# GENETIC ENGINEERING OF BETA-CAROTENE PRODUCTION IN HONEYDEW MELONS (*CUCUMIS MELO* L. *INODORUS*)

A Dissertation

by

YAN REN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Horticulture

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# ABSTRACT

Genetic Engineering of Beta-Carotene Production in Honeydew Melons (*Cucumis Melo* L. *inodorus*). (December 2011)

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Genetic transformation is a useful tool to incorporate novel genes, potentially allowing sexual incompatibility and interspecific barriers to be circumvented. The purpose of this study was to improve β-carotene levels in melon fruits by transferring a phytoene synthase (*PSY*) gene. At present, there are not sufficient regeneration and transformation studies reported on two commercially important melon types - western shipper cantaloupe and honeydew.

To establish a high efficiency shoot regeneration system, we evaluated three types of explants in our elite breeding lines. A shoot tip with a hypocotyl and cotyledon fragments, regenerated shoots whereas a shoot tip with a hypocotyl without cotyledon, did not produce regenerants. Murashige & Skoog (MS) basal medium with 1 mg $l^{-1}$ benzyladenine (BA), 0.26 mg $l^{-1}$ abscisic acid (ABA) and 0.8 mg $l^{-1}$ indole-3-acetic acid (IAA) was the best for regeneration from cotyledon explants in cantaloupe 'F39'. MS basal medium with 1 mg $l^{-1}$ BA and 0.26 mg $l^{-1}$ ABA was chosen for honeydew '150' to

solve a curving-up problem of explants. Fifty to sixty percent of regenerants were found to be polyploids.

To establish a reliable *Agrobacterium*-mediated transformation protocol, kanamycin sensitivity as well as Timentin™ and Clavamox® were evaluated. Kanamycin 200 and 150 mg $l^{-1}$ were chosen as the threshold levels for 'F39' and '150' respectively. No significant differences were found between Timentin™ and Clavamox® in 'F39'; however, Clavamox® reduced the incidence of vitrification and increased the frequency of shoot elongation in '150'. *A. tumefaciens* strain EHA105, harboring pCNL56 carrying *nptII* and *gusA* genes, was used to establish a transformation protocol. The transformation efficiency was 0.3% from 'F39' and 0.5% from '150'.

We introduced a watermelon *PSY-C* gene under the control of a fruit-specific promoter of a polygalacturonase gene into '150'. All the transgenic plants were tetraploids based on flow cytometry assays. Up to 32-fold of β-carotene was elevated in the rind tissue of transgenic honeydew including phytoene increase. This is a very promising result for a further investigation to increase β-carotene level in flesh tissue using the *PSY-C* gene with an appropriate promoter.

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# NOMENCLATURE

| | |
|---|---|
| ABA | Abscisic acid |
| BA | 6-Benzyladenine |
| HPLC | High performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| MS | Murashige and Skoog |
| NAA | Naphthaleneacetic acid |
| *nptII* | Neomycin phosphotransferase II |
| PCR | Polymerase chain reaction |
| GUS | β-Glucuronidase |

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## Introduction

Melon (*Cucumis melo* L.) is a group of high-value crops including cantaloupe (muskmelon), honeydew and casaba melon. This species has seven botanical variants: cantalupensis, reticulatus, inodorus, flexuosus, conomon, chito and dudaim. Only reticulatus and inodorus variants are commercially important in the United States. Cantaloupe is one of the most-consumed melons in the USA (about 50 kg per person per year (Flores 2005). Western-shipper cantaloupe (*Cucumis melo* var. *reticulatus*) refers to the type of cantaloupes originally grown in western states and shipped throughout the country. They usually have uniformly netted rinds, orange flesh and lack sutures. Nowadays, they are already adapted and grown all over the United States (Boyhan et al. 2009). Honeydews (*Cucumis melo* var. *inodorus*) usually have green or cream-colored rinds with pale-green flesh, and have been widely grown in the USA. New varieties of honeydews with orange flesh, which were created from a cross between netted orange-fleshed cantaloupe and non-netted green-fleshed honeydew, have been less popular in melon production (Lester 2008; Hodges and Lester 2006). As a large producer and consumer of melon, the United States has been involved in the improvement of melon

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This dissertation follows the style of Plant Cell, Tissue and Organ Culture.

quality to help increase melon profits and achieve more nutritional value for consumers. Cantaloupe and orange-fleshed honeydew are good sources of β-carotene, potassium and vitamin C. Pale green-fleshed honeydew lacks β-carotene but is rich in potassium, vitamin C and folate (data from online USDA National Nutrient Database for Standard Reference). In the past few years, melon breeders have worked on improving the nutritional quality of melon. "Consumers will get not only a better tasting, sweeter melon, but one that can help boost their intake of β-carotene and vitamin C," said Dr. Gene Lester, who has been working at the USDA in Weslaco since 1983 (Flores, 2005).

Generally, β-carotene is the prevalent carotene in yellow, orange, and green leafy fruits and vegetables, such as carrots, pumpkin, spinach, lettuce, pepper, sweet potatoes, broccoli, muskmelon, citrus, oranges, and winter squash (USDA National Nutrient Database for Standard Reference, Release 21). β-carotene is a natural food source of pro-vitamin A which may contribute to the prevention of some diseases such as eye problems, cardiovascular disease, and several cancers (Fraser et al. 2004). Heart disease and cancer have been identified as the top two diseases causing death in Americans. Therefore, consumption of foods rich in β -carotene is being recommended by the US National Cancer Institute and the USDA.

Although β-carotene accounts for 90% of the carotenoids found in orange-fleshed cantaloupe fruit (Karvouni et al. 1995), compared with other vegetables and fruits, its β-carotene content (20.2 μg $g^{-1}$ FW) is not considered to be very high. Moreover, pale green-flesh honeydew's β-carotene content is very low (0.3 μg $g^{-1}$ FW). Conventional plant breeding has been very successful at increasing productivity but has

focused less on improving the levels of health-promoting phytochemicals such as carotenoids. Genetic engineering (often termed genetic modification or genetic manipulation) is a faster and targeted method to transfer gene(s) compared with conventional breeding (Fraser et al. 2004). It is a useful procedure to incorporate novel genes within and across plant species. Sexual incompatibility and interspecific barriers to traditional plant breeding can therefore be circumvented. Several successful genetic engineering attempts to enhance carotenoids have been reported in crop plants including tomato (Fraser et al. 2001; 2002; Romer et al. 2000; Rosati et al. 2000; Ronen et al. 2000; Ralley et al. 2004; D'Ambrosio et al. 2004), carrot (reviewed from Fraser and Bramley 2004), rice (Ye et al. 2000; Paine et al. 2005), canola (Shewmaker et al. 1999), tobacco (Misawa et al. 1994; Ralley et al. 2004; Mann et al. 2000), lotus (Suzuki et al. 2007), *Arabidposis* seeds (Lindgren et al. 2003; Stalberg et al. 2003) and potato (Lopez et al. 2008; Diretto et al. 2007; Gerjets and Sandmann 2006; Ducreux et al. 2005). This study was undertaken to investigate the possibility of elevating β-carotene levels in commercially valuable melons via genetic transformation.

## Literature Review

### Melon *in vitro* culture

*In vitro* melon regeneration protocols have been reported in the past 25 years. Several factors impact regeneration efficiency, including genotype, explant type, and plant growth regulators (reviewed by Nuňez-Palenius et al. 2008). The main regeneration

pathways are adventitious shoot organogenesis and somatic embryogenesis. Adventitious shoot organogenesis has been used most often in tissue culture. The highest regeneration frequency of melon cotyledon can reach 100% (Ficcadenti and Rotino 1995). In the last decade, somatic embryogenesis has become a popular method for *in vitro* culture because it solved the problem of obtaining chimeric transformants (Akasaka-Kennedy et al. 2004), and it is an efficient method for production of diploid plants (Guis et al. 1997a). Akasaka-Kennedy et al. (2004) reported that an average of 116.7 and 130 of embryos were induced from each seed of Vedrantais and Earl's Favourite Fuyu A melon, respectively. Two weeks after being transferred to liquid MS medium without plant growth regulators, 75.9% and 23.3% of the embryos germinated to produce plants with shoots and roots.

Genotype is the most important determinant of melon regeneration ability. Galperin et al. (2003) screened 30 genotypes on 3 different media, including wild landraces, breeding lines and commercial cultivars. Twenty four out of 30 genotypes regenerated abnormal shoots while 5 genotypes had low frequency of regeneration. Only one genotype 'BU-21' showed a distinguished 100% regeneration frequency accompanied by normal shoot development. Gray et al. (1993) used 51 commercial varieties to test embryogenic regeneration frequency of cotyledons. The highest responding varieties reached 100% regeneration efficiency with 20 embryos per explant, while regeneration in the lowest responding varieties was only 5% with 0.1 embryos per explant. This genotype-dependence phenomenon is normal for melon *in vitro* culture,

therefore establishing an efficient regeneration system for genetic transformation of a specific melon cultivar is a necessary and important step.

Plant growth regulators have been used in melon tissue culture to optimize shoot regeneration. Both auxins and cytokinins are known to be essential for bud/shoot induction and the optimal auxin/cytokinin levels, critical for recovery of plants, are often genotype specific. The auxin indole-3-acetic acid (IAA) and cytokinin 6-benzyladenine (BA) are commonly used in organogenesis studies. The synthetic auxin 2,4-dichlorophenoxyacetic acid has been widely used to induce somatic embryogenesis (Akasaka-Kennedy et al. 2004; Gray et al. 1993; Guis et al. 1997a; Kintzios et al. 2004; Kintzios and Taravira 1997; Oridate and Oosawa 1986). In addition, 6-(γ,γ-dimethylallylamino)-purine (2iP), gibberellic acid ($GA_3$), kinetin, thidiazuron and α-naphthaleneacetic acid (NAA) have been used in melon culture (Fang and Grumet 1990; Ficcadenti and Rotino 1995; Guis et al. 2000; Souza et al. 2006; Yadav et al. 1996). The effects of these growth regulators on melon regeneration differed depending on plant genotype, explant type and culture conditions. Other media components also affect melon regeneration efficiency. For example, abscisic acid (ABA) and sucrose have been shown to enhance somatic embryogenesis in melon (Nakagawa et al. 2001). The anti-gibberellin analogue, ancymidol, was reported to promote shoot regeneration from cotyledonary explants of 'Galia' melon in combination with a low concentration of BA (0.44 μM), while the addition of $GA_3$ to this medium reduced the regeneration frequency 12-fold after 13 d of treatment (Gaba et al. 1996).

Different explant types have been used in melon regeneration including mature seed, cotyledon, hypocotyl, proximal zone of the hypocotyls, petiole, leaf, root, protoplast and shoot tip. All these explants proved to be able to regenerate shoots through either organogenesis or somatic embryogenesis. Cotyledon is the most-widely-used explant type for melon regeneration and transformation. Explants were excised from cotyledons at different ages such as unexpanded cotyledons on immature seeds (Adelberg et al. 1994) and mature seeds (Ezura and Oosawa 1994), as well as expanded cotyledons on seedlings. Age of the seedlings also varied from 2-day-old to 2-week-old (Nunez-Palenius et al., 2008). Usually, physiologically younger tissues were preferred for *in vitro* culture (Smith 2000). Molina and Nuez (1995) compared regeneration abilities from leaf, cotyledon and hypocotyl explants in three melon populations. They concluded that different types of explants had different regeneration capacities, which is controlled by a common genetic system. In their research, cotyledon and leaf explants had a similar regeneration frequency which was always higher than hypocotyl explants.

Shoot tip can be considered as an alternative explant type for melon regeneration (Ezura et al. 1997b) and transformation for three reasons. First, the shoot apical meristem is a potential target for direct gene transfer. Second, compared to plant regeneration from protoplasts, callus cultures, or directly from the explants via adventitious organogenesis or somatic embryogenesis, shoot apex has the potential to maintain cultivar integrity by escaping from culture-induced mutations (Park et al., 1998). Third, melon plants regenerated from cotyledons have a high tendency (approximately 80%) towards tetraploidy in tissue culture and genetic transformation of

melon using cotyledonary explants (approximate 80%) (Guis et al. 2000; Nuňez-Palenius et al. 2006 and 2008). On the other hand, plant regenerated from shoot primordium had a much lower frequency of tetraploidy (4%) (Ezura et al. 1992 and 1997b). Shoot primordium culture might be critical for shoot apex transformation. Tylicki et al. (2007) and Ogawa et al. (1997) indicated shoot primordia culture was an efficient system for maintaining genetic stability and a good system for transformation in melon.

Polyploidy is a common problem in melon *in vitro* culture. More than 80% of melon plants regenerated from 2-day-old cotyledon explants were tetraploids, whereas only 15% of the regenerated plants from leaf explants were tetraploids. Tetraploid melon has some phenotypic changes including short internodes, smaller fruit size, flatness of fruit and reduced productivity, which impede marketability due to the low fruit quality (Guis et al. 2000). Compared with plants regenerated from somatic embryos and adventitious shoots via nodes of elongated seedlings, those regenerated from shoot primordia had a much lower frequency of tetraploidy: somatic embryo 31%, adventitious shoots 30%, shoot primordial 4%; and the clonally propagated plants from axillary buds didn't produce tetraploidy (Ezura et al. 1992), which indicated the explant source as an important factor in reducing the number of tetraploid plants obtained from melon *in vitro* culture. Ezura et al. (1997b) examined the ploidy levels of shoot primordium cells and plants regenerated from the shoot tips. Both tissues were very stable in terms of ploidy, and the culture time (from 1 to 6 years) did not affect ploidy level. But Kathal et al. (1992) found that the polyploidy percentage of melon plants regenerated from leaf

explants increased as the time of *in vitro* culture increased. These opposing results indicate that polyploidy in tissue culture could be induced by many unknown factors. Further research on this phenomenon will be necessary to reduce this problem in melon *in vitro* culture.

In Chapters II and III, we evaluated the responsiveness of different explant types of our elite breeding lines on different regeneration media which have been reported to be optimal for different melon genotypes. Then we calculated the percentage of polyploidy in honeydew '150' regenerants, and observed the corresponding morphologies.

Genetic transformation in melons

In the past 20 years, melon transformation research has been mainly focused on two *C. melo* variants (*cantalupensis* and *reticulatus*) for disease resistance and longer shelf life. The genes used for these purposes included cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV) coat protein genes (Yoshioka et al. 1992; Fang and Grumet, 1993; Gonsalves et al. 1994; Clough and Ham, 1995; Fuchs et al. 1997; Yalçın-Mendi et al. 2004; Wu et al. 2009); and an antisense ACC oxidase gene (Ayub et al. 1996; Clendennen et al. 1999; Ezura et al. 1997a; Guis et al. 1997b, 2000; Shellie, 2001; Silva et al. 2004; Nuňez-Palenius et al. 2006; Hao et al., 2011). Increasing sugar content is another target trait for genetic engineering of melon. Two genes have been studied for regulating sucrose biosynthesis, which were an antisense acid invertase gene (anti-*MAI*1) (Fan et al. 2007) and an antisense sucrose

phosphate synthase gene (Tian et al. 2010). Yalçın-Mendi et al (2004) reported an *Agrobacterium*-mediated transfer of a ZYMV coat protein gene to a Turkey *inodorus* variant melon, cultivar "Kirkagac 637". They identified the frequency of gene escape and fruit quality characteristics from two transgenic lines (Yalçın-Mendi et al. 2010).

Despite the development of genetic transformation protocols for melon reported in the last two decades, transformation remains genotype-dependent and efficiencies are relatively low (0-12.5%) (Nuňez-Palenius et al. 2006; 2008). To date, the most successful methods for producing transgenic melon have been achieved in French (Fang and Grumet 1990), Israeli (Nuňez-Palenius et al. 2006) and Asian germplasm (Dong et al. 1991; Wu et al. 2009), which constitute a low percentage of the US consumer market. Successful transformation protocols are needed for improvement of commercial genotypes grown in the US, especially the western shipper cantaloupe (*C. melo* var. *reticulatus*) and honeydew (*C. melo* var. *inodorus*).

A review of pertinent literature reveals only a few studies on the tissue culture or transformation of western shipper and honeydew melons. At this writing, there were no transformation studies available for honeydew melon; however, a number of studies on shoot regeneration in culture have been reported (Ficcadenti and Rotino 1995; Keng and Hoong 2005; Kintzios and Taravira 1997; Oridate et al. 1992; Orts et al. 1987). In western shipper cantaloupe, only one microprojectile mediated transformation (Gonsalves et al. 1994) and two *Agrobacterium*-mediated transformations (Clough and Ham 1995; Fuchs et al. 1997) were reported.

