Genetics and Molecular Research

Overexpression of the *CmACS-3* gene in melon causes abnormal pollen development

H. Zhang[1] and F. Luan[2]

[1]Anhui Key Laboratory of Plant Resources and Biology, School of Life Science, Huaibei Normal University, Huaibei, Anhui, China
[2]College of Horticulture, Northeast Agricultural University, Harbin, China

Corresponding author: F. Luan
E-mail: luanfeishi@sina.com / zhhuijun@126.com

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ABSTRACT. Sexual diversity expressed by the Curcurbitaceae family is a primary example of developmental plasticity in plants. Most melon genotypes are andromonoecious, where an initial phase of male flowers is followed by a mixture of bisexual and male flowers. Overexpression of the *CmACS-3* gene in melon plants showed an increased number of flower buds, and increased femaleness as demonstrated by a larger number bisexual buds. Transformation of *CmACS-3* in melons showed earlier development of and an increased number of bisexual buds that matured to anthesis but also increased the rate of development of the bisexual buds to maturity. Field studies showed that *CmACS-3*-overexpressing melons had earlier mature bisexual flowers, earlier fruit set, and an increased number of fruits set on closely spaced nodes on the main stem.

Key words: Adventitious shoots induction; *Agrobacterium tumefaciens*; *CmACS-3*; Melon; Pollen; Transformation method

INTRODUCTION

Members of the Cucubitaceae family exhibit extensive diversity of sexual phenotypes, and thus have served as model systems for understanding sexual differentiation in plants. Depending on the genotype, individual plants will produce various combinations of male, female, and bisexual flowers (Kenigsbuch and Cohen, 1989; Roy and Saran, 1990; Perl-Treves, 1999). Watermelon is typically andromonoecious (Kenigsbuch and Cohen, 1989), with an initial phase of male flowers followed by a combination of bisexual and male flowers.

The effects of hormones on sex determination of cucurbits have received considerable attention (Rudich, 1990; Perl-Treves, 1999). Auxin, ethylene, brassinosteroids, and gibberellins can all influence sex expression. Ethylene, however, appears to be the primary hormonal factor, with other hormones acting via ethylene (Tolla and Peterson, 1979; Yin and Quinn, 1995; Trebitsh et al., 1997; Papadopoulou and Grumet, 2005). Ethylene is synthesized in plants from *S*-adenosyl methionine, which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and ACC is then oxidized to ethylene by ACC oxidase. In target tissue, ethylene is detected by a family of ethylene receptors showing similarity to the bacterial 2-component histidine kinase sensors. Five ethylene receptors have been characterized in *Arabidopsis* (Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998), which can be divided into 2 subfamilies based on their protein structures. Subfamily I receptors include ETR1 and ERS1, while subfamily II includes the receptors ETR2, ERS2, and EIN4 (Bleecker, 1999). All play a redundant function as negative regulators of ethylene signaling, and their double, triple, and quadruple mutants result in a constitutive response to ethylene (Hua and Meyerowitz, 1998; Wang et al., 2003).

Ethylene is synthesized in plant tissues via a 2-step process, converting *S*-adenosyl methionine into ACC by ACS, followed by oxidation of ACC to ethylene by ACC oxidase (Zarembinski and Theologis, 1994; Fluhr and Mattoo, 1996; Johnson and Ecker, 1998). Control of ethylene production can occur at various steps, including both synthesis and oxidation of ACC, and sequestration of ACC by conjugation (Fluhr and Mattoo, 1996). The availability of ACC is frequently the rate-limiting step of ethylene production (Zarembinski and Theologis, 1994; Johnson and Ecker, 1998).

Although melon is susceptible to *Agrobacterium* infection and is 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 tetraploid and false-positive regenerants are commonly observed.

