

A Single Insertion of ACC Oxidase Gene in Antisense Orientation Extends the Shelf Life in Muskmelon 'Galia' Hybrid Parental Line (*Cucumis melo* L. var. *reticulatus* Ser.)

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Abstract

'Galia' muskmelon is an Israeli melon hybrid, which has been the mainstay in much of the European market for more than 30 years. 'Galia' melons are harvested near peak maturity, which limits shelf life to two weeks or less. The male parental line of 'Galia' was transformed with the ACC oxidase gene in antisense orientation. A post-harvest evaluation of T₀ fruits from two completely diploid independent transgenic lines is described herein. Explants were transformed using *Agrobacterium tumefaciens* strain ABI, which contained a vector pCmACO1-AS plasmid, bearing an antisense gene of CMACO-1 and the CP4 *syn* gene (glyphosate-tolerance). Both CMACO-1 and CP4 *syn* genes were assessed by a polymerase chain reaction method. Flow cytometry analysis was performed to determine plant ploidy level of primary transformants. Two completely diploid independent transgenic lines were obtained, which were named TGM-AS-1 and TGM-AS-2. Southern blot and segregation analyses in the T₁ generation determined that each independent transgenic line had one single insertion of the transgene. Several post-harvest parameters were evaluated in transgenic (TGM-AS) and wild type (WT) fruits. WT fruits were harvested at 37, 42 and 50 days after pollination (DAP), whereas TGM-AS fruits were harvested at 42, 50 and 56 DAP. The harvest of TGM-AS fruits was delayed since they ripened slower than WT. Fruit weight, length, width, soluble solids, titratable acidity, pH, firmness, flesh thickness, seed cavity size and seed number parameters were not significantly different between the two genotypes at 42 and 50 DAP. ACC oxidase activity from 42 DAP TGM-AS fruits was three times less than WT fruits. Likewise, ethylene production from 42 DAP TGM-AS fruits was reduced 80% compared to WT. TGM-AS T₀ 'Galia' male parental line fruits had a delayed fruit ripening process by up to 12 days compared with WT fruits.

INTRODUCTION

'Galia' muskmelon was bred at the Newe Ya'ar research center and was the first Israeli melon F₁ hybrid released (Karchi, 2000). This muskmelon has the green-flesh characteristics of 'Ha'Ogen' type melon, which was used as the female parental line, and netted rind from 'Krymka' cultivar, which was used as the male parental line. Its exceptional characteristics such as yields from 35-50 t/ha in the field, excellent fruit quality with 13-15% total soluble solids (TSS), and bold flavor and aroma, increased Galia's local market popularity within few years. This commodity has been the mainstay in the European market for more than 30 years (Karchi, 2000). One disadvantage for this cultivar is its storage life, which is limited to two to three weeks. Timing of harvest is critical for 'Galia' fruit, because in order to develop peak flavor and aroma the melon should be picked at maturity (Karchi, 2000). Traditional breeding methods using long-shelf life melons have been used to develop 'Galia' muskmelons with a long-shelf life. However, this approach can result in a loss of favorable fruit quality characteristics.

Plant biotechnology has the potential to genetically transform plants and transfer novel characteristics. RNA antisense technology has permitted regulating the expression

of specific genes involved in fruit ripening in tomato (Chen et al., 1996) and melon (Ayub et al., 1996; Guis et al., 1997; Silva et al., 2004). Therefore, increasing 'Galia' muskmelon's shelf life by this approach is a feasible alternative. In the present study, experiments were conducted to compare fruit quality characteristics between transgenic ACC oxidase antisense (TGM-AS), azygous-AS (escapes), transgenic GUS (T-GUS) and wild type (WT) fruits from plants grown in greenhouse conditions.

MATERIALS AND METHOD

Plant Material

'Galia' muskmelon male (*Cucumis melo* L. var. *reticulatus* Ser. cv. 'Krymka') parental line transgenic plants, harboring either an ACC oxidase antisense (*CMACO-1*) (TGM-AS) or GUS (*uidA*) (T-GUS) gene, were obtained by a cotyledon-protocol previously described in Nunez-Palenius et al. (2005a). In vitro non-transgenic-AS 'escape' seedlings were obtained after *Agrobacterium tumefaciens*-mediated transformation was carried out. These plants were called azygous-AS to emphasize the absence of the ACC oxidase antisense transgene and they were used as control plants. Wild type (WT) muskmelon plants coming from seeds were used as control plants as well.