*A. tumefaciens*-mediated transformation has become the method of choice for melon transformation (Nuňez-Palenius et al. 2008). We opted to use *Agrobacterium* due to reports of low copy gene transfers, which help to reduce the chances of multi-gene triggered silencing in the transgenic plants. Unfortunately, previously reported procedures provided insufficient detail for us to replicate. Though various *A. tumefaciens* strains are available such as LBA4404, EHA105, ABI, C58B707, C58C1Rif® and GV3111SE, the strain LBA4404 was used in nearly half of melon transformation reports (Fang and Grumet 1990; Yoshioka et al. 1992; Fang and Grumet 1993; Vallés and Lasa 1994; Ayub et al. 1996; Bordas et al. 1997; Guis et al. 2000; Silva et al. 2004; Taler et al. 2004). In this study, we compared strains LBA4404 and EHA105 for their transformation efficiency.

Based on the regeneration system established for cantaloupe 'F39' and honeydew '150', we conducted kanamycin sensitivity assays to determine the concentration of the antibiotic for selecting transformed shoots. Furthermore, the effects of two antibiotics, Clavamox® and Timentin™, were compared on shoot regeneration. In the optimization of producing transformed shoots, we have studied the effect of light condition during co-cultivation. This has been neglected in designing transformation systems in melon but has proven to be important in other plant species such as *Phaseolus acutifolius* and *Arabidopsis thaliana* (Zambre et al. 2003). In addition, two strains of *A. tumefaciens*, EHA105 (Hood et al. 1993) and LBA4404 (Hoekema et al. 1983), were tested to see whether there were differences in their efficiency in producing transformed plants. Melon regeneration and transformation are still considered to be difficult due to several

factors such as: strong genotype dependence, a high percentage of polyploidy, high rates of 'escapes' and aberrant shoot development (Castelblanque et al. 2008; Chovelon et al. 2008; Dong et al. 1991; Akasaka-Kennedy et al. 2004; Wu et al. 2009). The content of Chapter IV describes establishment of a reliable genetic transformation system in these two elite breeding lines with commercial qualities.

## Carotenoid biosynthesis and regulation in plants

Carotenoids are pigments *de novo* synthesized by all photosynthetic organisms (higher plants and algae) and many non-photosynthetic organisms (some bacteria and fungi) (Bartley and Scolnik 1994). There are a series of gene expressions related to carotenoid biosynthesis controlling carotenoid accumulation. These gene expressions occur in the chloroplasts (photosynthetic tissues such as leaves) and in chromoplasts (nonphotosynthetic plant tissues such as fruits and flowers). Carotenoids in plants are isoprenoids formed from isopentenyl diphosphate (IPP) via the mevalonate-independent (MVA-independent) pathway in the plastid (Li et al. 2006). Figure 1 shows that Geranylgeranyl diphosphate (GGPP) deriving from IPP is the precursor of carotenoids. Phytoene synthase (PSY) catalyzes the conversion of GGPP to phytoene which is the first step in carotenoid biosynthesis. PSY enzyme, therefore, becomes the first key enzyme in this pathway. Lycopene, imparting a red or red-orange color to some fruits and vegetables, has dual roles in humans and plants as a free-radical scavenger (Collins et al. 2006). It is derived from phytoene in a series of dehydrogenation reactions, which is catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). Lycopene is

then converted to β-carotene and α-carotene by lycopene β-cyclase (Lcy-b) and lycopene ε-cyclase (Lcy-e), respectively (Fig. 1).

Although the carotenoid biosynthetic pathway in plants was elucidated in the 1950s and 1960s, the regulation of carotenoid biosynthesis at the gene and enzyme level is still poorly understood. Cauliflower Or gene (Li et al. 2001) and the apricot (Ap) tomato mutant have been identified, but no regulatory genes involved in carotenoid formation have been isolated yet. Since carotenoids play a central role in plant development and adaptation, their synthesis is consequently considered to be coordinated with other developmental processes such as plastid formation, flowering and fruit development. The partial knowledge of pathway regulation is responsible for the practical difficulties of working with carotenoids and their biosynthetic enzymes. It is believed that a single regulatory process is unlikely to control a branched pathway such as that of carotenoid formation from isoprenoid precursors. In contrast, each branch point is likely to be a control point and probably regulated on both transcriptional and post transcriptional levels (Fraser and Bramley 2004).

*Transcriptional Regulation*

Plastid differentiation, the development of chromoplasts and *de novo* carotenoid formation are processes which occur during tomato and pepper fruit ripening and flower development in daffodil, tomato and marigold (*Tagetes erecta*). Carotenoid formation is regulated by carotenoid genes, which have both up- and down-regulation of transcription. For example, expression of *PSY*-1 and *PDS* is increased while *LCY*-B and

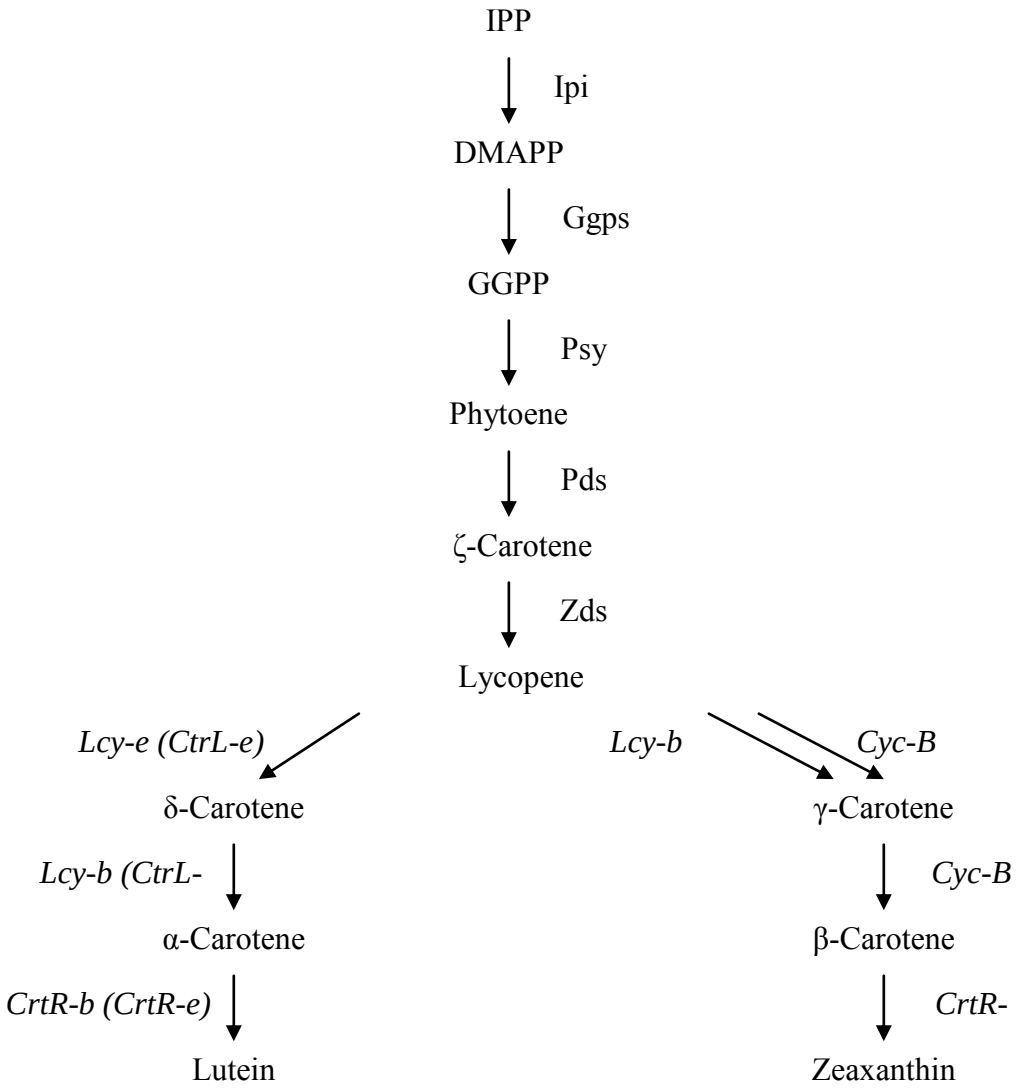


**Fig. 1** Carotenoid biosynthesis pathway in plants (Li et al. 2006)

*LCY*-E mRNAs are decreased during tomato fruit ripening (Ronen et al. 1999; Fraser et al. 1994). Enzyme activities in ripe fruit are also affected by these gene expression

changes. PSY-1 enzyme exerts the greatest control over pathway flux. The up-regulation of this synthase leads to the increase of acyclic carotenes and prevents the formation of the end products at the cyclization step, consequently it results in the accumulation of large amounts of lycopene in ripe tomato fruit. The elucidation of regulatory mechanisms of fruit ripening has progressed in tomato with color mutants and transgenic varieties (Ronen et al. 1999; Fray and Grierson 1993). There may be feedback inhibition that could operate within the pathway either from β-carotene or from a product of β-carotene according to the pigment analysis of the *crt*I transgenic tomato variety along with Lcy-b mutant Og, Ogc and high-beta. In contrast, phytoene-accumulating *immutans* mutant has no feedback inhibition of *PDS* gene expression (Fraser and Bramley, 2004). Increased enzyme activities resulting from transcriptional regulation have also been reported when pepper fruit is ripening (Römer et al. 1993).

Oxidative stress was reported as "a novel class of second messengers that mediates intense carotenoid synthesis during chromoplast differentiation" to up-regulate expression of carotenoid genes (Bouvier et al. 1998). Carotenoid gene expression is also up-regulated during flower development of daffodil (Schledz et al. 1996) and marigold (Moehs et al. 2001) as well as melon (Karvouni et al. 1995) and citrus fruit (Ikoma et al. 2001) development and ripening (Fraser and Bramley 2004).

Light intensity seems to be another factor which increases the expression of certain carotenoid biosynthetic genes to alter carotenoid formation. *PSY* expression of developing seedlings of white mustard (*Sinapis alba* L.) is upregulated with light while *PDS* and *GGPS* expression levels remain constant. *PSY* expression of *Arabidopsis*

*thaliana* also increased with both far-red and red light. *PSY* regulation is involved in both light-labile and light-stable phytochromes (Von Lintig et al. 1997). A 5-fold increase in the ratio between levels of *LCY-B* and *LCY-E* mRNAs in both *Arabidopsis thaliana* and tomato leaves was observed when plants were shifted from low light to strong light (Hirschberg, 2001).

*Post-transcriptional Regulation*

Fraser and Bramley (2004) mentioned that the key regulatory issue in the carotenoid biosynthesis pathway is what mechanisms control the partitioning of precursors into the branches of the pathway. The discovery of multigene families in the pathway has supported the theory of metabolic channeling between each branch in the pathway. The corresponding enzymes have been found in tomato (Fraser et al. 2000), pepper (Dogbo et al. 1987) and daffodil (Lützow and Beyer 1988). The metabolic branches are believed to be relatively independent from each other since there are relatively small changes of isoprenoids in transgenic plants in spite of increased enzyme activities. However, metabolite precursor pools such as GGPP pool can be diverted from one branch to the next (Fray et al. 1995). The fact that constitutive expression of *PSY*-1 caused dwarfism of tomato by redirecting metabolite availability shows metabolic cross talk does exist. *PSY* and *PDS* were post-transcriptionally regulated in chromoplasts of daffodil membrane association. In addition, light-induced membrane association and substrate specificity are also believed to regulate carotenoid biosynthesis.

*Carotenoid Sequestration Regulation*

This is a common form of regulation but differs in the way of sequestering the end-product carotenoids between chloroplasts and chromoplasts. Esterification of carotenoids was considered to be the mechanism of sequestration regulation in pepper, tomato and canola, and seems to be an effective method used by many flowers such as sunflower, daffodil and marigold (Fraser and Bramley 2004).

Genetic engineering to enhance carotenoids in crop plants

Genetic engineering of carotenoids in crops for nutritional enhancement has been studied for more than a decade. The first committed step in carotenoid biosynthesis is catalyzed by phytoene synthase (PSY), which converts GGPP into phytoene (Cunningham and Gantt 1998; Li et al. 2006). The overexpression of this gene, combined with or without the downstream pathway genes (PDS and Lcy-b), have enhanced carotenoids in crops such as tomato (Fraser et al. 2001; 2002), carrot (reviewed by Fraser and Bramley 2004), canola (Shewmaker et al. 2004), rice (Ye et al. 1999: 'Golden Rice'; Paine et al. 2005: 'Gold Rice 2'), potato (Diretto et al. 2007; Ducreux et al. 2005) and maize (Aluru et al. 2008). According to these reports, the total carotenoids of the transgenic crops have been increased by 2 to 50 folds in different species with 1.6 to 3600 folds in beta-carotene level, which resulted in the color changes to yellow or orange. So far, "Golden Rice" and "Golden Potato" are the best demonstrations for *PSY* gene function in carotenoid biosynthesis (Ye et al. 1999; Paine et al. 2005; Diretto et al. 2007).

*Tomato*

Tomato has become the most intensively studied crop in carotenoid metabolism engineering since its fruit and products represent an important source of carotenoids among diets. Phytoene synthase (PSY) is the most important rate-limiting enzyme manipulated by genetic engineering to regulate β-carotene levels in plants. The bacterium *Erwinia uredovora* phytoene synthase (*crt*B) gene has been over-expressed in tomato fruits. Total carotenoids of the transgenic tomatoes exhibited a 2- to 4-fold increase over the control: 2.4-fold (n=3) in phytoene, 1.8-fold (n=6) in lycopene and 2.2-fold (n=5) in β-carotene levels. Biochemical analysis showed that catalytically active CRTB protein was plastid-located with 5- to 10-fold (n=4) higher levels (Fraser et al. 2002). However, the additional *PSY* reduced the regulatory influence of this step over the pathway flux, which was suggested by Fraser and Bramley (2004).

Phytoene desaturation is another possible step that modifies carotenoid biosynthesis. An *Erwinia uredovora* PDS gene (*crt*I) with the CaMV 35S promoter has been transferred to tomato (CV. Ailsa Craig) plants (Römer et al. 2000). Total carotenoid levels were not changed in the transgenic tomatoes, but the β-carotene content increased about 3-fold (n=17), up to 45% of the total carotenoid content. The expression of the endogenous carotenoid genes behind *PDS* (*ZDS*, *LCY*-B) was also increased, but the *PSY* gene which is prior to PDS was decreased. Besides β-carotene, the content of other downstream pathway carotenoids such as neoxanthin, antheraxanthin, lutein, and zeaxanthin were increased. In contrast, the content of carotenoids prior to β-carotene such as phytoene, lycopene, γ- carotene and δ-carotene decreased. As a consequence, the total

amount of carotenoids decreased. The enhancement of PDS activity did not lead to the increase of lycopene. It was suggested that this result was due to *crt*I over-expression in tomato, which reduced the content of compounds prior to phytoene desaturation and induced endogenous lycopene cyclisation (Fraser and Bramley 2004). This phenotype of modified carotenoids has been found to be stable and reproducible over at least four generations.

Lycopene is a large precursor pool for β-carotene synthesis in ripe tomato fruit. Over-expression of the *Arabidopsis thaliana* lycopene β-cyclase (β-*Lcy*) in tomato fruit under the control of a fruit-specific promoter PDS has been achieved (Rosati et al. 2000), making lycopene convert into β-carotene. Transformants aimed at up-regulating β-*Lcy* gene expression exhibited a 5-fold increase in β-carotene. Other carotenoids were not changed in both fruits and leaf tissues, and endogenous carotenoid gene expression was not significantly changed. In contrast, transformants aimed at down-regulating β-*Lcy* gene showed up to 50% down-regulation of *β-Lcy* expression in ripe fruit, and consequently their lycopene content was slightly increased.

*Carrot*

Fraser and Bramley (2004) reported that the *Erwinia herbicola crt* genes were over-expressed in carrot, causing β-carotene levels to increase 2-5-fold in the root.

*Canola*

So far, the most dramatic increase in carotenoid levels was produced in transgenic canola. An *Erwinia uredovora* phytoene synthase gene *crt*B was over-expressed in a seed-specific manner. A 50-fold increase of carotenoids was found in the transgenic plant seeds (Shewmaker et al. 1999). The seed-specific expression of *crt*B leads to orange embryos in transgenic canola, predominantly containing α-carotene and β-carotene.

*Rice*

Rice is a staple food in most developing countries, however, it lacks β-carotene (pro-vitamin A). The first generation of the 'Gold Rice' (Golden Rice 1) was reported (Ye et al. 2000). Three biosynthetic genes were co-transformed via two vectors to enable the metabolism from GGPP to β-carotene. The PSY and lycopene β-cyclase cDNAs from daffodil (*Narcissus pseudonarcissus*) were expressed under the control of endosperm-specific glutelin promoter, and the *E. uredovora* phytoene desaturase gene *crt*I under the control of constitutive CaMV 35S promoter. As a result, total carotenoids accumulated to 1.6 μg $g^{-1}$ in endosperm (n=50). However, the limited production of β-carotene cannot be a solution for the vitamin A deficiency. Paine et al. (2005) launched the genetic engineering of a second generation of 'Golden Rice' (Golden Rice 2) by replacing the daffodil *PSY* with maize *PSY*, in combination with the *E. uredovora* phytoene desaturase gene *crt*I. The total carotenoids were increased to a maximum of 37 μg $g^{-1}$ with 86-89%

of β-carotene. In their study, *PSY* was found to still be the limiting step in the accumulation of carotenoids.