We report the development of a stable adventitious shoot induction transformation system for melon. This transformation system may be useful for introducing agronomically and biotechnologically relevant traits into melon. The production of transgenic melon with maternal inheritance of the transgene may be used to overcome limitations related to outcrossing between genetically modified crops and conventional crops or its wild relatives. Our results indicate that enhanced endogenous ethylene production hastens bisexual flower initiation, development of mature bisexual flowers, and fruit setting rate in melon.

MATERIAL AND METHODS

Transformation of melon by adventitious shoot induction

Melon seeds from mature embryos used for transformation were derived from viable seeds of the andromonoecious inbred melon line CM-15 (Watermelon & Melon Genetic Breeding Lab of Horticulture College at Northeast Agricultural University), which produce only male flowers in the main stem. Seeds soaking in 55-60°C water were mixed constantly with a glass rod, then soaked at room temperature for 12 h, and placed in a training dish for germination. After 2 days, if the cotyledon was not expanded, the 2 cotyledons were carefully removed between the apical buds. A wound was introduced using drops containing 1/3 MS + 2.0 mg/L 6-BA + 0.5% SilWet L-77, after 2 days, receptor material containing 1/3 MS + 1.5 mg/L 6-BA + 100 µM AS + 0.5% SilWet L-77 for 2 days of culture in the dark. The samples were cultured in light and induced by adding liquid. To determine the most useful antibiotic concentration for selecting transgenic buds, kanamycin at 9 different concentrations were used, including 0, 1000, 2000, 3000, 4000, 5000, 6000, 8000 mg/L. A smudge brush was used to introduce the solution on leaf surfaces, smearing 40 strains of each concentration of antibiotic solution 3 times a day for 3 consecutive days. Antibiotics were applied on the 3rd, 6th, and 9th day and the degree of leaf yellowing was observed.

A 20-µL suspension of *Agrobacterium tumefaciens* EHA105 (pBI121-cm) was cultured overnight in a reciprocal shaker (120 cycles/min) at 28°C in 50 mL liquid Luria-Bertani (LB) medium containing 50 mg/L kanamycin, 50 mg/L hygromycin (Wako Pure Chemical Industries, Osaka, Japan), and 50 mg/L rifampicin, while strain EHA105 (pBI121-cm) was cultured in liquid LB medium containing the same antibiotics. 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 + 0.5‰ SilWet L-77. The plants were then dipped into the bacterial suspension. After inoculation, cultivation of moisturizing, drops were applied twice on the 2nd day (Figure 1).

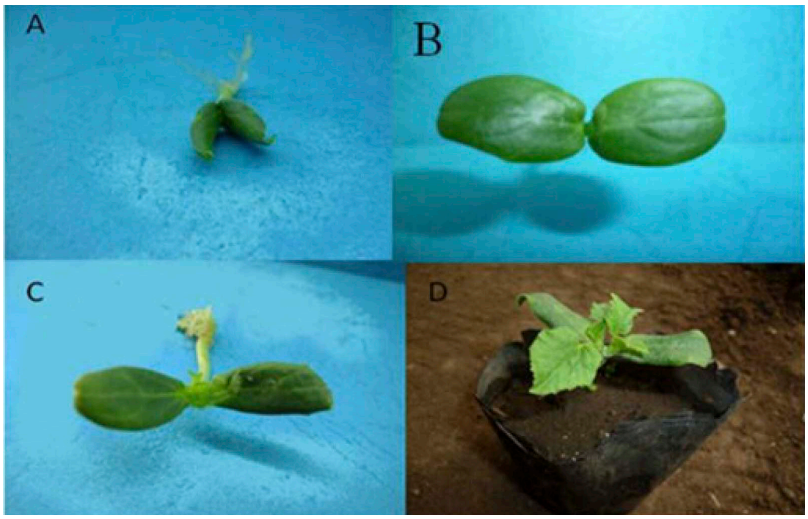

Figure 1. Culture and shoot induction. **A.** Culture for 2 days; **B.** shoot induction; **C.** shoot elongation; **D.** transplant.