Detection of Transgenes and Southern Blot Assay

Total DNA isolation from wild-type and putative transgenic melon leaflets was performed using a modified CTAB protocol (Doyle and Doyle, 1987, 1990). Both, PCR assay of putative transgenic plants and flow cytometry analysis of PCR-positive plants were carried out by means of protocols previously described (Nunez-Palenius et al., 2005b). A Southern blot hybridization assay, using the *CP4 syn* gene PCR-product as a probe, was carried out in PCR-positive and -negative T₁ seedlings according to Nunez-Palenius et al. (2005a).

Production of Melon Plants in the Greenhouse

Plants were grown inside an evaporative-cooled fan and pad glasshouse in Gainesville, FL, 28°C day and 20°C night. Plants were grown in plastic pots (11.3 L) filled with soilless media (COURSE-grade perlite) and following common growing practices recommended by Rodriguez and Cantliffe (2001). Complementary light was supplied by Metalarc^R lamps with a light intensity of 350-530 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 18 h per day. Training, pruning, and fertigation of plants, as well as application of fungicides, were performed according to those recommended by Rodriguez and Cantliffe (2001).

An integrated pest management program (IPM) was used to keep pests under control (<http://www.hos.ufl.edu/protectedag/>).

In order to increase plant numbers, lateral branch cuttings from each T₀ transgenic and non-transgenic lines were rooted by exogenous application of rooting hormone (0.1% indole-3-butyric acid, Sigma, Co.) on severed stem bases. Ploidy was confirmed by flow cytometry analysis according to the procedure described in Nuñez-Palenius et al. (2005a). Only diploid plants were used to set fruit. Non-transformed WT and azygous-AS, and T-GUS 'Galia' male parental plants were used as controls.

A sample of 10 fruit from wild type, azygous-AS and T-GUS were harvested at 37, 42 and 50 days after pollination (DAP), corresponding to zero-, half- and full-slip developmental stages, respectively. TGM-AS-1 and TGM-AS-2 fruits were harvested at 42, 50 and 56 DAP, corresponding to zero-, half- and full-slip developmental stages, respectively.

Postharvest Evaluation

Thirteen post-harvest parameters, such as weight, length, width, flesh size, seed cavity size, soluble solids, titratable acidity, pH, seed number, ethylene evolution, ACC oxidase activity in vivo, rind color and firmness, were evaluated in T₀ transgenic (TT) and wild type (WT) fruits according to protocols previously described (Nunez-Palenius et al., 2005b).

RESULTS AND DISCUSSION

Lateral branch cuttings from TGM-AS, T-GUS, and Azygous-AS plants rooted extensively and developed healthy plants after exogenous application of 0.1% indole-3-butyric acid (rooting hormone) on severed stem bases (Fig. 1). Flow cytometry analysis was performed on leaves to determine plant ploidy level (data not shown), and only completely diploid plants were used to set fruit. Overall, muskmelon genotype did not have an effect on rooting and development of diploid muskmelon plants.

PCR assays aided in detecting the presence of *CP4 syn* and ACC oxidase antisense genes in TGM-AS plants, as well as the presence of *uidA* gene in T-GUS plants (Fig. 2). Using that PCR assay we were able to observe the existence of 1.4, 0.5, and 0.3 kb bands which belonged to the *CP4 syn*, native ACC oxidase and engineered ACC oxidase genes respectively in TGM-AS plants, and the presence of a 0.75 Kb fragment, which belonged to the GUS gene. In summary, transgenic plants were identified by means of PCR assay.

A segregation analysis on T₁ muskmelon seedlings was performed for TGM-AS-1 and TGM-AS-2 lines in order to reveal the copy number of the inserted ACC oxidase antisense gene into the transgenic lines and to determine the transgene distribution inheritance pattern. On a population of 62 T₁ plants (Fig. 4) randomly chosen from each transgenic line, TGM-AS-1 and TGM-AS-2, DNA was extracted individually, at least three times, and a PCR assay was carried out. The observed distribution of the *CMACO*-antisense and *CP4 syn* genes was consistent with a 3:1 ratio (X^2 value for TGM-AS-1 and TGM-AS-2 was 0.193 and 0.021, with a probability of 66% and 88%, respectively), which corresponded to the segregation of one single copy insertion of T-DNA. Moreover, the southern blot assay supported that one single copy insertion of T-DNA was present in both TGM-AS lines (data not shown).