*Tobacco*

The *E. uredovora* phytoene desaturase (*crt*I) overexpression in tobacco plants led to a large amount of the CRTI proteins which correlates with the *crt*I mRNA levels produced in the transformants. Carotenoid analysis of transgenic tobacco leaves suggested changes in the composition of leaf carotenoids. β-carotene levels were increased and the level of lutein was reduced, while the total amount of carotenoids was not significantly altered (Misawa et al. 1994).

*Potato*

In recent years, genetic manipulation of carotenoids has been addressed in potato. Ducreux et al. (2003) over-expressed *E. uredovora* phytoene synthase gene *crtB* in potato tubers, which increased carotenoids from 5.6 μg $g^{-1}$ to 35 μg $g^{-1}$ in the potato tubers, and their β-carotene reached to 11 μg $g^{-1}$ compared to negligible amounts in the controls. Later on, Diretto et al. (2007) reported 'Golden Potato' with 114 μg $g^{-1}$ of carotenoids and 47 μg $g^{-1}$ of β-carotene in the tubers by introducing three pathway genes (*crtB*, *crtI* and *crtY*) from *E. uredovora* together. Lopez et al. (2008) incorporated a non-pathway gene *Or*, isolated from an orange cauliflower mutant, into potato genome. This transgene elevated carotenoids to 31 μg $g^{-1}$ and β-carotene to 4-5 μg $g^{-1}$ in the potato tubers.

Phytoene synthase gene (*PSY*)

A *PSY* gene family with at least two genes (*PSY1* and *PSY2*) has been identified in *C. melo* (Qin et al. 2011; Karvouni et al. 1995). Controversial results on melon PSY1 expression during fruit ripening have been reported. Qin et al. (2011) compared the gene expressions between *CmPSY1* and *CmPSY2* in cantaloupe melon tissues. *CmPSY1* expressed in most tissues (leaf, stem, flower and fruit) except the root where only *CmPSY2* was present. The highest expression of *CmPSY1* in the fruits was at 40 DAP, which was consistent with the previous report from Karvouni et al. (1995) that the expression of melon *PSY1* (MEL5) mRNA dramatically increased and reached its highest level during the period when cantaloupe melon flesh color changed from green to orange, which was assumed to coincide with the increase in carotenoid and beta-carotene accumulation. However, Aggelis et al. (1997) examined four ripening-related mRNAs including *MEL5* identified by Karvouni et al. (1995), in seven varieties exhibiting differences in their ripening behavior. *MEL5* mRNA level was lower than other ripening-related mRNAs (*MEL1*, *MEL2* and *MEL7*), and its expression pattern during ripening varied in different varieties. *MEL5* decreased (from 100% to less than 20% of maximum signal) in an *inodorus* casaba melon 'Marygold' (green-flesh, very slow ripening); stayed unchanged in the *cantalupensis* melons 'Delada' (green flesh, long shelf-life), 'Viva' (orange flesh, normal shelf-life) and 'Alpha' (orange flesh, normal ripening); increased till 40 days after anthesis (DPA) and decreased significantly afterwards in *cantalupensis* melons 'Tornado' and 'Sirio' (both were orange flesh and slow ripening); and continuously increased in *cantalupensis* melon 'Topper' (orange

flesh, long shelf-life). These results suggested that *PSY1* (*MEL5*) expression was not necessarily correlated with melon fruit color change. It is possible that coordination of multiple genes regulated carotenoid accumulation leading to the color change, or other functional PSY gene(s) existed in melons like the *PSY* gene families in tomato, maize, sorghum and rice (Fray and Grierson, 1993; Gallagher et al. 2004; Li et al. 2008).

Melon *PSY1* (*MEL5*) was homologous to the *PSY* clones in tomato, pepper and *Arabidposis* (Karvouni et al. 1995). Bang et al. (2006) identified a *PSY* gene family (*PSY-A*, *PSY-B* and *PSY-C*) in red-fleshed watermelon and found that *PSY-C* had the highest homology to tomato *PSY1*. Both nucleotide and amino acid sequences of *PSY-C* (unpublished data) showed 94% similarity to *C. melo PSY1* (GenBank: Z37543). It was presumed that *PSY-C* may have a critical function in the watermelon carotenoid pathway converting GGPP to phytoene as compared to *PSY-A* and *PSY-B*. Therefore, we have selected this gene to study how carotenoid compositions in honeydew melon are regulated by the transgene *PSY-C*; and moreover, to gain a novel genotype of honeydew.

# CHAPTER II

# ESTABLISHMENT OF REGENERATION SYSTEMS FOR CANTALOUPE AND CASABA MELONS*

## Materials and Methods

### Plant material

Three melon breeding lines, 'F39', '141' and 'TMS' were initially selected to test published melon regeneration protocols. 'F39' and '141' are inbred lines of western shipper cantaloupe (*C. melo* var. *reticulatus*), which produce high quality orange-fleshed fruits with netted surface and have been inbred for over 10 generations at Texas AgriLife Research Center, Weslaco, TX. In addition, 'F39' has medium to high resistances to multiple diseases. 'TMS' is an elite white-fleshed casaba melon (*C. melo* var. *inodorus*) with smooth surface.

### Explant preparation

Seeds were surface sterilized in a 50% commercial bleach solution (3% sodium hypochlorite) containing a drop of Tween-20 for 30 min and rinsed three times with

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* Part of this chapter is reprinted with permission from "*Agrobacterium* -mediated transformation and shoot regeneration in elite breeding lines of western shipper cantaloupe and honeydew melons (*Cucumis melo* L.)" by Ren Y, Bang H, Curtis IS, Gould G, Patil BS, Crosby KM (2011) Plant Cell Tiss Organ Cult Online First™, 8 Sep 2011 , Copyrigtht 2011 by Springer.

sterile water. After soaking seeds in sterile water for 4-8 h, the seed coats were removed to expose each embryo. De-coated embryos were sterilized in 70% ethanol for 30 s followed by a 5% commercial bleach solution (0.3% sodium hypochlorite) containing a drop of Tween-20 for 10 min. Embryos were rinsed three times with sterile water and cultured in the dark on a germination medium, consisting of Murashige and Skoog (MS) (1962) salts medium supplemented with 30 g $l^{-1}$ sucrose and solidified by 5 g $l^{-1}$ agar, pH 5.7-5.8. Three explants types were prepared for regeneration experiments as follows.

*Cotyledonary explants*

Seven days later, cotyledons were excised 2 mm from the cotyledonary nodes (conjunction sites between cotyledons and hypocotyls). Each cotyledon was then cut into 6 equally-sized explants of approximately 3 mm × 2 mm, and was placed adaxially on a regeneration medium in a 100 mm ×15 mm Petri dish (12 explants/dish, 5 replicates/treatment).

*Shoot tip with hypocotyl and cotyledon explant (STHC) vs. shoot tip with hypocotyl explant (STH)*

After cotyledonary explants were removed from the 7-day-old seedlings, the rest of the tissue, a shoot tip connected with 2 mm-long proximal part of cotyledons and a 3 mm-long hypocotyl (Fig. 2a and b), were longitudinally bisected into two halves (Fig. 2c and d). This explant type was named shoot tip with hypocotyl and cotyledon (STHC). Explant type shoot tip with hypocotyl (STH) referred to STHC without cotyledon

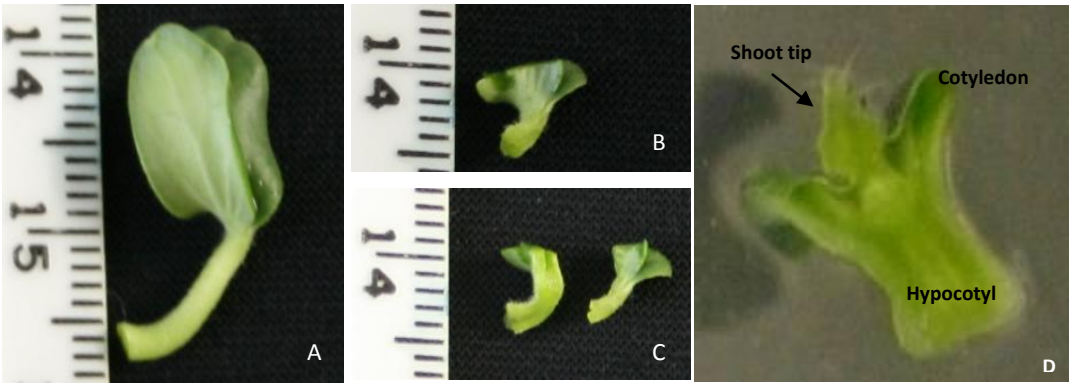


**Fig. 2** Preparation of a shoot tip with hypocotyl and cotyledon (STHC) explant. **a** A 7-day-old seedling removed from medium, **b** a STHC explant consisting of a shoot tip attached with 2 mm-long proximal part of cotyledons and a 3 mm-long hypocotyl, **c** bisected STHC explants, **d** enlarged look of a bisected STHC explant

fragments attached (cotyledons were removed from STHC). Explants were then placed into 100 mm × 15 mm Petri dishes (STHC: 6 explants/dish, 4 replicates/treatment; STH: 6 explants/dish, 2 replicates/treatment).

*Shoot tip explant*

2-3 mm-long shoot tips were isolated from 5-day-old seedlings and placed into 100×15 mm Petri dishes (6 explants/dish, 2 replicates/treatment). For bisected shoot apical explants, three- to five-day pre-cultured intact shoot tips were longitudinally (from apex to base) split into two asymmetrical tissues by a sterile blade, and the bisected shoot tips were then placed back onto 100×15 mm Petri dishes (6 explants/dish, 4 replicates/treatment).

Shoot regenerations

Six regeneration media, RM1 (Ficcadenti and Rotino 1995), RM2 (Guis et al. 2000), RM3 (Yadav et al. 1996), RM4 (Fang and Grumet 1990), RM5 (Souza et al. 2006), and RM6 (Bordas et al. 1997), were evaluated with the cotyledonary explants of '141', 'F39' and 'TMS' (Table 1). Each medium represented the optimal composition that resulted in the highest regeneration frequency in each study reported. All media were based on MS salts supplemented with 30 g $l^{-1}$ sucrose and various combinations of plant growth regulators (BA, ABA, IAA, 2iP and kinetin). Media were solidified using agar 7 g $l^{-1}$ except in RM3 where agar was replaced by 2.6 g $l^{-1}$ Phytagel™.

Liquid shoot primoridum induction media, SPI1 and SPI2 (Table 1), were reported to induce shoot primordium aggregates and shoot proliferation from *C. melo* 'Prince', respectively (Ezura et al. 1997b). In our study, we evaluated shoot regeneration of both intact and bisected shoot tip explants of '141', 'F39' and 'TMS' on the solidified media SPI1 and SPI2 by adding 8 mg $l^{-1}$ agar.

**Table 1** Evaluation of six media on the regeneration of melon '141', 'F39' and 'TMS'

| Medium | MS vitamins | Myo-inositol | IAA | BA | 2iP | Kinetin | ABA | $AgNO_3$ | $CuSO_4 \cdot 5H_2O$ | NAA | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RM1 | - | | | 0.63 | | | 0.26 | | | | Ficcadenti and Rotino 1995 |
| RM2 | - | | | 0.20 | 0.20 | | | | | | Guis et al. 2000 |
| RM3 | + | | 0.80 | 1.00 | | | 0.26 | 5.40 | | | Yadav et al. 1996 |
| RM4 | + | | 0.88 | 1.13 | | | 0.26 | | | | Fang and Grumet 1990 |
| RM5 | - | 100 | 1.50 | 1.00 | | | | | 1.00 | | Souza et al. 2006 |
| RM6 | - | 100 | 1.50 | | | 6.00 | | | 1.00 | | Bordas et al. 1997 |
| SPI1 | + | | | 1.00 | | | | | | 0.01 | Ezura et al. 1997b |
| SPI2 | + | | | 1.00 | | | | | | | Ezura et al. 1997b |

Basal medium was Murashige and Skoog (1962) salts. Data is presented as mg $l^{-1}$

Tissue cultures were placed in a room maintained at 25±2 ºC under cool white fluorescent lights with 16 h light/8 h dark photoperiod and 60-80 μmol $m^{-2}$ $s^{-1}$ light intensity. After 4 weeks, the induction of calli and shoot primordia was recorded and scored from 0 to 100% (Table 2).

**Table 2** Scoring method used to estimate callus and shoot primordium induction from cotyledonary explants of melon '141', 'F39' and 'TMS'

| Score | Responding area of total cut surfaces of an explant (%)* |
|---|---|
| 0 | 0 |
| 0.125 | <12.5 |
| 0.25 | 12.6-25 |
| 0.50 | 26-50 |
| 1 | 51-100 |

* Cut surfaces produced calli and/or shoot primordia. See Fig. 2

Shoot elongation and rooting

Shoots or shoot primordia regenerated from cotyledonary explants were transferred to shoot elongation medium, which consisted of MS basal medium supplemented with 30 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar and BA at different concentrations (0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$). Shoots that failed to root on elongation medium were transferred to a rooting medium (MS basal medium supplemented with 30 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar).

Statistical analysis

Cotyledon regeneration experiments were conducted with two factors (genotype × medium) in a randomized complete block design (12 explants/dish; 5 replicates/treatment) and analyzed by two-way analysis of variance (ANOVA). Shoot

apex regeneration experiments were conducted with three factors (genotype × medium × bisection) in a randomized complete block design (6 explants/dish; 2 or 3 replicates/treatment) and analyzed by generalized linear model. Cotyledonary-node regeneration experiments were conducted with one-way ANOVA. Differences between the means were performed using Duncan's Multiple Range Test where the 5% probability level was considered significant. Kanamycin sensitivity was tested in a randomized complete block design (4 explants/dish; 8 replicates/treatment) and analyzed by one-way analysis of variance. Each dish was considered as a replicate in all the experimental designs.

**Results and Discussion**

Plant regeneration

*Cotyledonary explants*

Six media, previously reported to be effective from other melon regeneration and/or transformation protocols, were screened for our three different genotypes (Fig. 3). Calli and shoot primordia were usually formed within 3-4 weeks on the initial regeneration media. Shoots developed from shoot primordia approximately 2-3 weeks later. Significant differences appeared in the frequency of explants producing calli and shoot primordia on the various media, and an interaction between genotype and medium composition was also observed (Table 3). Overall, media RM3, RM4 and RM1 were best for shoot regeneration in '141', 'F39' and 'TMS', respectively (Fig. 4a, b and c).

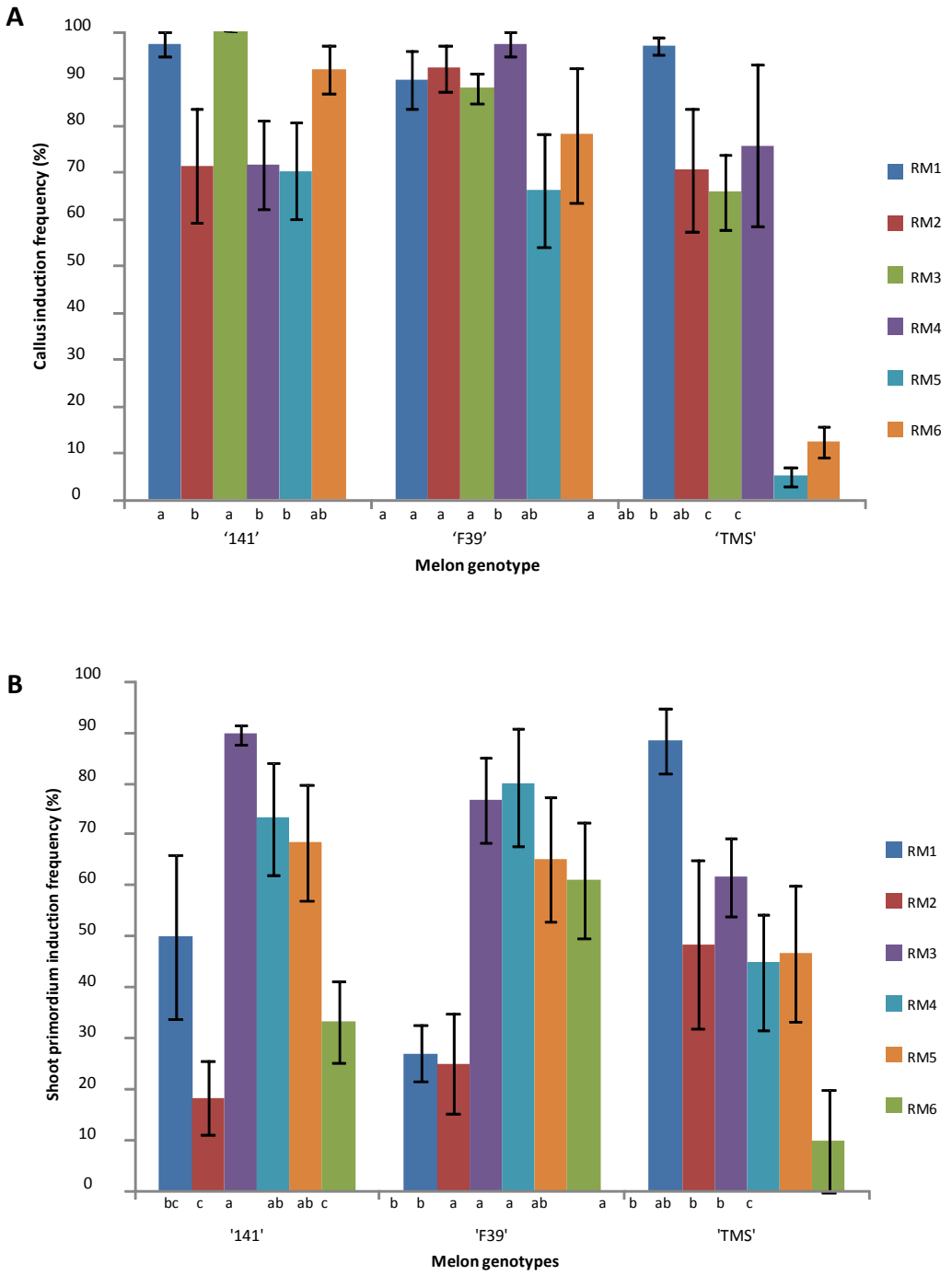


**Fig. 3** Effect of six different media (RM1-RM6) on the production of calli and shoot primordia from cotyledonary explants of three melon genotypes '141, 'F39" and 'TMS'. **a** The frequency of explants producing calli, **b** the frequency of explants producing shoot primordia. Bars with same letters are not significantly different by Duncan's Multiple Range Test at 5% probability level. *Vertical bars* show standard errors

RM3 and RM4 induced the highest frequency of both callus and shoot primordium regeneration in ‘F39’; however, RM4 performed better in terms of producing fewer vitrified shoots (Fig. 4d and e).


