random selected and both male and female flowers were isolated by covering them with bags. The following morning, the flowers were pollinated and at 1, 6, 12, and 24 h after pollination the upper style was excised from the ovary. The style was fixed with 8:1 to describe the ratio of ethanol to acetic acid, and 24 h later, it was briefly rinsed with water. The ovary was slit using a blade to remove the style. It was then softened with ethanol:HCl (1:1) for 1-5 min and washed with distilled water for 1.5 h. Finally, it was stained with water-soluble aniline blue for 2-3 h, observed through an Olympus fluorescence microscope (Japan OLYMPUS), and photographed.

For the transformation, the inbred M-3 melon seedlings were grown in greenhouses and managed in the field with regular pruning. In the afternoon, male and female flowering melon plants were random selected and isolated by covering them with bags. The subsequent

morning, the flowers were pollinated and 24 h after pollination the upper part of the stigma was cut. The flowers were then immediately injected with a solution containing 100 ng plasmid DNA, using a microsyringe with the needle kept in a vertical position. In total, 50 flowers were injected with 100 ng DNA each. When the fruits ripened, they were picked according to a well-marked single fruit, and the seeds were collected.

### **Transformation of melon seeds using a lethal concentration of kanamycin**

For converting the melon seeds, a lethal concentration of kanamycin (Km) was used to screen and select 100 fully grown M-3 seeds that sprouted at 28°C. The seeds were placed on Petri dishes on filter paper, and a 20-mL solution containing different concentrations of Km was added. For the preliminary screening, the gradient was set at 0, 100, 200, 300, 500, 1000, and 1200 mg/L. From impregnation to the emergence of the leaves, seeds were placed in antibiotics. The fruits were harvested using the pollen-tube pathway method. From each fruit, 20 seeds were removed and a Km-resistance screening was performed. Melon seeds can germinate on Petri dishes, but after the emergence of the cotyledon, they will gradually turn yellow, and subsequently die. Transgenic plants grow normally. However, when the plant growth was robust, they were transplanted to the greenhouse.

### **Extraction of genomic DNA and PCR**

Genomic DNA was isolated from young leaves (0.2 g) of the putative transgenic plantlets as well as the non-transgenic melon plants. They were stored at -80°C until DNA isolation, as recommended by Luan et al. (2008). The presence of the *CmACS-7* gene in the genome of the transformed lines was confirmed using PCR amplification on total DNA samples. The specific primers 5'-CAGAAAACAAGGATTTCTTTTTCTTTTCCTCAG-3' and 5'-TTCAACAAATCTTCAGTTCAATTTCTCTC-3' were used for the amplification of the *CmACS-7* gene, respectively. The PCR was performed in a 50- $\mu$ L reaction mixture containing 100 ng DNA, 2  $\mu$ L each primer (10 mM), 2.5  $\mu$ L dNTPs (10 mM), 5  $\mu$ L 10X Taq buffer (Takara, Dalian, China), 1.5  $\mu$ L MgCl<sub>2</sub> (25 mM), 1  $\mu$ L Taq DNA polymerase (Takara), and ddH<sub>2</sub>O. The PCR was run under the following conditions: 94°C for 5 min; 40 cycles of 94°C for 30 s, 58°C of annealing for 1 min, 72°C of extension for 1 min; and a 10-min final extension at 72°C. The amplification products were electrophoresed on 1% agarose gel and detected using ethidium bromide staining.

## **RESULTS**

### **Determination of the lethal concentration of Km**

The results showed that, in the concentrations of Km, the seeds of the melon strains could germinate only after the cotyledons had expanded and the cotyledon and hypocotyl surface had gradually turned yellow, with the cotyledon showing varying degrees of yellow. In the end, the whole plant turned yellow. After 10 days, 1200 mg/L Km was found sufficient to kill the melon seedlings (Table 1). The experiment was repeated thrice and the results were similar (data not shown). Therefore, it can be concluded that normal melon seedlings can be inhibited using a Km concentration of at least 1200 mg/L.