Genetic manipulation

We first isolated the melon 1-aminocyclopropane-1-carboxylate (*CmACS-3*) (FJ383171) coding sequence by reverse transcription-polymerase chain reaction (PCR) with the following primers: 5'-GATCCACAGCCTACTGATGATC-3' and 5'-AAGCCACTACTAA TCCCACAAT3-'. The *CmACS-3* CDS 1335-base pair construct preceded by a GUS leader as a *Sac*I-*Hid*III fragment was transferred to the binary vector pBI121 to generate the pBI-ACS-3' untranslated region. *A. tumefaciens* EHA105 was transformed with pBI121-*CmACS-3* by electroporation with the binary vector pBI121-cm neomycin phosphotransferase II, while pBIcdS contained the *CmACS-3* deaminase gene (Figure 2).

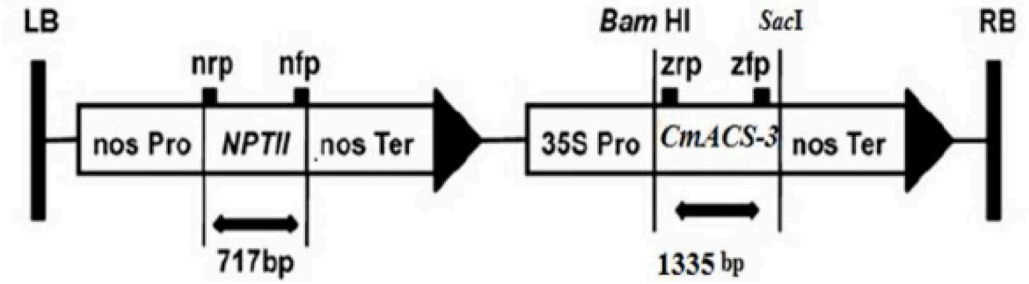

Figure 2. T-DNA construct of the binary vector plasmids used for genetic transformation in melon. EHA105 (pBI121-cm); RB = right border, LB = left border, nptII neomycin phosphotransferase gene, *CmACS-3* = 1-aminocyclopropane-1-carboxylate deaminase, *NPTII* = neomycin phosphotransferase resistance gene.

PCR analyses of the transformants

Genomic DNA was isolated from 0.2 g young leaves of the putative transgenic plantlets and non-transgenic melon plants using the CTAB method (Luan et al., 2008). The presence of the *CmACS-3* gene in the genome of the transformed lines was confirmed by PCR amplification of total DNA samples. Specific primers (5'-GATCCACAGCCTACTGATGATC-3' and 5'-AAGCCACTACTAATCCCACAAT-3') were used to amplify the *CmACS-3* gene. PCR was carried out in 50 μL containing 100 ng DNA, 1 μL of each 10 mM primer, 0.5 μL 10 mM dNTPs, 2.5 μL 10X Taq buffer (Takara, Dalian, China), 1.5 μL 25 mM $MgCl_2$, 1 μL Taq DNA polymerase (Takara), and ddH_2O under the following conditions: 94°C for 5 min, then 40 cycles of 94°C for 30 s, 58°C of annealing for 1 min, 72°C of extension for 1 min, and 10 min for final extension at 72°C. Amplification products were electrophoresed on a 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 1 recognition site in the T-DNA region), transferred from the gel to a nylon membrane using the vacuum method, and hybridized with a digoxigenin-labeled beta (500

base pairs) probe. Hybridization was performed according to the manufacturer instructions for the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer, Ingelheim, Germany). After digestion, 260 mL digestion reaction was loaded into the deep wells of a 1% agarose gel and separated at 30 V for 16-18 h. Washing and detection were performed according to the instruction manual of the DIG labeling and detection system (Roche Diagnostics). To detect the hybridization signals, the membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics) for 1 h.