**Fig. 4** Callus production and shoot regeneration on the optimal medium from cotyledonary explants of three different melon genotypes after four weeks of culture. **a** ‘141’ on RM3 [MS basal supplemented with 1mg $l^{-1}$ BA, 0.26 mg $l^{-1}$ ABA, 0.8 mg $l^{-1}$ IAA and 5.4 mg $l^{-1}$ $AgNO_3$], **b** ‘F39’ on RM4 [MS basal supplemented with 1.13 mg $l^{-1}$ BA, 0.26 mg $l^{-1}$ ABA and 0.88 mg $l^{-1}$ IAA], **c** ‘TMS’ on RM1 [MS salt supplemented with 0.63 mg $l^{-1}$ BA and 0.26 mg $l^{-1}$ ABA], **d** ‘F39’ on RM4 (less vitrification), **e** ‘F39’ on RM3 (more vitrification) (bars = 1cm)

Subsequently, we compared shoot primordium regeneration efficiency from each region of the cotyledonary explants: proximal, middle and distal. No significant differences were detected (data not shown), which was not in accord with the results reported by Gonsalves et al. (1994). Their research showed that regeneration frequency of the proximal side of the melon explant was significantly higher than that of the distal region.

Regenerated shoots proliferated but did not elongate on MS basal medium supplemented with BA levels higher than 0.1 mg $l^{-1}$. A series of low BA concentrations, 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$, was examined on 'F39' for shoot elongation. Low

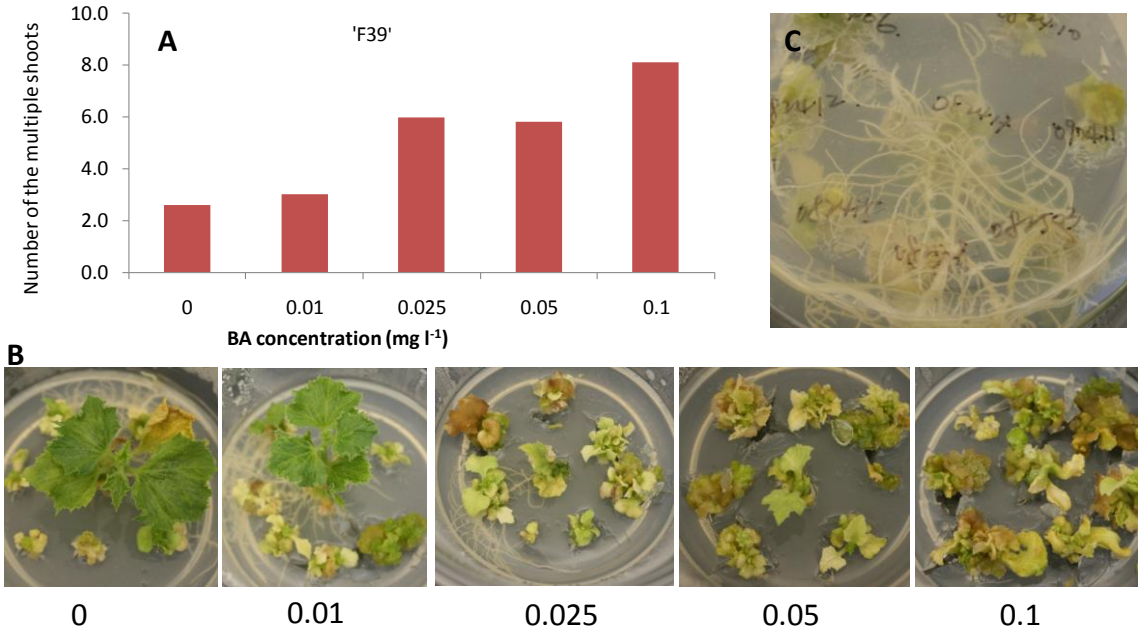


**Fig. 5** Multiple shoot regeneration, shoot elongation and rooting of melon 'F39' on MS basal medium supplemented with various BA concentrations. **a** Multiple shoot regeneration on BA 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$, **b** shoot elongation on BA 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$, **c** rooting on BA 0.1 mg $l^{-1}$

levels of BA (0 and 0.01 mg $l^{-1}$) treatments reduced the prevalence of multiple shoots (Fig. 5a) and allowed shoots to elongate and produce roots (Fig. 5 b and c).

*Shoot tip explants*

Shoot primordium induction (SPI) media, SPI1 and SPI2, were compared between intact shoot tips and bisected shoot tips of our three genotypes (Table 3). Shoot primordia and/or shoots were formed within 4 weeks on both media. Overall, both media can induce shoot primordia and shoots from both shoot tip explant types; however, SPI2 was better for developing normal shoots. Genotypic differences were shown in shoot primordium induction, where '141' responded best while 'TMS' had the lowest response. An interaction between medium composition and genotype was also observed in shoot primordium and/or shoot induction. For '141', no significant differences were found in media and explant types. SPI2 performed better than SPI1 to induce shoot primordia and/or shoots in 'F39'. Bisection of shoot tips did not significantly affect shoot regeneration in '141' and 'F39' but in 'TMS'. Bisection of shoot tips exposes shoot apical meristem to *Agrobacterium* to facilitate inoculation, which will improve the *Agrobacterium* –mediated transformation efficiency (personal communication with Dr. Jean Gould). Based on our result, bisected shoot tips could be considered as an explant type for *Agrobacterium*-mediated transformation in '141' and 'F39'.

**Table 3** Regeneration of shoot primordia and shoots from shoot tip explants of '141', 'F39' and 'TMS'

| Genotype | Medium | Shoot tip type | Shoot primordium induction percentage (%) | Shoot regeneration percentage (%) | Average number of shoots/explant |
|---|---|---|---|---|---|
| 141 | SPI1 | Intact | 91.7±8.3 | 60.0±27.3 | 1.5±1.1 |
| | | Bisected | 95.0±5.0 | 40.0±0 | 0.6±0.1 |
| | SPI2 | Intact | 70.2±13.1 | 63.1±20.2 | 2.0±0.3 |
| | | Bisected | 65.0±12.6 $^{NS}$ | 55.0±9.6 $^{NS}$ | 3.7±1.5 $^{NS}$ |
| F39 | SPI1 | Intact | 63.3±1.7 b | 53.3±6.7 ab | 1.2±0.3 |
| | | Bisected | 50.0±11.8 b | 12.5±7.9 b | 0.3±0.2 |
| | SPI2 | Intact | 100.0±0.0 a | 66.7±8.3 a | 1.6±0.2 |
| | | Bisected | 62.5±14.2 ab | 58.3±10.8 a | 1.8±0.7 $^{NS}$ |
| TMS | SPI1 | Intact | 29.8±13.1 | 76.2±9.5 a | 1.1±0.1 a |
| | | Bisected | 19.7±8.5 | 20.1±3.4 b | 0.3±0.1 b |
| | SPI2 | Intact | 39.3±10.7 | 61.9±4.8 a | 0.8±0.2 ab |
| | | Bisected | 25.0±6.8 $^{NS}$ | 16.9±8.3 b | 0.3±0.1 b |
| Genotype | | | *** | NS | * |
| Medium | | | NS | NS | * |
| Shoot tip type | | | NS | *** | NS |
| Genotype × Medium | | | ** | * | NS |
| Medium × Shoot tip type | | | NS | NS | NS |
| Genotype × Shoot tip type | | | NS | NS | NS |
| Genotype × Medium × Shoot tip type | | | NS | NS | NS |

Values are presented as means ± SE, where n=4. Means within the same columns within each genotype followed by same letters are not significantly different by Duncan's Multiple Range Test at 5% probability level. The percentage data were transformed by an arcsin function before analysis to normalize the distribution. $^{NS}$ No significant difference. $^{***}$, $^{**}$, $^{*}$ Significant at 0.1%, 1% and 5% probability level, respectively. Data were recorded after 4 weeks of culture

*Shoot tip with hypocotyl and cotyledon (STHC) explants vs. shoot tip with hypocotyls (STH)*

Preliminary tests showed that explant STHC of 'F39' responded better than that of '141' and 'TMS' on the six media (data not shown). Thus, 'F39' was tested for shoot regeneration from STHC and STH explants (Figs. 6 and 7). Two types of shoots regenerated from STHC, primary shoots and adventitious shoots, which emerged from the first week and the second week of the culture, respectively. Significant differences

were found in the frequencies of primary and adventitious shoot regenerations on various media (Fig. 6a and c; Fig. 7a). Medium RM3 produced the most primary shoots (83%) but the least adventitious shoots (8%) while medium RM4 induced the most adventitious shoots (90%). This indicated that BA and IAA were necessary for adventitious shoot induction but the addition of $AgNO_3$ severely inhibited adventitious shoot regeneration from this type of explant. Although less STHC explants produced primary shoots on medium RM2 (46%), these primary shoots grew faster and regenerated more axillary shoots than other media. Average length of the primary shoots was more than 2.5 cm, and the average number of the axillary shoots grown on the primary shoots was six (Fig. 6e-f). Axillary shoots were reported to be used as another good explant type to maintain original ploidy in melon *in vitro* culture (Ezura et al. 1992). Therefore, RM2 and RM4 are considered to be a good medium for primary shoot regeneration and adventitious shoot regeneration, respectively.

An interesting phenomenon was observed: very few primary and adventitious shoots regenerated and could not normally grow when cotyledon fragments were removed from STHC explants (Fig. 6b and d; Fig. 7b). Similar results were reported in *Vigna* (Sen and Guha-Mukherjee 1998), melon (Curuk et al. 2002) and squash (Ananthakrishnan et al. 2003). The hypocotyl explant with a fragment of cotyledon attached could produce a high percentage of regenerated shoots while removal of cotyledons caused either zero or a very low frequency of shoot regeneration. The plausible reason could be the loss of certain signals and / or hormones existing in

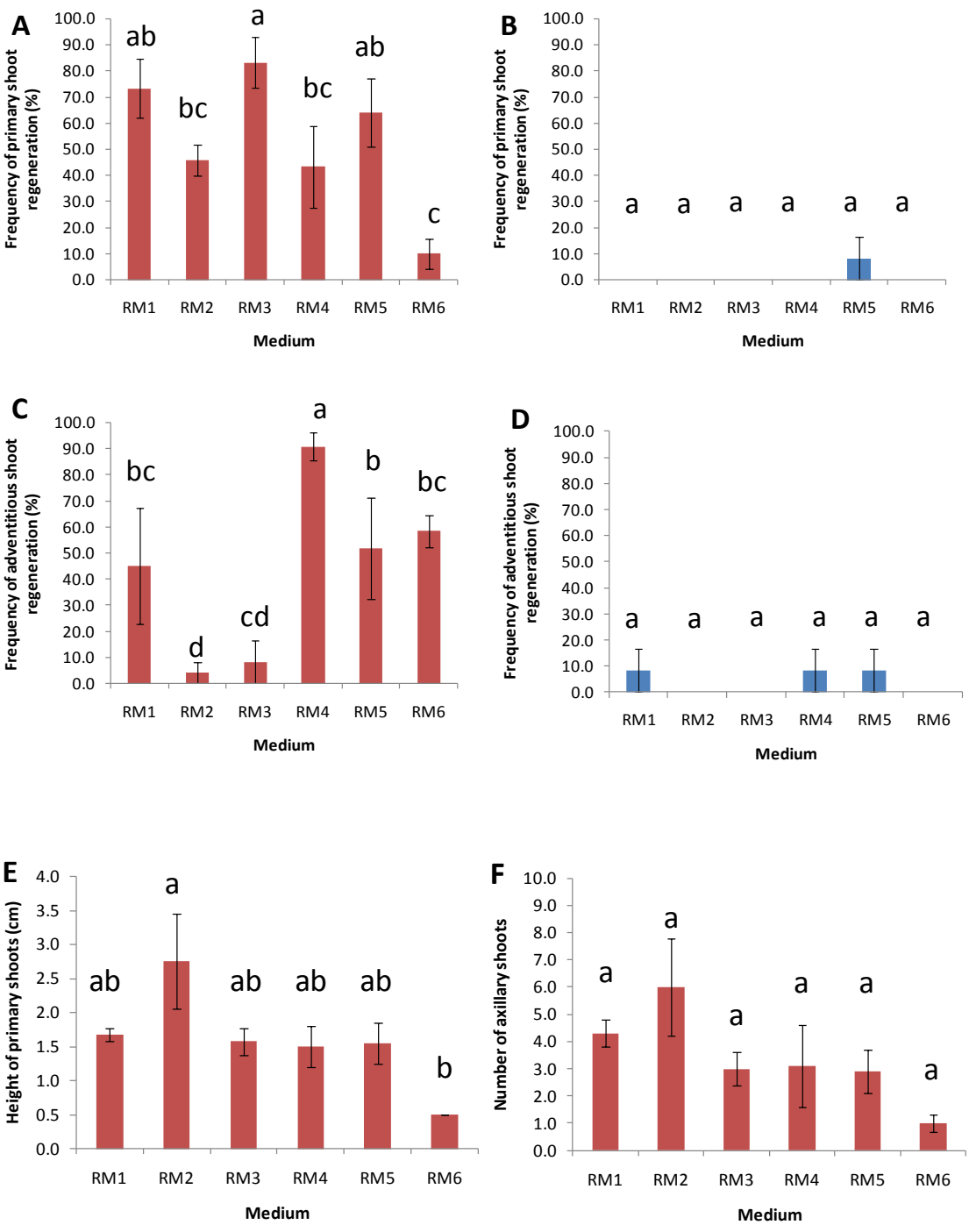


**Fig. 6** Shoot regeneration and development from shoot tip with hypocotyl and cotyledon fragments explants (A, C, E and F) as well as shoot tip with hypocotyl explants (B and D) of melon 'F39' on six different media (RM1-RM6)