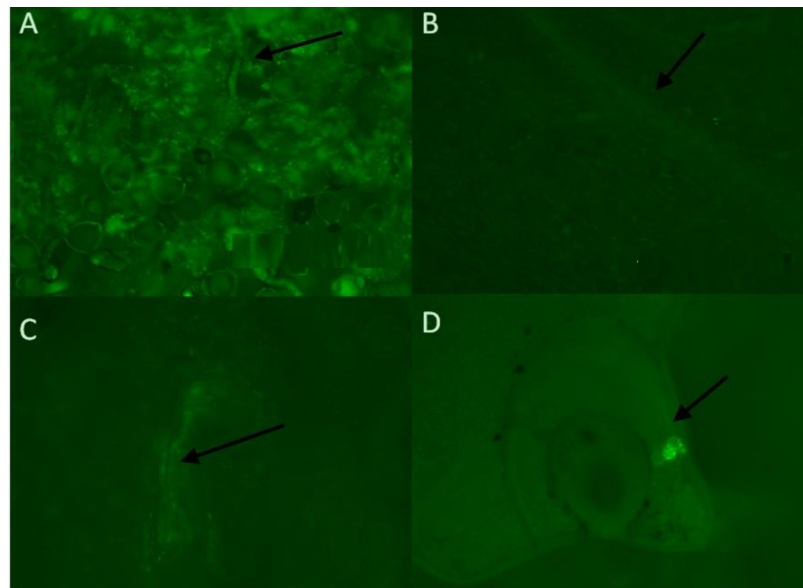
**Table 1.** Effects of various kanamycin (Km) concentrations on wild-type *Cucumis melo* L. using the Cm-23 receptor material.

Km concentration (mg/L)	Green seedling rate (%)
0	100
100	70
200	40
300	20
400	10
1000	0
1200	0

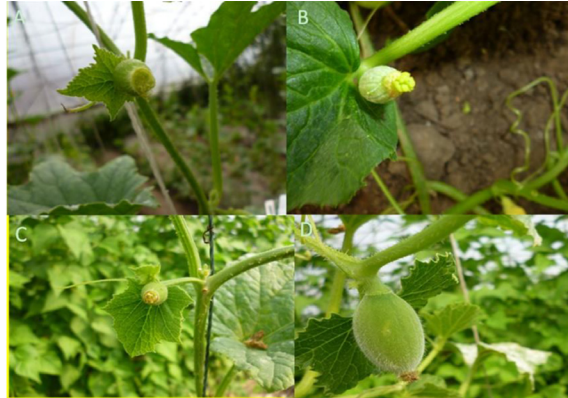
### Melon pollen-tube pathway used for the determination of appropriate conversion time

The inbred M-23 melons were observed using fluorescence microscopy. At 1 h after pollination, the pollens had begun to germinate; after 6 h, the pollen tubes were growing towards the style; after 12 h, the pollen tubes had reached the base of the style; and after 24 h, the pollen tubes protruded into the ovule (Figure 2). These results indicate that the pollens can reach the embryo sac 24 h after pollination, considering the time required for double fertilization. Hence, we suggest that injections to the melon ovary should be made at 24 h after pollination.

As can be seen in Figure 3, the injection of ovary strain M-23 using a microsyringe resulted in damage to the ovary and in visible flower size differences, which directly affects the fruit set. Of the 50 DNA injected flowers, nine (18%) reached the fruiting stage (Table 2 and Figure 3).



**Figure 2.** Melon pollen-tube growth in ovary dynamics. **A.** Pollen germinated on stigma (1 h after pollination); **B.** pollen-tube bundle toward the ovary (6 h after pollination); **C.** most of the pollen tubes reached the styles (12 h after pollination); **D.** embryo sac has been fertilized (24 h after pollination).



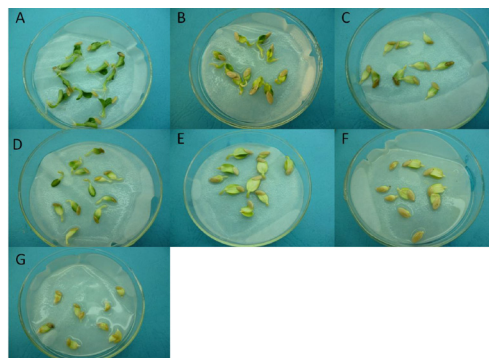
**Figure 3.** Pollen-tube pathway method of plants in different growth stages. **A.** Pollen-tube with the pistil cut; **B.** normal female flower; **C.** ovary wilting after DNA injection; **D.** normal ovary development after DNA injection.