A thorough evaluation was carried out for TGM-AS, WT, T-GUS and Azygous-AS fruits, in order to compare their performance concerning thirteen different postharvest parameters. In order to simplify results the WT, T-GUS and Azygous-AS fruits were considered as WT. Among those postharvest parameters, the seed number (full and empty) is very important, because 'Krymka' muskmelon cultivar is highly susceptible to develop 'empty seeds' (Fig. 3) when the average minimum-night temperature is lower than 12°C (Rylski and Aloni, 1990) or the 'Admire' insecticide is applied to muskmelon plants (Nunez-Palenius et al., unpublished results). The greatest number of empty seeds was observed in Azygous-AS at 42 DAP, consequently, the lowest number of full seeds was recorded in that genotype and date (Table 1). Conversely, TGM-AS-2 on 50 DAP had the greatest number of full seeds. Finally, no significant differences were observed among all different treatments for full and total seed number by Tukey's mean separation test (data not shown). It seems that the ACC oxidase antisense gene did not have a significant effect on full and total seed number in fruits for both TGM-AS-1 and TGM-AS-2 lines. Fruit weight, length, width, soluble solids, titratable acidity, pH, firmness, flesh thickness, and seed cavity size parameters were not significantly different between the two genotypes at 42 and 50 DAP (data not shown). ACC oxidase activity from 42 DAP TGM-AS fruits was three times less than WT fruits. Likewise, ethylene production from 42 DAP TGM-AS fruits was reduced 80% compared to WT (data not shown). TGM-AS T₀ 'Galia' male parental line fruits had a delayed fruit ripening process by up to 12 days compared with WT fruits.

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Tables

Table 1. Percentage of empty and full seeds in 'Krymka' muskmelon fruits.

Genotype	DAP ^z	Empty seed (%)	Full seed (%)
Wild type	37	15.0 ± 3.8 ^y	85.0 ± 3.75
	42	15.3 ± 10.0	84.7 ± 9.97
	50	16.6 ± 6.5	83.4 ± 6.51
TGM-AS-1	42	24.1 ± 18.6	76.0 ± 18.58
	50	22.4 ± 17.1	77.6 ± 17.07
	56	21.6 ± 14.5	78.4 ± 14.48
TGM-AS-2	42	17.4 ± 10.1	82.6 ± 10.07
	50	10.3 ± 6.2	89.7 ± 6.17
	56	20.6 ± 12.3	79.4 ± 12.28
Azygous-AS	37	14.4 ± 10.2	85.6 ± 10.19
	42	30.4 ± 24.5	69.6 ± 24.48
	50	16.5 ± 10.2	83.5 ± 10.22
T-GUS	37	24.1 ± 12.9	75.9 ± 12.89
	42	11.3 ± 3.5	88.7 ± 3.54
	50	16.4 ± 14.5	83.6 ± 14.46

^zDAP: days after pollination.

^yData are the average of ten fruits ± SE.

Figures

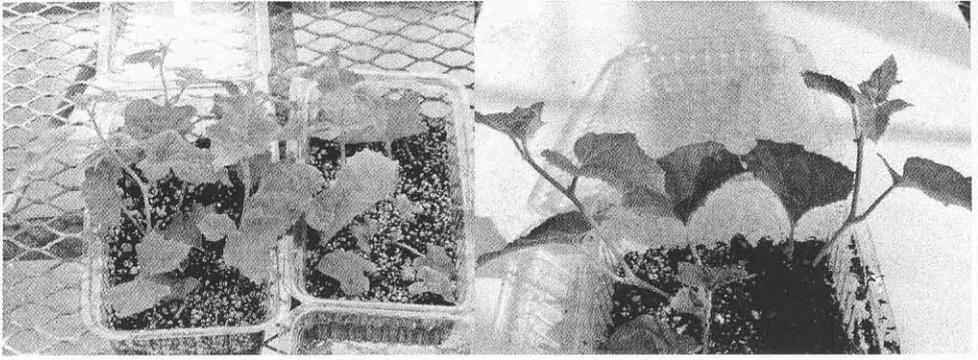


Fig. 1. Healthy muskmelon plants obtained after rooting of lateral branch cuttings from TGM-AS, T-GUS and Azygous-AS plants.