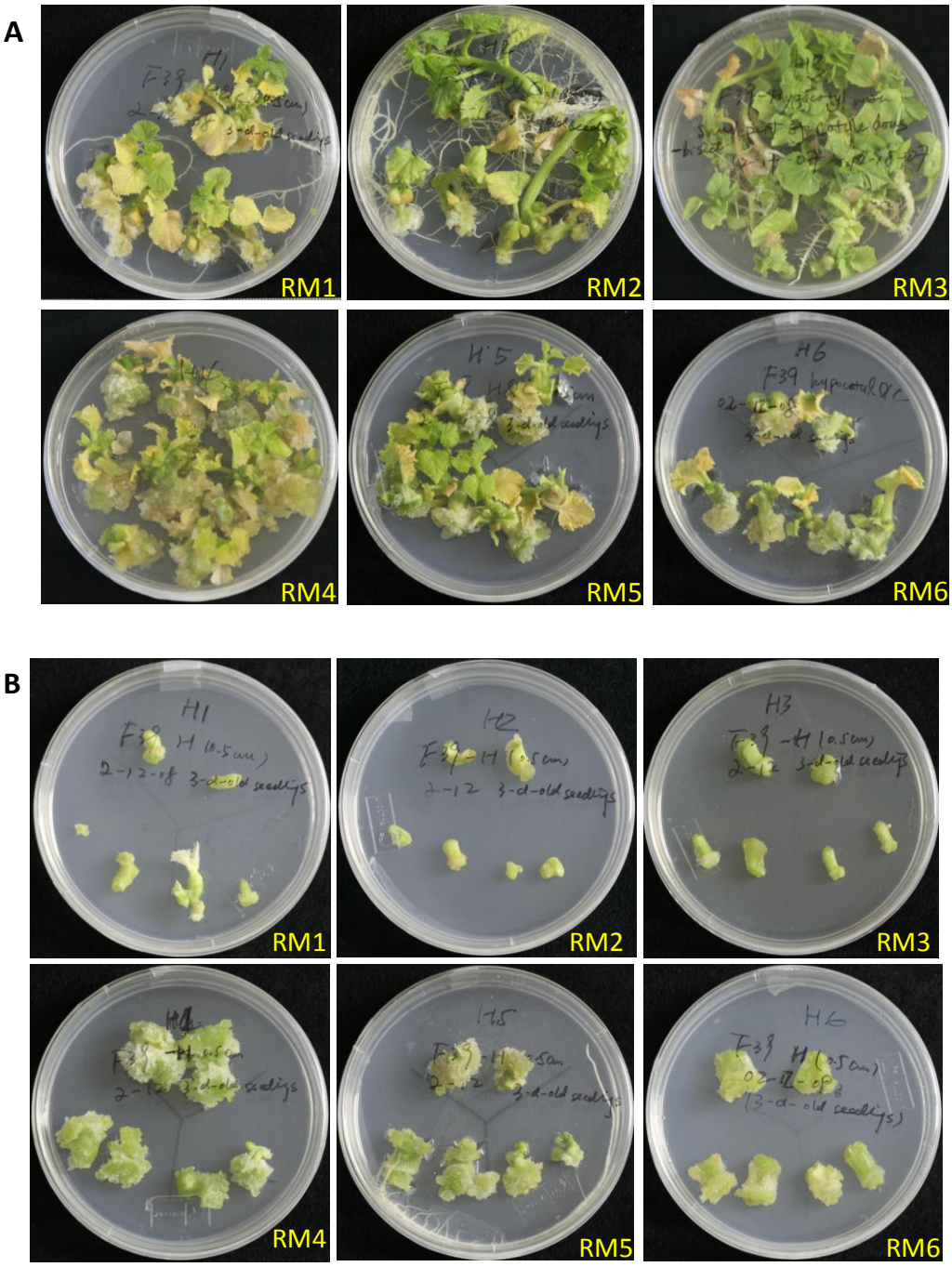


**Fig. 7** Comparison of shoot regeneration from shoot tip with hypocotyl and cotyledon explants (A) as well as shoot tip with hypocotyl explants (B) of melon 'F39' on six different media (RM1-RM6)

cotyledons for shoot regeneration, which was also supported by other authors (Sen and Guha-Mukherjee 1998; Curuk et al. 2002).

## Conclusion

We tested shoot regeneration ability with three different explant types of our elite breeding cantaloupe lines, ‘141’ and ‘F39’, as well as casaba melon line ‘TMS’, on the previously reported media. Each type of explant had a high efficiency of regeneration on its optimal medium. Interaction between melon genotype and medium composition was found in all the regeneration experiments. Medium RM4 is the best medium for shoot regeneration from cotyledonary explants of ‘F39’. Bisected shoot tip and bisected shoot tip with hypocotyl and cotyledons are also good explant sources for regeneration and transformation in ‘F39’. Thus, our research proceeded to establish a transformation protocol for cantaloupe ‘F39’ based on the high efficiency shoot regeneration systems.

# CHAPTER III

# SHOOT REGENERATION AND PLOIDY VARIATION IN TISSUE CULTURE OF HONEYDEW MELON

## Materials and Methods

### Plant material

Honeydew (*C. melo* var. *inodorus*) '150' is an elite inbred line with pale green flesh and smooth rind surface, which has been inbred for more than 10 generations at Texas AgriLife Research Center, Weslaco, TX.

### Plant regeneration

Preliminary tests showed that the frequency of regeneration in '150' was very high on the medium RM4 (MS basal+1.13 mg $l^{-1}$ BA+0.26 mg $l^{-1}$ ABA+0.88 mg $l^{-1}$ IAA+30 g $l^{-1}$ sucrose +8 g $l^{-1}$ agar). A regeneration medium test was then conducted based on this result. All four media were prepared by MS basal supplemented with 30 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, and additions of different combinations of 1 mg $l^{-1}$ BA, 0.26 mg $l^{-1}$ ABA and 0.8 mg $l^{-1}$ IAA (pH 5.8) (Table 4). Cotyledonary explants were prepared as for other melons described in Chapter II.

### Shoot elongation and rooting

The culture procedure was the same as the "Shoot elongation and rooting" described in Materials and Methods in Chapter II.

**Table 4** Evaluation of four media for shoot regeneration of honeydew '150'

| Medium | 1 mg $l^{-1}$ BA | 0.26 mg $l^{-1}$ ABA | 0.8 mg $l^{-1}$ IAA |
|---|---|---|---|
| M1 | + | - | - |
| M2 | + | + | - |
| M3 | + | - | + |
| M4 | + | + | + |

Basal medium was Murashige and Skoog (1962) basal

Ploidy determination

Ploidy level was determined by flow cytometry. Samples were prepared from the third leaf below shoot apex of plants acclimatized in pots using a commercial kit CyStain PI absolute P (PATTEC, Germany) following the manufacturer's instruction. Plant nuclei were analyzed on a FASCSalibur (Becton Dickinson Immunocytometry System, San Jose, CA) flow cytometer, equipped with a 15mW air-cooled argonlaser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter. A minimum of 5,000 events, defined by a region for single nuclei in a plot of propidium iodide area versus width, were measured for each sample. Data analysis was performed in FlowJo (version 8.8.7, Treestar, Inc., Ashland, OR).

Statistical analysis

Regeneration was conducted with one factor (medium composition) in a randomized complete block design (4 explants/dish; 6 replicates/treatment) and analyzed by one-way analysis of variance. Each dish was considered as a replicate. Mean separations were

performed using Duncan's Multiple Range Test, and differences at the 5% probability level were considered significant.

**Results and Discussion**

Medium optimization for shoot regeneration

Shoot regeneration has been previously tested on medium M4 which was an optimal regeneration medium reported by Fang and Grumet (1990). The regeneration was highly efficient on this medium (Ren et al. 2011). However, we observed that the cotyledonary explants kept curving up away from the medium surface no matter which side of the explant was in touch with the medium. This would cause the 'escape' problem on the selection medium for transformation (Ren et al. 2011). Therefore, efforts have been made to solve this problem.

Based on the result of the addition of NAA into the best medium for multiple shoot induction (MS + 8 mg $l^{-1}$ BA), Keng and Hoong (2006) indicated that NAA was not necessary for multiple shoot induction from the nodal segments of honeydew. Ficcadenti and Rotino (1995) did a massive screening of multiple melon genotypes including *inodorus* variants across MS and B5 medium supplemented with BA, ABA and TDZ (thidiazuron). They reported that BA can produced shoots in honeydew, but the combination of BA and ABA significantly increased the number of shoots regenerated from cotyledonary explants regardless of genotypes. To solve the curving problem as well as investigate the effects of plant growth regulators BA, ABA and IAA on shoot

regeneration from cotyledonary explants of our honeydew genotype, we conducted a regeneration test with four different combinations of BA, ABA and IAA in MS basal medium (Table 4).

On the media without IAA (M1 and M2), cotyledonary explants did not curve up from the medium surface, which would help to increase the efficiency of kanamycin selection to prevent false positive shoots from regenerating on selection medium. Media with ABA (M2 and M4) had produced more shoots than media without ABA (M1 and M3). This result was in agreement with Ficcadenti and Rotino (1995) that addition of ABA remarkably induced more shoots regeneration from cotyledons. Furthermore, addition of IAA increased formation of white friable callus which hampered shoot regeneration and effective selection by kanamycin due to thickened explants (Table 5 and Fig. 8).

**Table 5** The frequency of initial shoot regeneration in the first 3 weeks on the different media and the polyploidy estimation of the regenerants

| Medium[z] | No. of the explant tested | Percentage of responding explant | No. of shoots per responding explant | Percentage of white friable callus on explants | No. of the shoots tested for ploidy | Percentage of tetraploidy or mixoploidy |
|---|---|---|---|---|---|---|
| M1 | 24 | 75 b | 2.4 b | 62.5 bc | 7 | 57.1 |
| M2 | 24 | 95.8 a | 15.1 a | 33.3 c | 12 | 50.0 |
| M3 | 24 | 91.7 ab | 5.9 b | 95.8 a | 5 | 60.0 |
| M4 | 24 | 100 a | 13.4 a | 79.2 ab | 8 | 50.0 |

[z] M1 (MS basal+1 mg $l^{-1}$ BA), M2 (MS basal+1 mg $l^{-1}$ BA+0.26 mg $l^{-1}$ ABA), M3 (MS basal+1 mg $l^{-1}$ BA+0.8 mg $l^{-1}$ IAA) and M4 (MS basal+1 mg $l^{-1}$ BA+0.26 mg $l^{-1}$ ABA+0.8 mg $l^{-1}$ IAA)

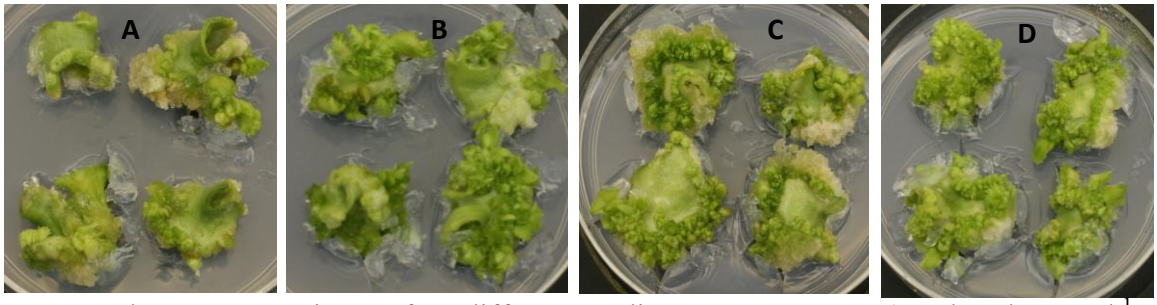


**Fig. 8** Shoot regeneration on four different media: M1 - M4. **a** M1 (MS basal+1 mg $l^{-1}$ BA), **b** M2 (MS basal+1 mg $l^{-1}$ BA+0.26 mg $l^{-1}$ ABA), **c** M3 (MS basal+1 mg $l^{-1}$ BA+0.8 mg $l^{-1}$ IAA), **d** M4 (MS basal+1 mg $l^{-1}$ BA+0.26 mg $l^{-1}$ ABA+0.8 mg $l^{-1}$ IAA)

Shoot elongation and rooting

Like cantaloupe 'F39', regenerated shoots of honeydew '150' on medium M4 also proliferated but did not elongate on MS basal medium supplemented with BA levels higher than 0.1 mg $l^{-1}$ (see Chapter II). The same series of low BA concentrations, 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$, was examined on '150' for shoot elongation. Treatments below 0.1 mg $l^{-1}$ of BA prohibited the regeneration of multiple shoots. BA 0.01 mg $l^{-1}$ allowed shoots to elongate (Fig. 9) and produce roots in 2-3 weeks.

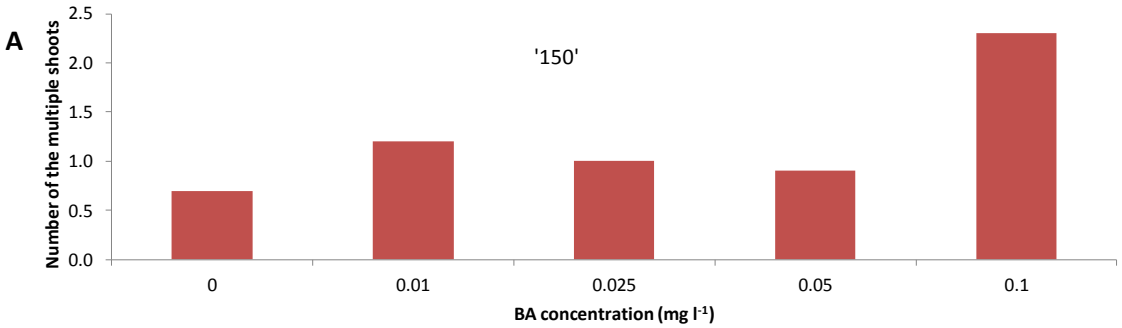


**Fig. 9** Shoot elongation and rooting of melon '150' on MS basal medium supplemented with various BA concentrations. **a** Multiple shoot regeneration on BA 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$, **b** shoot elongation on BA 0, 0.01, 0.025, 0.05 and 0.1 mg $l^{-1}$

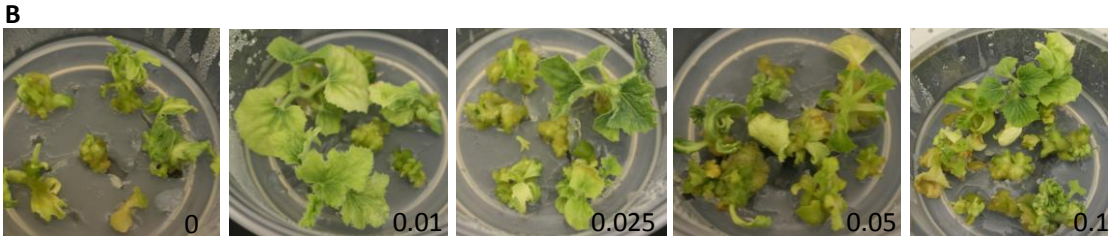


**Fig. 9** Continued

Ploidy determination of regenerants

Polyploidy was a common problem of plants regenerated from cotyledon explants of diploid melon from *in vitro* culture. After acclimatizing the regenerated plants in soils, we randomly chose 32 plants to analyze their ploidy levels using flow cytometry. Fifty to sixty percent of regenerants from each medium treatment were polyploid (Table 5). Overall, only 15 (46.9%) regenerants identified as a diploid and the rest 17 (53.1%) plants appeared to be a tetraploid or a mixoploid (diploid+tetraploid or tetraploid+octoploid) (Fig. 10). A diploid histogram was identical to a seed-grown diploid control plant having more than 50% of diploid cells (Fig. 11a-b). A tetraploid was scored when having more than 50% of tetraploid cells (Fig. 11c). Mixoploid was determined by the percentage of diploid, tetraploid and octoploid cells (Fig. 11d-e). The 4C peak in a diploid (Fig. 11a-b) probably represented the dividing 2C cells; the 8C peaks in a tetraploid and a mixoploid (2C+4C) (Fig. 11c-d) were possibly from dividing 4C cells. To confirm the ploidy level of a mixoploid (4C+8C) sample, the cytometry was reset to detect higher ploidy peaks (Fig. 11e). No 16C peak was found in this sample (Fig. 11f).