**Table 2.** Survey statistics of harvested rate by pollen tube.

Receptor material	No. of flowers dealt	Fruit number	Fruiting rate (%)
<i>Cm-23</i>	50	9	18

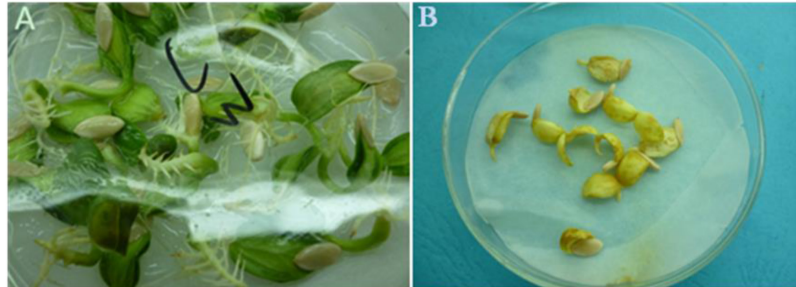
### Screening of transgenic plant-resistant seeds

From the pollen-tube pathway method, nine melon fruit M-23 strains were obtained. The Km screening test was performed at the seedling stage (Figure 3). Untransformed seeds were treated with 1200 mg/L Km, which does not result in normal germination. A strain-resistant plant was eventually obtained through this screening and was used for the subsequent tests (Figures 4 and 5). The plants were resistant to PCR testing. Preliminary evidence showed that four of the 100 resistant plants were PCR positive, suggesting that these genes had been integrated into the genome. Finally, four transgenic fruits (4% of the initial 100 plants) were harvested (Figure 6). Under the same conditions (e.g., temperature, light, hormones), our results indicated that genetically modified (gm) and observed under transgenic melon plant phenotype, its survival plant observations are as follows: 3 transgenic melon in the female flowers (Figure 7 and Table 3).

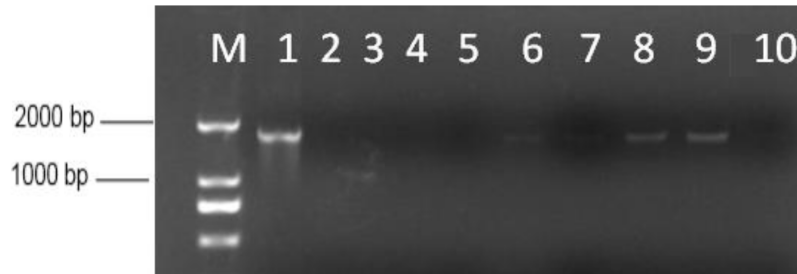


**Figure 4.** Results from the analysis of the critical concentration of kanamycin screening of the seed pollen-tube pathway. **A.** 0 mg/L; **B.** 100 mg/L; **C.** 200 mg/L; **D.** 300 mg/L; **E.** 500 mg/L; **F.** 1000 mg/L **G.** 1200 mg/L.

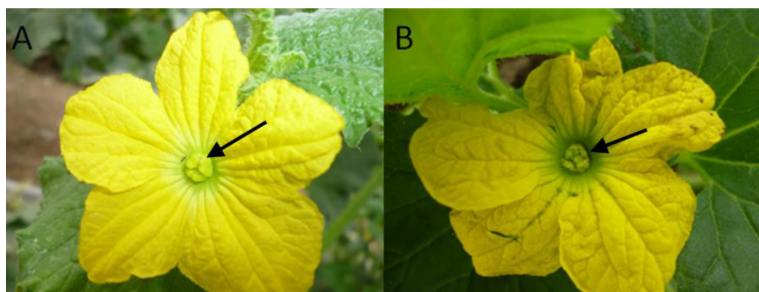




**Figure 5.** Selection of transgenic plant seeds. **A.** Transgenic plant seeds; **B.** non-transgenic plant seeds.



**Figure 6.** PCR analysis of transgenic melon lines. *Lane M:* ladder, 2 kbp; *lane 1:* plasmid; *lanes 6-10:* transgenic lines; *lanes 2-5:* non-transgenic plants.



**Figure 7.** Flower morphology observations for transgenic melon plants. **A.** Transgenic melon andromonoecious inbred lines have female flowers; **B.** non-transgenic melon andromonoecious inbred lines have bisexual flowers.