Scanning electron microscopy

Pollen grains were observed under a fluorescence microscope (Leica, Solms, Germany) coupled to a CCD camera. Three independent experiments were conducted with 200-300 pollen grains per genotype. Scanning electron microscopy was also used to measure stomata. Pre-treatment of the flower was the same as the method used for fluorescence microscopy. In brief, flowers were fixed in a solution containing 2.5% glutaraldehyde and 4% para-formaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 3 days; after rinsing with 0.1 M phosphate buffered saline, the samples were dehydrated, mounted onto standard aluminum stubs for the scanning electron microscopy (Hitachi, Tokyo, Japan), and then sputter-coated with approximately 30-nm gold using a sputter coater (K550, EMITech, Fall River, MA, USA). Images were viewed using an S2400 scanning electron microscope (Hitachi) with an accelerating voltage of 1.5 kV. For statistical analysis of pollen, 3 independent experiments were conducted using 200-300 pollen grains per genotype.

RESULTS

Regeneration of transgenic melon

Examination of the melon leaf showed that melon had some resistance to kanamycin with varying degrees of growth inhibition observed; differentiation of adventitious buds was inhibited in a concentration-dependent manner. Shoot survival decreased at kanamycin concentration of 4000-5000 mg/L, and the growth of new leaves was completely inhibited when the kanamycin concentration exceeded 6000 mg/L. Therefore, we added 6000 mg/L kanamycin to the regeneration medium for selection of transformed cells. Transformation of melon with *A. tumefaciens* EAH105 containing pBI121-cm was performed adopting the optimized method. Lateral and apical buds of the melon were removed at 3 days of germination, and infected with the construct shown in Figure 2. After *Agrobacterium* infection, adventitious shoot differentiation and growth were induced every 2 days in the wound plus adventitious bud formation was induced using liquid drops. Two drops were used each time. After 7-10 days of differentiation, at the developmental point of expansion and adventitious buds differentiation, adventitious buds continued to grow and buds began elongating. When the new leaves showed bud elongation with 2 leaves, kanamycin was applied and the plants were observed for 9 days. Large areas showing a color change

to yellow were considered to be kanamycin-susceptible. We used scissors to discard these negative samples from the base of the plant, until the filter on the same receptor on the plant leaves showed no change in resistance. Smudge-resistant strains to go through for the first time in turn produce different parts of 3 filters, and were considered kanamycin-resistant. Kanamycin-resistant plants were transplanted into soil cultivated in a greenhouse (Figure 1). Following the hardening process, all plantlets from these 30 lines were stable and had normal shoot and root systems in the greenhouse, and thus were used for further studies. The plantlets from individual shoots that were PCR-confirmed and regenerated from explants were selected as plantlets for further assays. The established T_1 transgenic melon lines, which appeared to be morphologically normal, were analyzed using PCR and Southern blotting.

Analysis of transgenic plants

PCR detection of the genomic DNA from the 4 putative transgenic melon lines using the transgene-specific primer pair resulted in an expected 1.7-kb product (Figure 3). The PCR amplified a 0.8-kb product with the NPTT specific primer pair, corroborating this data (data not shown). Southern blotting analysis was carried out using a probe on 2 transplants to verify the homoplasmic condition and transgene integration. Southern blot hybridization analysis revealed 1.7-kb fragments in the transplanted plants, when a DNA blot was probed with the probe-targeting sequence. Fragments were detected in 4 transplant lines and no hybridization signal was detected in non-transformed plants by DNA blot probing (Figure 4). To test the expression of the *CmACS-3* gene, after several subcultures on rooting medium, 10 transgenic plants, 2 from each transgene, were analyzed by Southern hybridization as described above. Variable hybridization patterns were observed among transgenic plants, indicating the expected random integration of the *CmACS-3* gene into the melon genome. Lines 2-6 infected with EHA105 (pBI121-cm) showed a single copy of T-DNA integrated in the plant genome.