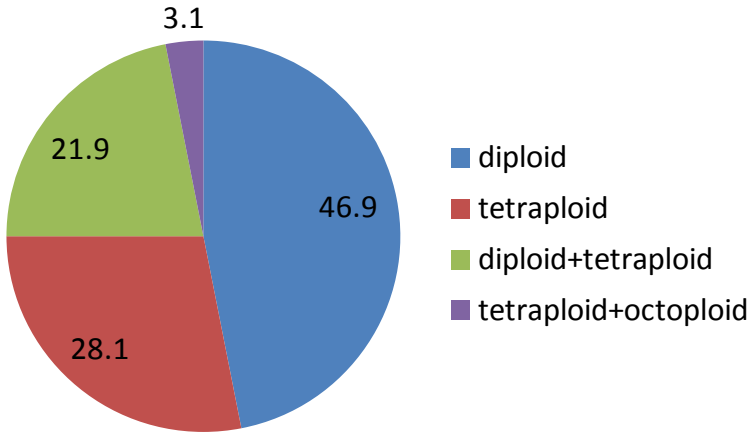


**Fig. 10** The polyploidy percentage of plants regenerated from cotyledonary explants of honeydew '150'

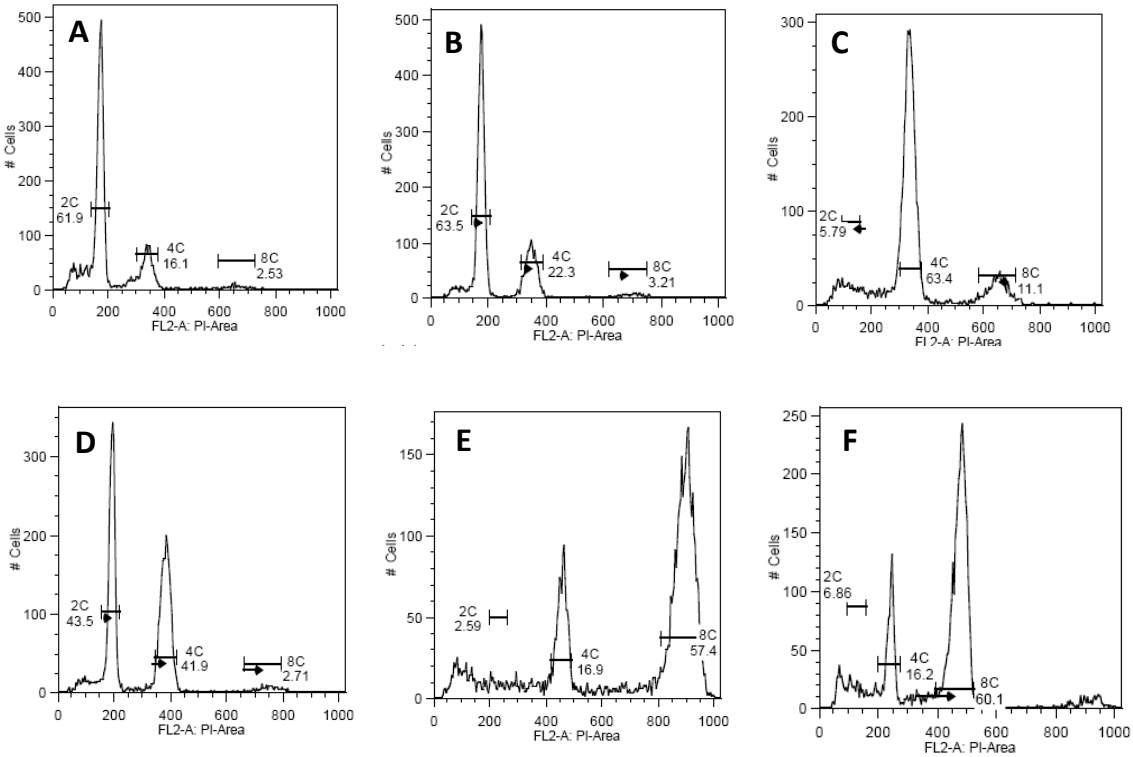


**Fig. 11** Flow cytometry histograms of leaf tissues from *in vitro* regenerated honeydew '150' plants. **a** Diploid from seed-grown plant, **b** diploid, **c** tetraploid, **d** 2C+4C mixoploid, **e** 4C+8C mixoploid, **f** reset cytometry to detect 16C cells in the 4C+8C mixoploid

Higher polyploidy percentage in melon regenerants was reported by Guis et al. (2000). Plants regenerated from 2-day-old cotyledon explants of *Cucumis melo* L. var. *Cantalupensis* (cv. Védrantais) had more than 80% of tetraploids. Compared with plants regenerated from somatic embryos and adventitious shoots, those regenerated from shoot primordia had a much lower frequency of tetraploidy: somatic embryo 31%, adventitious shoots 30%, and shoot primordia 4% (Ezura et al. 1992 and 1997b). Our results showed that cotyledon tissue of honeydew melon also has a high tendency to induce polyploid shoots during the tissue culture process. To maintain ploidy stability, shoot tip as another explant type for honeydew regeneration needs to be investigated.

Morphology of polyploid regenerants

Extreme morphologic changes didn't occur in the polyploid plants except that thickened leaves have been observed from some of them (Table 6 and Fig. 12). Nuňez-Palenius et al. (2008) described many characteristics that appeared in tetraploid melon plants, such as "large male and hermaphrodite flowers, protruding stigmas, thickend and leathery leaves, short internodes, flat fruits". In our study, large flowers and protruding stigmas appeared on both tetraploid and diploid regenerated plants as well as diploid seed-grown control plants, which may be caused by the environment. We found a high percentage of empty-embryo seeds (80-90%) formed in the fruits from both diploid and polyploid regenerated plants but not from the seed-grown control plant. The polyploid fruits' shape, size and total soluble solid (TSS) contents were similar to diploid fruits (Table 6 and Fig. 12b). Round-shaped seeds were observed in both diploid and polyploid

**Table 6** Morphological characteristics of diploid, tetraploid and mixoploid regenerants of honeydew '150'

| Ploidy | No. of the plants observed | No. of plants having thickened leaves | No. of plants having large flowers | No. of the fruits observed | No. of fruits having round seeds | TSS range (Brix) [means] |
|---|---|---|---|---|---|---|
| Diploid | 15 | 0 | 7 | 13 | 2 | 7.5 - 13.5 [10.4] |
| Tetraploid | 7 | 3 | 2 | 6 | 3 | 8.5 - 11.0 [9.9] |
| Mixoploid (2C+4C and 4C+8C) | 8 | 1 | 0 | 8 | 3 | 6.5 - 13.0 [9.9] |

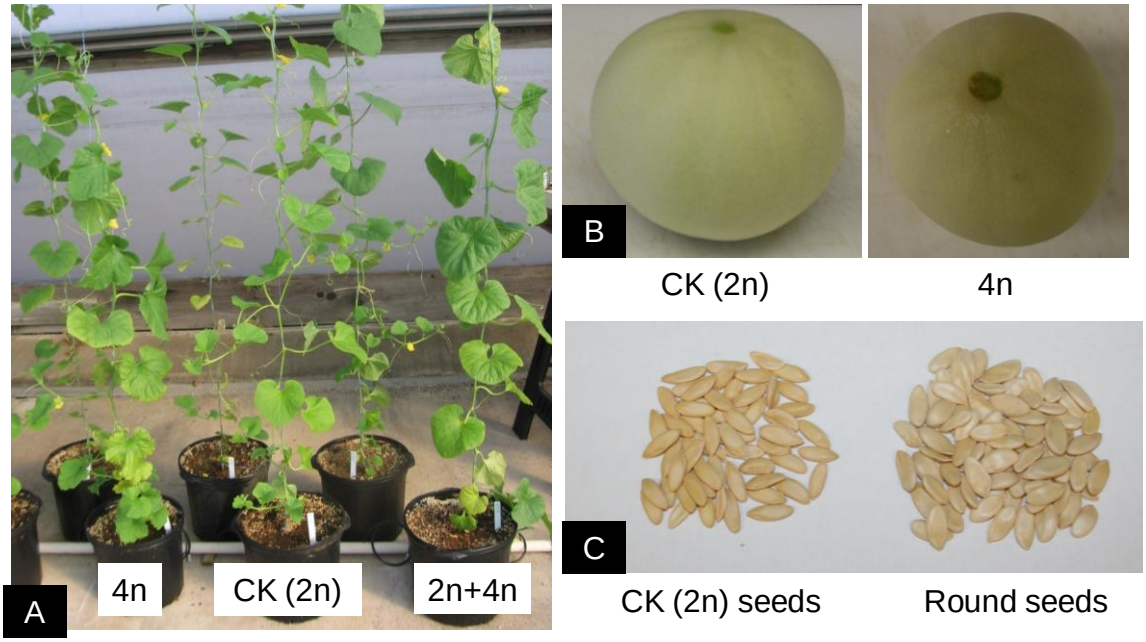


**Fig. 12** Morphological characteristics of polyploid regenerants, their fruits and seeds. **a** Regenerated plants growing in a greenhouse, **b** a fruit harvested from a diploid plant (left) and a tetraploid plant (right), **c** seeds harvested from a diploid plant (left) and regenerated plants (right). CK (2n) is diploid seed-grown plant as a control

regenerated plants (Fig. 12c), which was possibly due to the tissue culture process and/or environments. Normal fruit characteristics of polyploid honeydew may help their

acceptability in the market, not like other polyploid *C. melo* variants having reduced productivity and impeded marketability due to the low fruit quality (Guis et al. 2000).

## Conclusion

We solved the curving problem of cotyledonary explants during tissue culture by removing IAA from the medium without reducing the frequency of regeneration. Thus, an optimal regeneration system has been established for honeydew '150'. Our research proceeded to develop a transformation protocol for honeydew '150' based on this high efficiency shoot regeneration system. In addition, we observed the ploidy level of the regenerated plants and the morphologies of polyploid plants. Although 50-60% of regenerated plants has an increased ploidy level, no extremely aberrant morphology was found to impact the growth and development of polyploid plants. Further studies are needed to maintain ploidy level in honeydew regeneration by using other types of explants.

# CHAPTER IV

# ESTABLISHMENT OF TRANSFORMATION SYSTEMS FOR CANTALOUPE LINE 'F39' AND HONEYDEW LINE '150'*

## Materials and Methods

### Plant materials and explant preparation

Genotypes and their cotyledonary explants were prepared by the same method described in Chapter II.

### Preparation of *Agrobacterium tumefaciens*

*A.tumefaciens* strains EHA105 and LBA4404 carrying pCNL56, harboring a CaMV 35S promoter for constitutive gene expression of neomycin phosphotransferase II (*nptII*) and *gusA*/intron genes (Li et al. 1992), as well as *A.tumefaciens* strain EHA105 carrying pBI121, harboring a CaMV 35S promoter for constitutive gene expression of neomycin phosphotransferase II (*nptII*) and *gusA* genes (Clontech), were both used in transformation studies. A single colony of *A. tumefaciens* was inoculated in 3 ml of YEP liquid medium supplemented with 50 mg $l^{-1}$ kanamycin and 20 mg $l^{-1}$ rifampicin and

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* Part of this chapter is reprinted with permission from "*Agrobacterium* -mediated transformation and shoot regeneration in elite breeding lines of western shipper cantaloupe and honeydew melons (*Cucumis melo* L.)" by Ren Y, Bang H, Curtis IS, Gould G, Patil BS, Crosby KM (2011) Plant Cell Tiss Organ Cult Online First™, 8 Sep 2011 , Copyrigtht 2011 by Springer.

grown at 200 rpm at 28 ºC for 24 h. The 3 ml culture was added to 20 ml of YEP liquid medium supplemented with the same concentrations of antibiotics and cultured for 3 to 5 h until an $OD_{600}$ reached between 0.7 and 1.0. Bacterial cells were collected using centrifugation at 4000 rpm for 10 min at room temperature and then re-suspended in liquid RM4 medium supplemented with 100 µM acetosyringone.

Inoculation, co-cultivation and light test

Cotyledonary explants were pre-cultured on RM4 medium for 2 d and then inoculated by immersion in the *A. tumefaciens* suspension for 10 min. They were removed from the suspension, dried on sterile filter paper and then transferred to RM4 medium supplemented with 100 µM acetosyringone (pH 5.5). The effect of light during co-cultivation was compared by maintaining inoculated explants on co-cultivation medium for 3 d in dark (22 ºC) or for 3 d in light (24 ºC, fluorescent desk lamp). Seven days after inoculation, GUS transient expression in cotyledonary explants was assessed.

Antibiotic testing and plant regeneration

To determine sensitivity of cotyledonary explants to kanamycin, both cantaloupe 'F39' and honeydew '150' genotypes were tested in RM4 medium with various kanamycin concentrations: 0, 100, 125, 150, 175 and 200 mg $l^{-1}$. Appropriate concentrations were determined for each genotype. To eliminate *A. tumefaciens* overgrowth during selection, the effects of Clavamox® (amoxicillin trihydrate/clavulanate potassium tablets, Pfizer Animal Health) and Timentin™ (ticarcillin disodium/potassium clavulanate powders,

Duchefa Direct, St. Louis) on the regeneration of both genotypes were compared by supplementing media with 250 mg $l^{-1}$ Clavamox® or 300 mg $l^{-1}$ Timentin™ throughout the regeneration process (shoot regeneration, shoot elongation and rooting).

After co-cultivation at 22 ºC in the dark for 3 d, explants were transferred to medium RM4 containing kanamycin and 250 mg $l^{-1}$ Clavamox®, and sub-cultured every 14 d. Three to four weeks later, regenerated shoot primordium aggregates were excised into small pieces (3 mm × 3 mm) and transferred onto shoot elongation medium supplemented with 50 mg $l^{-1}$ kanamycin and 250 mg $l^{-1}$ Clavamox®. Some elongated shoots produced roots in 14 to 28 d on this medium. Large shoots that failed to produce roots were transferred to rooting medium supplemented with 50 mg $l^{-1}$ kanamycin as described by Compton et al. (2004). When root systems were well developed, plants were transferred to soil and acclimatized in the tissue culture room (24 ± 2 ºC, 16 h light/8 h dark photoperiod). Plants were then transferred to a greenhouse where they were allowed to grow to maturity.

Histochemical GUS assay, PCR and Southern blot analyses

To examine the expression of the gusA gene in putative transformed regenerants, tissues were incubated in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) staining solution at 37 ºC overnight followed by washing with 95% ethanol to remove chlorophyll (Jefferson et al. 1987).

Genomic DNA was isolated from leaf tissues of wild type and putative transformants by the method of Skroch and Nienhuis (1995). As an initial screen for

presence of the transferred gene, PCR primers were designed to amplify fragments from *nptII* under the following conditions: forward and reverse primers were 5'-CCC GGT TCT TTT TGT CAA GAC CGA CCT-3' and 5'-GTT TGC GCG CTA TAT TTT GTT TTC TAT CGC-3', respectively. The PCR reaction mixture contained 50 ng of genomic DNA, 1× PCR buffer, 200 μM dNTP, 0.2 μM forward and reverse primers and 1 μl of polymerase mix (Clontech Laboratories, Inc. Mountain View, CA, USA) in a total volume of 50 μl. The reaction consisted of an initial denaturation at 94 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 68 ºC for 30 s, and elongation at 72 ºC for 2 min, and a final elongation at 72 ºC for 10 min. Amplified products were visualized on 1% (w/v) agarose gels.

Twenty micrograms of genomic DNA was digested with *EcoR*I at 37 ºC overnight and separated by electrophoresis on a 1% (w/v) agarose gel at 24 V overnight. DNA fragments were then denatured and transferred onto an $N^+$ Hybond nylon membrane (Amersham Hybond™-N, GE Healthcare Life Sciences). A *gusA* probe was labeled using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany). The membrane was then hybridized with the DIG-labeled probe in DIG Easy Hyb solution (Roche Applied Science, Mannheim, Germany) at 45 ºC overnight. DIG-labeled nucleic acids were detected with CDP-Star (Roche Applied Science, Mannheim, Germany) by exposing the membrane under a LAS-4000 Chemiluminescent Image System (Fuji Film Life Science, USA). Probe labeling, hybridization, washing and detection were conducted according to the manufacturer's instruction.

Statistical analysis

Differences between the means were identified using Duncan's Multiple Range Test where the 5% probability level was considered significant. Kanamycin sensitivity was tested in a randomized complete block design (4 explants/dish; 8 replicates/treatment) and analyzed by one-way analysis of variance. Each dish was considered as a replicate in all the experimental designs.

## Results and Discussion

Selection for transformation

To determine the concentration of kanamycin for the selection of transformed shoots, explants were cultured on regeneration media containing a range of antibiotic concentrations (Fig. 13). *In vitro* shoot regeneration frequencies of both genotypes were approximately 90% in medium RM4 without kanamycin. Some tissues produced calli and shoot primordia on 100-150 mg $l^{-1}$ kanamycin, but failed to develop normal shoots. In 'F39' and '150', shoot regeneration was completely inhibited on 200 and 150 mg $l^{-1}$ kanamycin, respectively. These two concentrations were chosen as the selection thresholds for those genotypes.

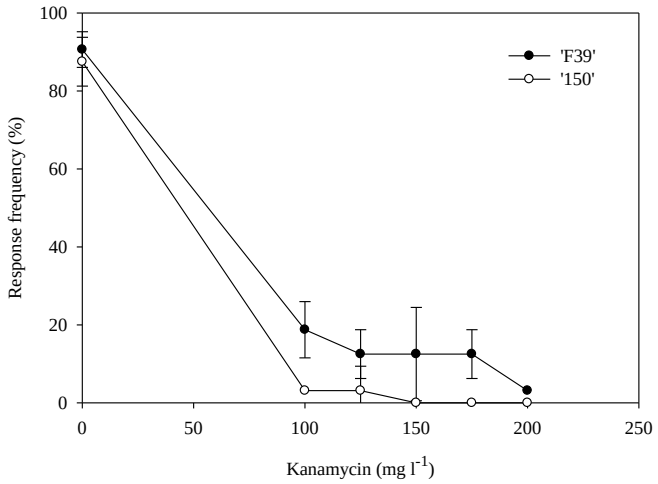


**Fig. 13** Kanamycin sensitivity assay to determine selection thresholds using non-inoculated explants of melon 'F39' and '150'. *Vertical bars* show standard errors

The beta-lactam antibiotics are used to eliminate *A. tumefaciens* from inoculated tissues. Timentin™ is one of the most expensive antibiotics used widely in transformation studies (Ieamkhang et al 2005; Slater et al 2011; Thiruvengadam et al 2011). Clavamox® is relatively inexpensive and appears to work effectively at a low dose (250 mg $l^{-1}$). Even a high one time dose of 10,000 mg $l^{-1}$ was reported to be non-toxic to plant tissues (Gould and Magallens-Cedeno 1998). Clavamox® tablets are sterile and individually packaged. The antibiotic is stable in this form and convenient to use since there is no need to prepare a stock solution. Cheng et al. (1998) reported that Timentin™ stock solution was stable only for four weeks at -20 °C or -80 °C, which is another disadvantage compared to Clavamox®. Our results indicated that Clavamox® was superior to Timentin™ with regard to the impact on tissue regeneration efficiency. Clavamox® has been compared with two other commonly used beta-lactam antibiotics,

carbenicillin and cefotaxime, on the growth of *A. tumefaciens* strain LBA4404 and the shoot regeneration of tomato. No significant differences were found (Hussain et al. 2008).