**Table 3.** Survey of female transgenic plant rates.

Inbred <i>Cm-3</i>	Total number of flowers	Number of female flowers	Female flower rate (%)
CK non-transgenic	85	0	0
6	67	8	11.9
7	75	6	8
8	69	9	13
9	80	0	0

## DISCUSSION

The pollen-tube pathway method can only be used in flowering plants. This is a type of plant tissue culture method used for genetic transformation. It is restricted by the small receptor gene type and high conversion efficiency, and has the advantage of combining traditional breeding and genetic engineering. Although the pollen-tube pathway method of genetic transformation at the molecular level has been tested previously, there are only relatively few published reports (Hao et al., 2011). The potential of this minimal cassette technology in the transformation of plants using particle bombardment has been explored in *C. melo*, as well as in other plants such as potato, grapevine, wheat, soybean, and grass (*Paspalum notatum* Flugge) (Sandhu and Altpeter, 2008; Yang et al., 2009). Melon is a complex plant that includes seven sex combinations. Unlike the production of most thin-skinned cultivars, thick-skinned melon cultivars all have male flowers, flower homologous strains, completely as a bisexual flower itself has a pistil, as well as stamens. Artificial emasculation is a widely used method in the process of producing melon hybrid seeds. Artificial emasculation can easily damage the flower. However, the removal of the pistil will result in bad pollination, a drop in the rate of fruit set, a cost increase in seed production, and difficulty in guaranteeing seed purity, resulting in general inconvenience in the production of hybrids. According to previously published data, the cost of producing F<sub>1</sub> melon hybrid seeds is generally 12-30 times that of conventional varieties. The use of female or different male and female flowers of homologous strains for pollination without emasculation greatly reduces the cost of producing hybrid seeds. However, the production of hybrids using the methods described has disadvantages such as lengthy cycles and heavy workloads.

In contrast to *Agrobacterium*-mediated gene transfer, biolistic gene transfer of linear transgene expression constructs of the promoter, the coding sequence, and the untranslated polyadenylation signal avoids the integration of the vector backbone sequences into the recipient genome (Fu et al., 2000). However, the processes of *in vitro* culturing and plant regeneration are required for this method, which are both time-consuming and complex. The pollen-tube pathway transformation method does not suffer from these problems. Zhang et al. (2005) reported that the transgenic plants derived from the pollen-tube method developed normally, flowered, and, in most cases were indistinguishable from non-transgenic plants. In contrast, those produced by particle bombardment or *Agrobacterium*-mediated transformation often showed phenotypic abnormalities and reduced fertility (Zhang et al., 2005). Recently, maize was transformed using linear transgene cassettes lacking the vector backbone sequences using the pollen-tube pathway, which yielded transgenic plants with low transgene copy numbers. This resulted in a simple integration pattern and efficient expression (Yang et al., 2009). In the present study, the same method was used for melon 24 h after pollination. The exogenous plasmid DNA was injected into the pollen tube at the beginning of the fertilization process. Double fertilization occurred under the effect of the drawing of the embryo sac, enabling better integration of the exogenous gene into the melon genome. In this experiment, after the injection of the melon, we get the melon seeds, the test to deal with the 50 flowers for experiment. In the first generation, nine fruits were produced, and the seeds from these fruits were used to test the resistance to Km out of M-2 inbred line, via PCR screening. Finally, we were able to harvest four transgenic fruits from the T<sub>1</sub> generation. To scale up melon production and to lay the direction of future transgenic melon systems, a foundation for genetic improvement of gender-specific materials was established.



In conclusion, we have established a melon transformation system based on the pollen-tube method in this study. This process does not include *in vitro* culturing or plant regeneration, making the transformation a cost-efficient method for obtaining transgenic melons. It is expected that this simple but efficient method can soon be implemented in other species. Our study illustrates that this method is particularly suitable for the transformation of melon crops. This is because the melon flowers are big and easy to handle, the ovary has big ovules, and many seeds can be obtained from a single flower. However, the pollen-tube method also has certain limitations. It can only be used for flowering plants and in commercial flowers. This study establishes an efficient and stable genetic transformation system for melon. The pollen-tube pathway facilitates the emergence of excellent sex-related genetic strains in melon and, thus, provides a new melon germplasm resource.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

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