In general, the first bisexual flowers occurred earlier in the field (nodes 5-8) in the transformed plants than in the non-transformed plants (Figure 5), likely because of the more favorable environmental conditions, including 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 in the *CmACS-3* transgenic lines compared to non-transformed plants (Figure 5), the first bisexual flowers appeared significantly earlier in the main stem than in the non-transformed plants. The transgenic *CmACS-3* lines also produced a significantly greater number of mature bisexual flowers in the first 5-7 nodes of the main stem (Figure 5). *In vitro* pollen germination experiments showed a low germination rate (Table 1), and transmission electron microscopy revealed 2 main reasons for this, including a reduced number of pollen in anthers and pollen abnormalities (Figure 6). Transgenic pollen abnormalities are caused by pollen wall deformity, and malformation likely results from the defective synthesis of the pollen outside wall. Single small spores were observed in the tapetum of cytoplasmic vacuoles in transgenic plants, which also showed deformities and pollen grains in the vacuole.

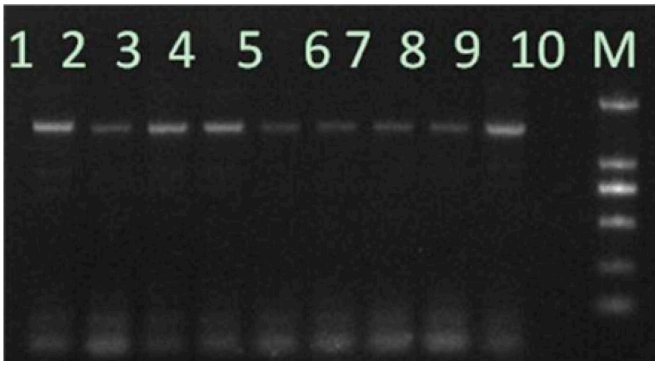

Figure 3. Detection of transgenic plants of *Arabidopsis* by PCR amplification. *Lane M* = molecular markers. *Lane 1* = positive plasmid; *lane 10* = transgenic plants using 35s-PB121 (negative control); *lanes 2-9* = results using transgenic plants.

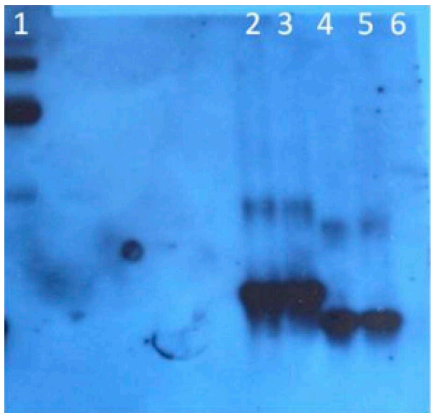

Figure 4. Southern hybridization performed as described in the text. Genomic DNA from each lane was digested with *Eco*RI and probed with *CmACS-3* gene fragment. *Lane 1* = plasmid PBI121; *lane 6* = non-trasformed planted; *lanes 2-5* = transformed plant tests. The bands represent the transgene insertions.

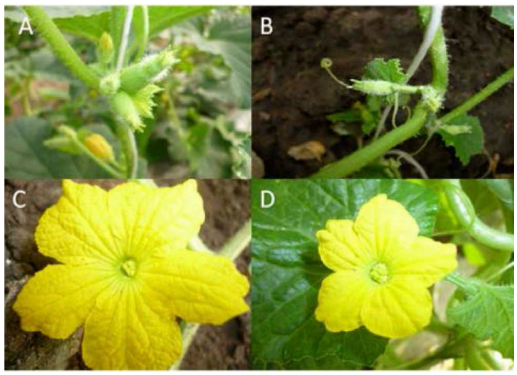

Figure 5. Evaluation of transgenic oriental melon lines carrying the ACS-3 transgene. **A.** Non-transgenic melon CM-23; **B.** transgenic melon CM-23; **C.** non-transgenic andromonoecious lines of bisexual flowers; **D.** female flower of the transgenic melon of the CM-23 andromonoecious line.

Table 1. Comparison of pollen in transformed and untransformed plant lines.