Clavamox® and Timentin™ were tested on non-inoculated explants of 'F39' and '150' to examine possible adverse effects on plant regeneration. In '150', no significant differences were detected during the early stages of shoot regeneration (first four weeks); however, inclusion of Timentin™ resulted in vitrification and/or aberrant morphology in 70% of the shoots over time (Fig. 14 and Table 7). This vitrification severely reduced shoot elongation frequency and ability to produce roots. In contrast, only 5% of the shoots cultured on the medium containing Clavamox® became vitrified and/or abnormal. This result was similar to results seen in the non-antibiotic treatments. For 'F39', Clavamox® and Timentin™ treatments failed to show any significant differences in shoot regeneration and production of abnormal types, suggesting the effects of these antibiotics could be genotype-dependent.

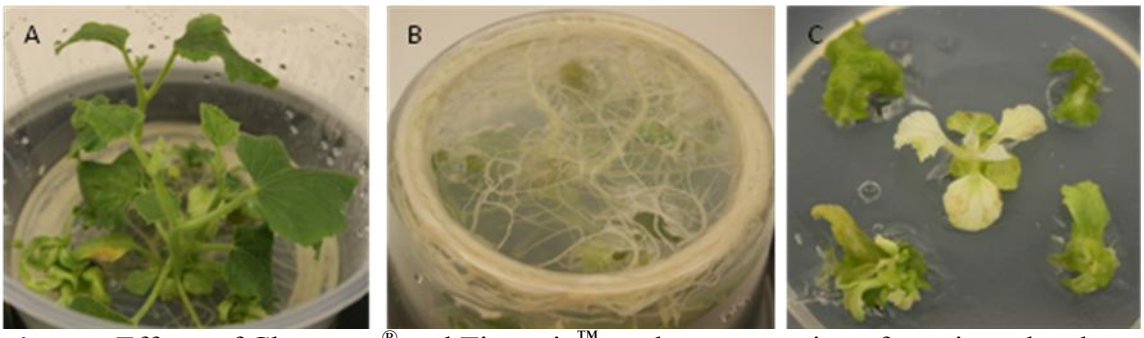


**Fig. 14** Effects of Clavamox® and Timentin™ on the regeneration of non-inoculated explants of honeydew '150' after 75 days of culture. **a** & **b** Shoot growth and rooting on the regeneration medium supplemented with 250 mg $l^{-1}$ Clavamox®, **c** with 300 mg $l^{-1}$ Timentin™

**Table 7** Effects of Clavamox® and Timentin™ on shoot development and elongation of non-inoculated explants of melon 'F39' and '150'

| Genotype | Antibiotic | No. of explants | Regeneration frequency (%) | No. of shoots per explant | Percentage of elongated /rooted shoots (%) | Aberrant /vitrified shoot (%) |
|---|---|---|---|---|---|---|
| 'F39' | Clavamox® | 16 | 97.5 a | 4.6 b | 31.1 a | 40.0 a |
| | Timentin™ | 16 | 90.0 a | 11.4 a | 30.0 a | 34.4 a |
| '150' | Clavamox® | 40 | 62.5 a | 6.8 a | 40.0 a | 5.0 b |
| | Timentin™ | 40 | 56.3 a | 8.2 a | 5.0 b | 70.0 a |

Means within the same columns within each genotype followed by different letters are significantly different by Duncan's Multiple Range Test at 5% probability level. The percentage data were transformed by an arcsin function before analysis to normalize the distribution

Like many other melon transformation reports (Dong et al. 1991; Akasaka-Kennedy et al. 2004; Wu et al. 2009), the common problem of 'escapes' was also identified in our study. By reducing explant size (6 explants per cotyledon, 3 mm × 2 mm) and increasing the volume of selection medium per Petri dish (45 ml), we were able to reduce the curling of cotyledonary explants and the incidence of 'escapes'. A similar result was obtained by Wu et al. (2009) in oriental melon transformation.

Inoculation and co-cultivation

Initially, *A. tumefaciens* strains LBA4404 and EHA105 were tested for their ability to transform explants based on GUS histochemical staining. Both strains showed fairly good inoculation efficiencies demonstrated by GUS assays (Fig. 15). EHA105 was used in subsequent studies as it was reported to be more efficient in producing stable transformants compared to LBA4404 (Galperin et al. 2003).

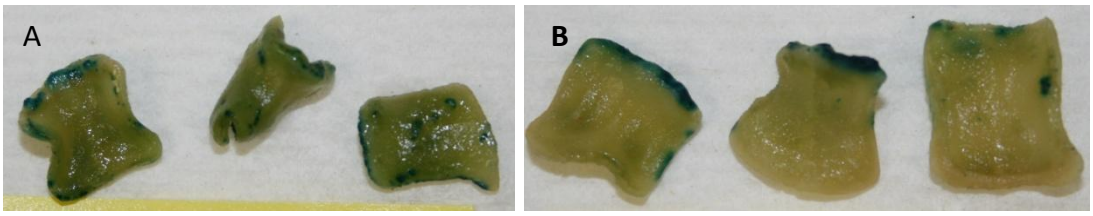


**Fig. 15** Transient GUS expression of explants inoculated with different *Agrobacterium tumefaciens* strains carrying pCNL56, 7 days after inoculation. **a** strain EHA105, **b** strain LBA4404

Previous reports of melon transformation indicated optimal inoculation times of 20 sec to 30 min (Ayub et al. 1996; Guis et al. 2000; Vallés and Lasa 1994) depending on the *Agrobacterium* strain used, concentration of the bacterial suspension, plant species, explant size and thickness. We evaluated 10, 15 and 20 min inoculation times. Three days after inoculation, the explants inoculated for 10 min remained healthy, but the longer inoculation treatments resulted in the explants becoming brown or necrotic in some cases (data not shown). Therefore, an inoculation period of 10 min was adopted.

Co-cultivation is one of the most important steps in a transformation procedure. Factors during co-cultivation can enhance transformation efficiency, i.e., temperature (Fullner and Nester 1996; Yasmin and Debener 2010; Sharma et al. 2011; Seo et al. 2011), lighting conditions (Zambre et al. 2003), co-cultivation period (Fang and Grumet 1990; Shilpa et al. 2010; Seo et al. 2011), addition of acetosyringone (Costa et al. 2006; Afroz et al. 2010; Sharma et al. 2011) and antioxidants (Dan et al. 2009; Olhoft and Somers 2001; Ostergaard and Yanofsky 2004; Toldi et al. 2002; Zheng et al. 2005; Kumar et al. 2011; Dutt et al. 2011). Co-cultivation periods of 2 to 6 d were reported to be optimal for melons (Galperin et al. 2003; Vallés and Lasa 1994; Akasaka-Kennedy et

al. 2004; Dong et al. 1991). Under our co-cultivation conditions at 22 ºC, *A. tumefaciens* strain EHA105 began to overgrow the explant 3 d following the inoculation (data not shown).

Zambre et al. (2003) reported a positive effect of light on gene transfer from *A. tumefaciens* to callus explants of *Phaseolus acutifolius* (tepary bean) and root explants of *Arabidopsis thaliana*. Our results showed there were no significant differences in transformation rates between light and dark treatments ($P \leq 0.05$) during co-cultivation (Table 8). Plausible reasons for this result may be: 1) the light/dark influence on *Agrobacterium*-mediated transformation may be genotype dependent; 2) the light/dark influence may depend on explant types (callus and/or root); 3) the co-cultivation temperatures of our light and dark treatments of 24 °C and 22 °C respectively, may have influenced our results. Temperatures of 19-22 ºC have been reported to be critical for high efficiency of *Agrobacterium*-mediated transformation while temperatures higher than 22 ºC dramatically decreased this efficiency (Fullner and Nester 1996).

**Table 8** Effect of light condition on GUS transient expression during the co-cultivation period in cotyledonary explants of 'F39' and '150'

| Genotype | Light condition | No. of explants analyzed | No. of explants stained blue | GUS positives (%) |
|---|---|---|---|---|
| 'F39' | Light | 80 | 18 | 22.8 a |
| | Dark | 80 | 30 | 38.5 a |
| '150' | Light | 80 | 74 | 92.5 a |
| | Dark | 80 | 70 | 87.5 a |

Means within the same columns within each genotype followed by same letters are not significantly different by Duncan's Multiple Range Test at 5% probability level. The percentage data were transformed by an arcsin function before analysis to normalize the distribution

GUS staining, PCR and Southern blot analyses

Cotyledonary explants were inoculated with *A. tumefaciens* strain EHA105. A total of 1075 explants of 'F39' (from two experiments) and 1205 explants of '150' (from three experiments) were used. GUS histochemical analysis was conducted throughout the selection procedure to identify putative transformants, as well as non-transformed or

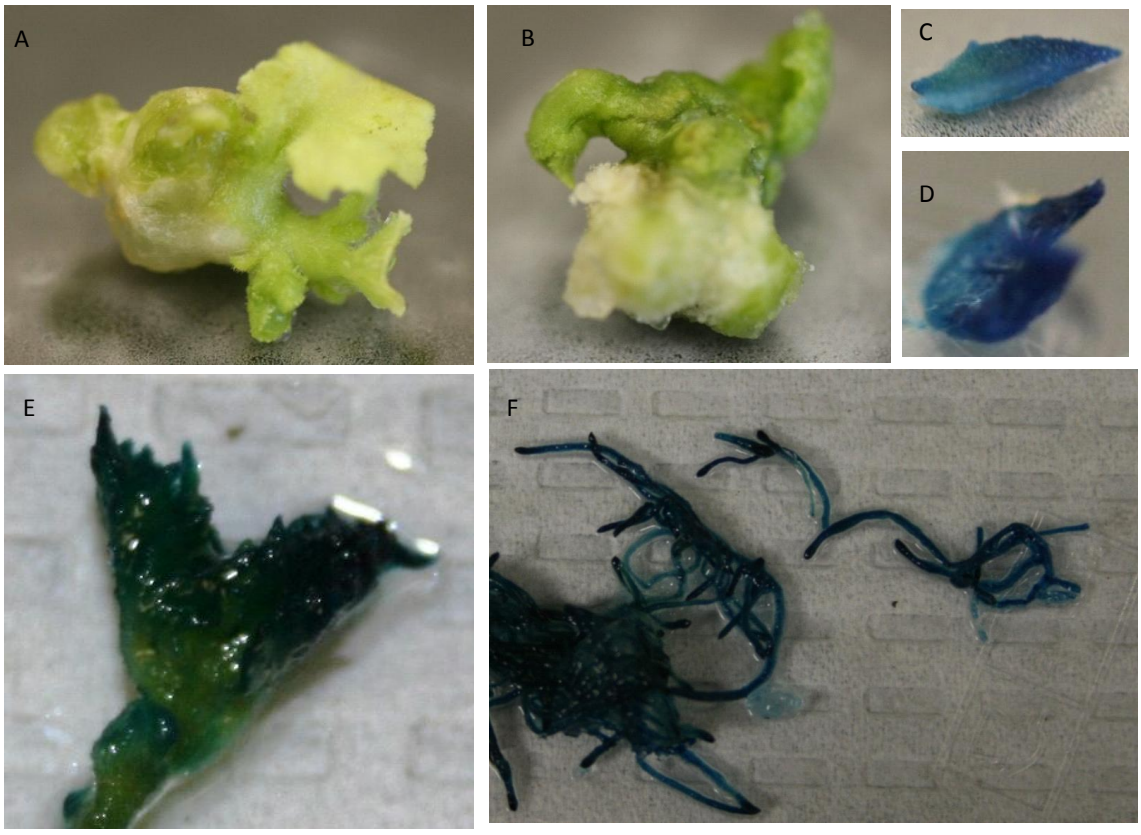


**Fig. 16** Callus and shoot development from explants and GUS histochemical assay. **a** & **c** a regenerated shoot of cantaloupe 'F39' before (a) and after (c) GUS staining, **b** & **d** a cotyledonary explant with callus producing shoot primordia of honeydew '150' before (b) and after (d) GUS staining, **e** an apical shoot of a GUS-positive plant of '150', **f** GUS expression in roots of a kanamycin-resistant plant of '150'

chimeric tissues. Isolated pieces of leaf tissue from regenerating shoots were examined for GUS activity (Figs. 16a-d). All GUS positive tissues were then analyzed further for expression of the GUS gene in apical shoots (Fig. 16e) and roots (Fig. 16f). During large scale GUS assays, we found a large proportion of chimeras in both genotypes (9 out of 12 samples of 'F39' and 9 out of 13 samples of '150'). Chimeric shoots exhibited blue sectors of various sizes following GUS histochemical staining as reported in other crop species (Moore 1995; Mollel et al. 2004; Kathiravan et al. 2006). Most chimeric shoots did not survive kanamycin selection and died before or during rooting.

The binary plasmid pCNL56 harbors both *nptII* and *gusA* genes. All GUS-positive plants that rooted on media containing kanamycin were selected for PCR analysis to screen for the presence of *nptII*. Genomic DNA from all rooted GUS-positive plants yielded a 1.2-kb fragment identical to the expected fragment amplified from the *nptII* gene (Fig. 17a). Plants regenerated from non-inoculated explants failed to produce a fragment. Most non-transformed shoots were killed by kanamycin selection during regeneration, elongation and rooting. Although some shoots survived kanamycin (150 and 200 mg $l^{-1}$), many failed to root in the rooting medium containing 50 mg $l^{-1}$ kanamycin. Based on PCR analysis, we found only one 'F39' and two '150' regenerated plants to be non-transgenic escapes.

Southern blot analysis was performed on all PCR-positive plants to identify genomic integration of *gusA* (Fig. 17b). Transformation efficiency was estimated using the total number of plants exhibiting genomic incorporation, divided by the total number of inoculated explants. The transformation efficiency for 'F39' ranged between 0.2%

and 0.4% (average efficiency was 0.3 ± 0.1%), and for '150' the range was between 0.2% and 0.8% (average was 0.5 ± 0.3%).