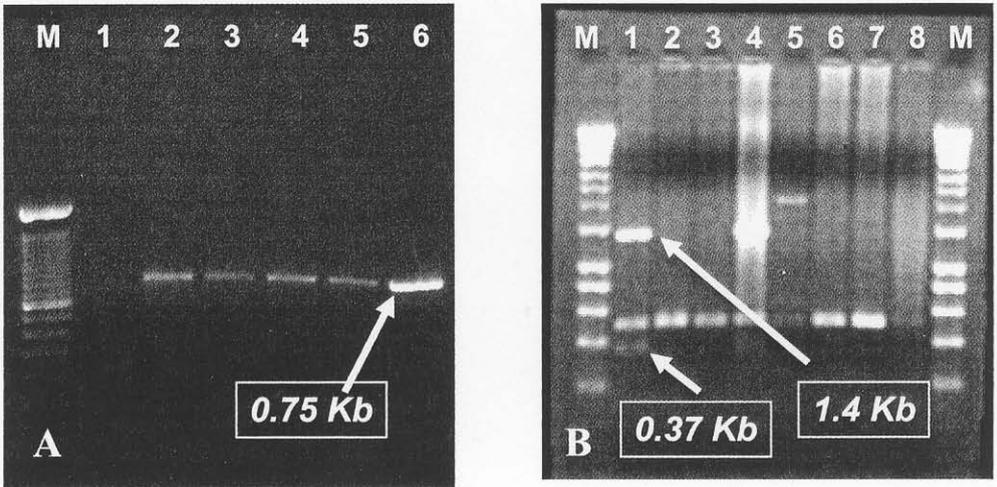


Fig. 2. PCR assay for putative transgenic 'Galia' muskmelon male line plants. A. The amplification product for *uidA* gene was 0.75 Kb. M: 100 bp Ladder, 1: negative control (DNA from wild type melon plant), 2 and 3: DNA from 'Galia' male GUS positive plant, 4 and 5: DNA from 'Galia' female GUS positive plant, 6: positive control (DNA from GUS plasmid). B. The amplification product for *CP4syn*, native ACO-1, and engineered ACO-1 genes were 1.4 Kb, 0.5 Kb, and 0.3 Kb, respectively. M: HyperLadder (Biolone), 1 through 6: putative transgenic plants, 7: DNA from negative plant, 8: PCR reaction mixture.



Fig. 3. 'Krymka' muskmelon 'empty seeds' were obtained after melon seedlings were treated with 'Admire' to control insect pests. Notice how the testa is completely developed but embryo abortion has occurred.

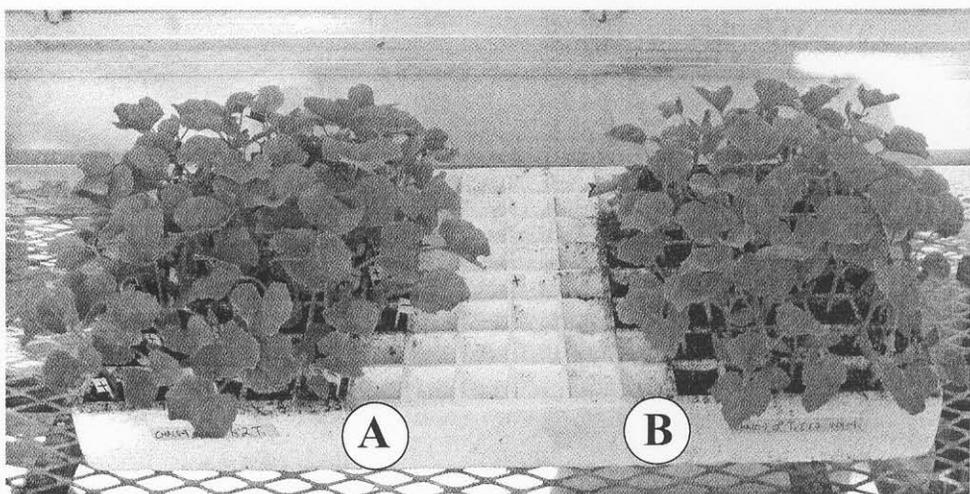


Fig. 4. TGM-AS-1 (A) and TGM-AS-2 (B) seedlings were used to extract DNA and run a PCR assay to determine the transgene distribution inheritance pattern.