Sample No.	No. of pollen	No. of abnormal pollen	Ratio of abnormality
1	323.33	238.33	76.15 ± 13.87^{B}
2	312.67	241.33	77.23 ± 1.78^{B}
3	318.33	225.00	71.23 ± 4.65^{B}
4	236.33	170.67	72.54 ± 5.95^{B}
CK	396	25	6.32 ± 0.67^{A}

Different superscript letters in the table denote a significant difference.

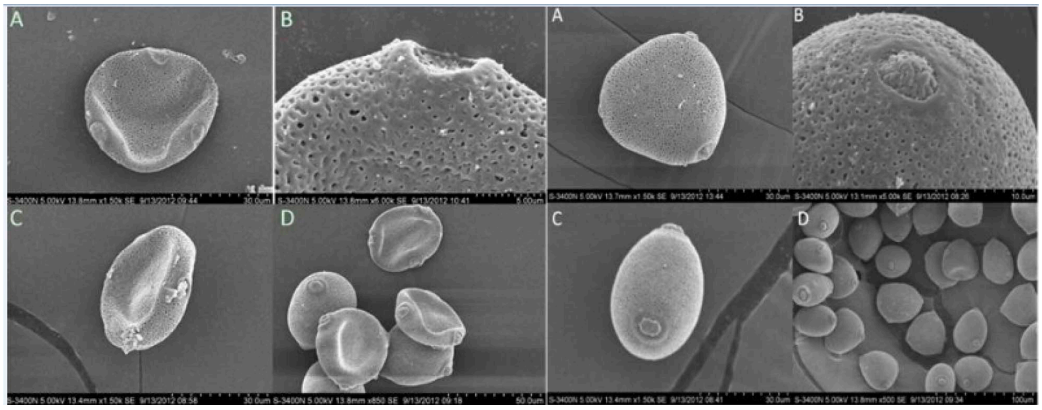

Figure 6. Comparison of pollen in transformation plant and non-transformation plant lines. **a.** Non-transgenic plant pollen. **b.** Transgenic plant pollen. A = pollen polar view; B = local amplification; C = pollen equatorial side; D = pollen grains.

DISCUSSION

Tissue culture methods for transgenic melons require a specific bacteria-free environment, complicated protocols, and are labor-intensive for explants with high processing requirements. Most methods used for culturing of melon explants cause much damage, such as leaf cutting, hypocotyl cutting, damage induced by the air used for culture conditions, an artificially created environment for plant tissue culture to achieve normal growth of the explants with trauma, as well as methods for the differentiation of adventitious buds of explants. Thus, the culture conditions used to reduce trauma of explants are important factors for the efficiency of genetic modification. Because of the diversity of melon genotypes and the specific culture conditions required for different genotypes, transformation of melon genotypes is greatly restricted. Various aspects of the transformation process, such as subculture methods and removal of bacterial contamination, also affect the final transformation efficiency.

Studies aiming to develop a new transgenic method have faced several constraints in maintaining the integrity of melon, and to enable it to grow normally and induce the development of adventitious buds so that transgenic plants can be stably maintained. Because of the specific requirements for culture conditions and the need for maintaining a sterile, bacterium-free environment, creating conditions suitable for the growth of melon is essential for facilitating transformation with *Agrobacterium*. For differentiation, the transformed cells require light and humidity, proper cultivation methods, optimal humidity conditions, and hydroponic liquid nutrient control. The spotting method of infection using liquid including hormone solutions

is one potential method for achieving transgenic plants while maintaining the integrity of the melon and induce the formation of adventitious buds for targeted screening.

Studies examining methods for establishing transgenic melons showed that the time required from infection to the PCR test is 35-40 days, and 3-4 months are required for the entire seed cycle. In addition, a high seed number is required because bud regeneration in melon does not occur from a multiple-cell origin (Bordas et al., 1997; Choi et al., 2012). Thus, to maintain future generations of transgenic plants from the T0 generation will require regular herbicide treatments and filtering of plants to eliminate chimeric plants.

Factors influencing plant reproduction, such as sex determination and floral development, are critical determinants of species survival and crop yield. In cucurbit species, exogenous ethylene and gene expression studies have suggested a key role for ethylene in the sex determination process (Perl-Treves, 1999; Tanurdzic and Banks, 2004). Phenotypic evaluation of transgenic melon plants carrying the *CmACS-3* transgene under control of the constitutive CaMV 35S promoter showed increasing endogenous ethylene production in leaves and flower buds relative to non-transgenic plants. This indicated that increased *CmACS-3* expression resulted in elevated ethylene production in vegetative and reproductive tissues. These results in the 35S:*CmACS-3* melons indicate that ethylene production plays a central role in sex determination and female flower organ development in melon. Constitutive expression of an additional copy of the *CmACS-3* genes in transgenic andromonoecious melon plants induced earlier and increased formation of female flower buds, resulting in earlier fruit setting.

Arrest of stamen development in female flower buds occurs later than the arrest of carpels in male buds (Yamasaki et al., 2001; Bai et al., 2004). In female buds, both stamens and carpels continue to develop during stage 6; the arrest of stamen development occurs at stage 7, just after differentiation between the anther and filament of the stamen (Bai et al., 2004). Thus, suppression of stamens may occur in carpel-bearing flowers if ethylene levels are sufficiently high or if the stamen (or petal) primordia are sufficiently receptive. At sufficiently high concentrations, exogenous ethylene has been shown to inhibit stamens in andromonoecious melons. Overexpression of *CmACS-3* in stamens in the flowers may result from elevated levels of ethylene produced by the flower buds. Thus, ethylene produced in the stamens/petals would allow for initial carpel development; the elevated ethylene could, in turn, subsequently inhibit stamen development.

This suggests that ethylene perception plays a positive role in the sustained maturation of the carpel and provides support for the observations made in transgenic melon constitutively overexpressing *CmACS-3*, and further suggests that ethylene enhances carpel maturation (Papadopoulou et al., 2005). The 35S:*ACS-3* melon plants exhibited increased ethylene production, had an earlier and increased production of carpel-bearing buds, and a marked increase in the proportion of carpel-bearing buds that reached anthesis. These results are consistent with inhibited ovary and carpel development observed on bisexual melon flowers or female zucchini flowers following inhibition of ethylene production or perception (Payán et al., 2006). Thus, ethylene may have independent functions for sex determination and promotion of subsequent maturation of carpel bearing buds. This is an interesting and unexpected effect of the *ACS* gene on flower bud maturity. The first bisexual bud to reach maturity, rather than abscising prior to anthesis, occurred earlier on the transgenic plants, and a higher total number of bisexual buds on the main stem reached maturity. The early appearance and increase in mature bisexual flowers did not result from earlier bisexual bud formation, as there was a reduced delay in the number of nodes from the first bisexual bud to the first mature bisexual flower on

the *CmACS-3* plants, and a higher percentage of bisexual buds on the *CmACS-3* plants reached maturity. These phenotypes were observed in both the greenhouse and in the field, suggesting that ethylene may be important not only for promoting pistil development at the time of sex determination but also for sustaining further development of the female organ.

Transgenic plants showed normal vegetative growth, with a low amount of pollen on the anthers and not on the set fruit. Thus, *CmACS-3* may be expressed during the development of the tapetum in the later stage of meiosis and serve a regulatory function in the proliferation of the tapetum cells to help maintain normal growth of the pollen. In conclusion, we demonstrated the feasibility of transformation of melon and adventitious shoot induction by using 6-BA and selecting transformed shoots in the presence of kanamycin. All of the germinated seedlings of melon were used as explants. The transformation system maintained the integrity of melon, thereby overcoming the limitations of traditional tissue culture. The transformation was conducted in a non-sterile environment. Using this procedure, fertile transgenic plants generating transgenic progenies were also obtained. These results provide further support for a dual role of ethylene in both sex determination and subsequent carpel maturation.

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