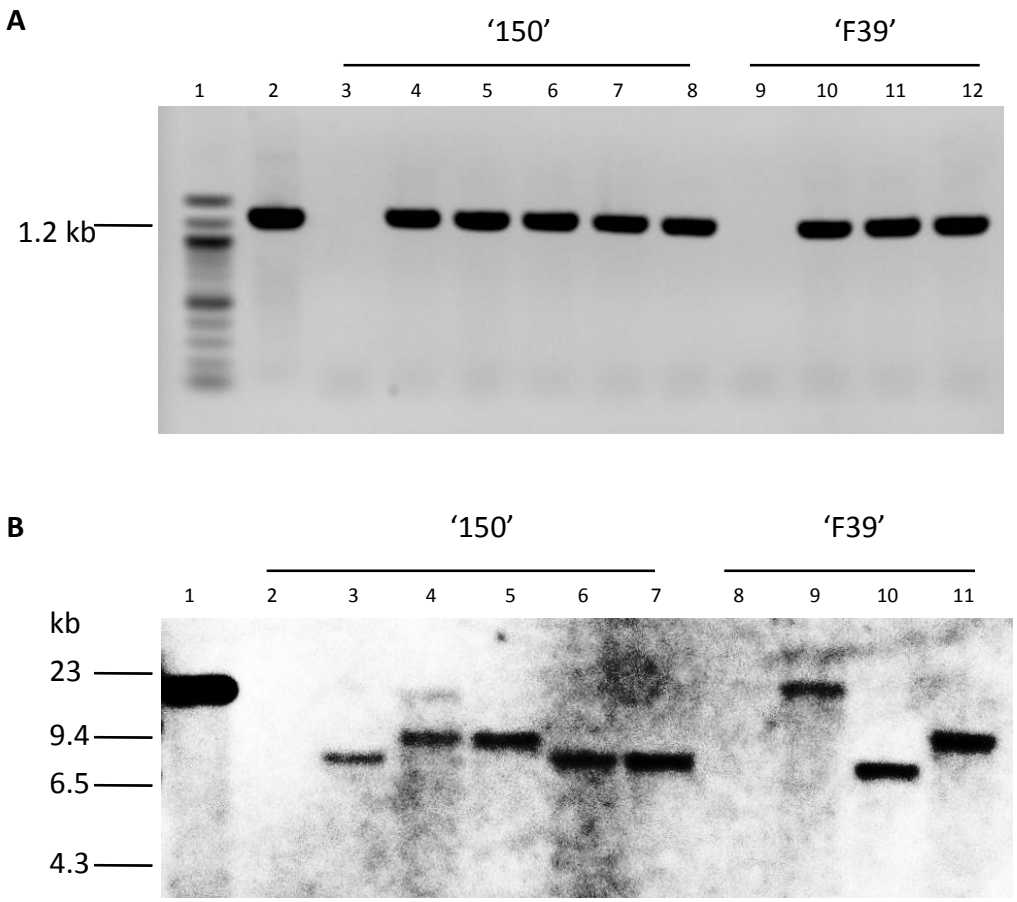


**Fig. 17** PCR and southern blot analyses of putative transformants. **a** preliminary screen for detecting the presence of the *nptII* gene in putative transformants using PCR. Lane 1: 100-bp ladder; lane 2: pCNL56 (positive control); lane 3: wild type honeydew '150' (negative control); lanes 4-8: GUS-positive plants of honeydew '150'; lane 9: wild type cantaloupe 'F39' (negative control); lanes 10-12: GUS-positive plants of cantaloupe 'F39', **b** integration of the *gusA* gene as detected by Southern blotting. Lane 1: pCNL56 (positive control); lane 2: wild type honeydew '150' (negative control); lanes 3-7: PCR-positive plants of honeydew '150'; lane 8: wild type cantaloupe 'F39' (negative control); lanes 9-11: PCR-positive plants of cantaloupe 'F39'

Phenotypes of regenerated transformants

Abnormal morphological characteristics were observed in the transgenic melon plants (Fig. 18a-g). The most severe phenotype was a lack of apical dominance (Fig. 18a)

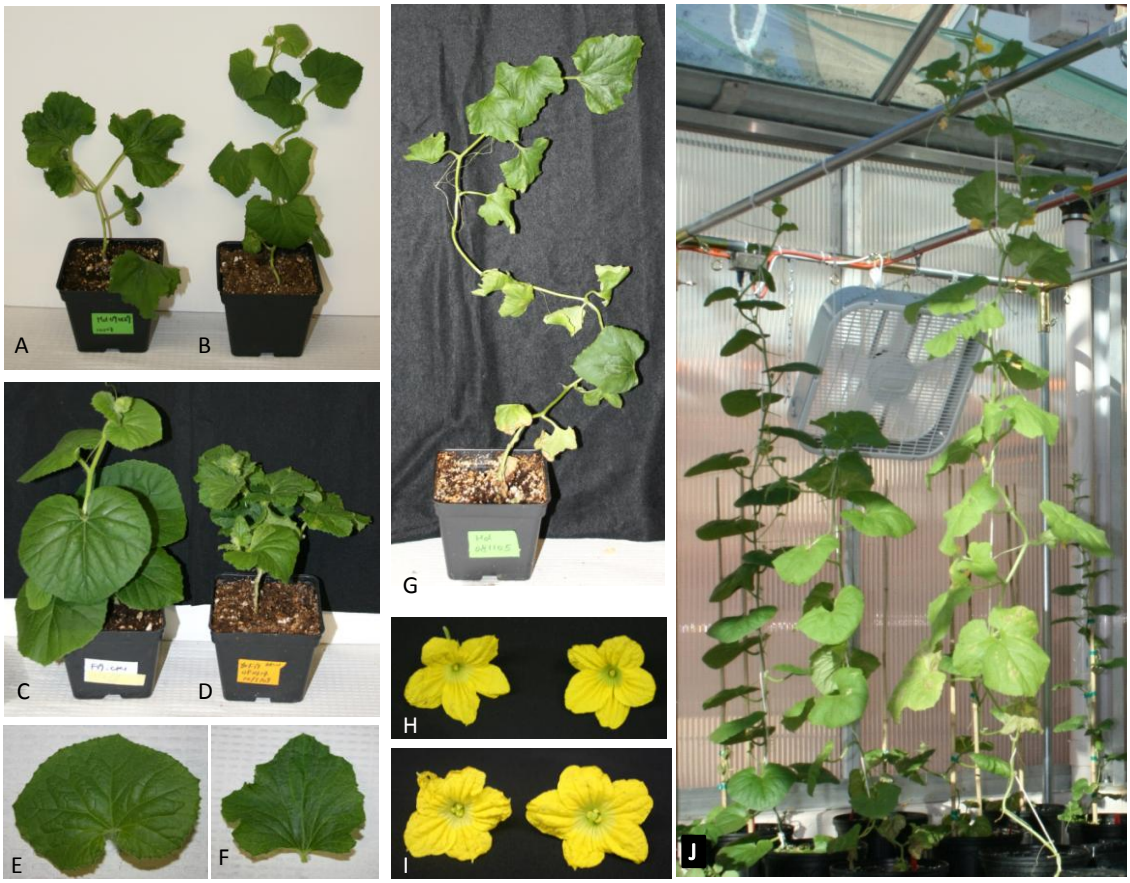


**Fig. 18** Morphological characteristics of transgenic melon plants. **a** transgenic honeydew line '090129' showing a lack of apical dominance, **b** wild type honeydew '150', **c** wild type cantaloupe 'F39', **d** transgenic cantaloupe line '082417', **e** a leaf of wild type cantaloupe 'F39', **f** a leaf of transgenic cantaloupe line '082417', **g** transgenic honeydew line '081105', **h** male flowers of wild type (left) and transgenic (right) honeydew '150', **i** perfect flowers of wild type (left) and transgenic (right) honeydew '150', **j** transgenic melon plants in the greenhouse

resulting in abnormal growth. Other aberrant morphologies included shorter (Fig. 18d) and longer (Fig. 18g) internodal growth and irregular leaf shapes (Fig. 18f). Plants exhibiting lack of apical dominance and shorter/longer internodes died prematurely in the greenhouse. Morphologically normal transgenic plants produced both male (Fig. 18h) and perfect (Fig. 18i) flowers.

Similar abnormalities reported in western shipper melon 'Topmark' and other melon types were suggested to be caused by a high kanamycin concentration (150 mg $l^{-1}$) in the selection medium (Gonsalves et al. 1994). In our study, kanamycin at 200 and 150 mg $l^{-1}$ was used for *in vitro* selection of 'F39' and '150' respectively, similar to the levels reported by Gonsalves et al. (1994). However, in our study, abnormal *in vitro* regenerated wild-type plants were also observed in the absence of kanamycin (data not shown). This observation suggests that the tissue culture process and plant growth regulator effects may induce aberrant morphology in regenerated melon shoots (Larkin and Scowcroft 1981).

Pre-culture test with pBI121

We used EHA105 harboring the binary vector pCNL56 for the protocol development. However, the gene of interest for our future study was cloned into the binary vector pBI121 harbored by EHA105. By testing the protocol with this construct, we found that GUS transient expression levels in both melon lines were much lower than those inoculated with pCNL56 (Fig. 19a-b). Similar results were reported for *Agrobacterium*-mediated transformation in finger millet (Sharma et al. 2010). To adjust our protocol for

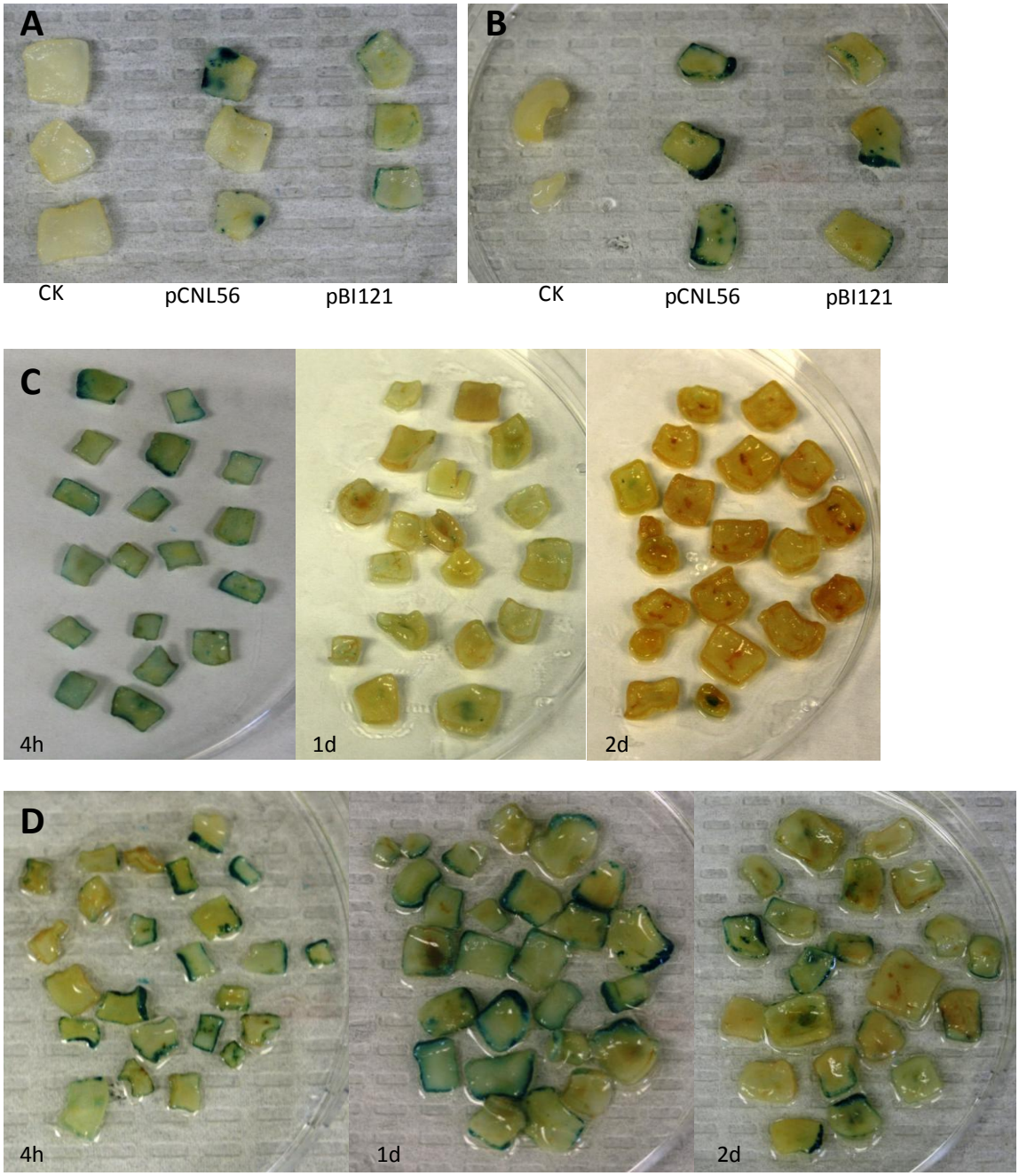


**Fig 19** Transient GUS expression of cotyledonary explants of 'F39' and '150' inoculated with *Agrobacterium tumefaciens* strain EHA105 carrying two different plasmids. Data was recorded 8 days after inoculation. **a** Plasmid pCNL56 vs. pBI121 in 'F39', **b** plasmid pCNL56 vs. pBI121 in '150', **c** 'F39' inoculated with pBI121 for 4 h, 1 d and 2 d, **d** '150' inoculated with pBI121 for 4 h, 1 d and 2 d

pBI121 use, different pre-culture conditions were tested using cotyledonary explants of both lines: 1 d hardening (growing the seedlings under lights on the germination medium for 1 d before preparing cotyledonary explants) + 4 h on liquid RM4 medium, 1 d and 2 d on solid RM4. Based on the degree of the GUS transient expression (Fig. 20), 4 h pre-culture was remarkably better than 1 d and 2 d pre-cultures for 'F39' (Fig. 19c), but 1 d was better than 4 h and 2 d treatments for '150' (Fig. 19d and Table 9). Overall, GUS transient expression level in '150' was higher than that in 'F39' when inoculated with EHA105 carrying both plasmids pCNL56 and pBI121 (Fig. 19). This result indicated binary vector, plant genotype and pre-culture condition could also significantly affect melon transformation efficiency. In addition, 'F39' is a climacteric type while '150' is a non-climacteric type. Low inoculation efficiency of *A. tumefaciens* may be due to ethylene production in wounded tissues of 'F39' (Nonaka et al. 2008).

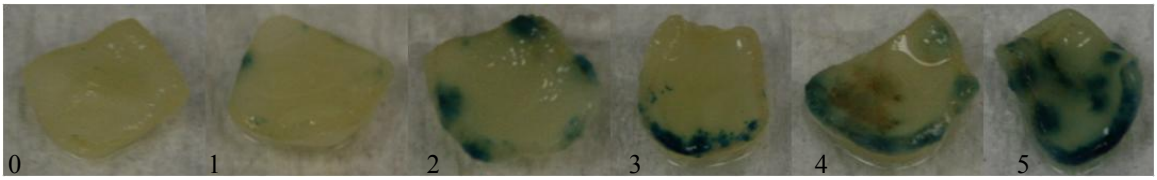


**Fig. 20** Evaluation of the degree of GUS transient expression in cotyledonary explants of 'F39'. Degree index: 0-5

**Table 9** Effects of different pre-culture periods on GUS transient expression in cotyledonary explants of '150'

| Pre-culture period | No. of explants examined | No. of GUS-positive explants | No. of the explants showing GUS expression | | | | | | Infection frequency (%) | Infection index[y] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | | |
| 4 h | 24 | 23 | 1 | 2 | 1 | 5 | 7 | 8 | 96 | 3.63 |
| 1d | 26 | 26 | 0 | 1 | 0 | 2 | 8 | 15 | 100 | 4.38 |
| 2d | 21 | 20 | 1 | 3 | 4 | 5 | 4 | 4 | 95 | 2.95 |

[z] The intensity of GUS transient expression was categorized into 5 classes (see Fig 20): degree index 0, explants without a blue area on cut surface; index 1, explants with blue dots on cut surface; index 2, explants with small blue spots on cut surface; index 3, explants with blue patches on cut surface; index 4, explants of which the entire cut surface was stained blue; index 5, explant of which the entire cut surface was strongly stained blue. GUS staining was scored only in the cutting region. [y] The infection index was calculated by the formula: [Σ (No. of the infected explants × their corresponding degree of GUS expression)] / No. of GUS-positive explants

## Conclusion

We developed a genetic transformation protocol for two elite breeding lines of melon: western shipper cantaloupe 'F39' and honeydew '150'. The protocol developed here served to initiate a successful transformation of '150' with a phytoene synthase gene to increase β-carotene concentration in fruit. However, the protocol presented here cannot be considered routine. Further studies are needed to minimize the incidence of chimeras and abnormal plant development.

# CHAPTER V

# GENETIC ENGINEERING OF BETA-CAROTENE PRODUCTION IN HONEYDEW

## Materials and Methods

### Agrobacterium inoculation and plant transformation

*Agrobacterium tumefaciens* strain EHA105 carrying a binary vector, PSYC/pRD12, harboring an *nptII* and a *PSY-C* genes was used. We isolated a *PSY-C* gene from a watermelon flesh and ligated into pRD12 having a 4.8-kb polygalacturonase (PG) promoter and a 1.8-kb PG terminator isolated from ripe tomato fruits (Lau et al. 2009, GenBank: FJ465170.1). The binary vector pRD12 was provided by Dr. Jim Giovannoni at Cornell University (Fig. 21).

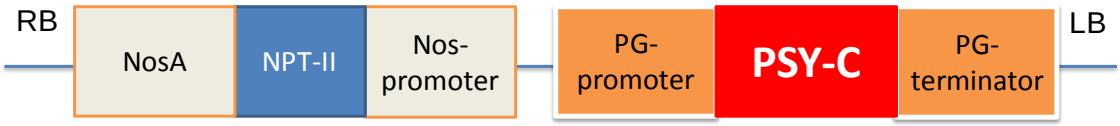


**Fig. 21** T-DNA region of binary vector PSYC/pRD12

### Ploidy determination

Ploidy level was determined by flow cytometry. Broccoli leaf sample was used as an internal control. Samples were prepared from the third leaf below shoot apex of acclimatized plants in pots using a commercial kit CyStain PI absolute P (PATTEC, Germany). Plant nuclei were analyzed on a FASCSalibur (Becton Dickinson Immunocytometry System, San Jose, CA) flow cytometer, equipped with a 15mW air-

cooled argonlaser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter. A minimum of 5,000 events, defined by a region for single nuclei in a plot of propidium iodide area versus width, were measured for each sample. Data analysis was performed in FlowJo (version 8.8.7, Treestar, Inc., Ashland, OR).

Phytoene and β-carotene extraction and analysis

Carotenoid profile was analyzed by HPLC. Rind, flesh and placental tissues were frozen at -80 °C before analysis. Approximately 50 g of frozen tissue were pulverized with a rubber mallet and mixed thoroughly while the tissue remained frozen. Two to three gram slurries were ground to fine powders in a mortar and pestle with liquid nitrogen. Powders were washed with acetone on filter paper until tissues became colorless. Hexane (30 ml) was added to the acetone extracts and distilled water was added to separate the hexane layer from the acetone/water layer. All carotenoids were collected in the hexane layer and stored at -80 °C. Hexane extracts (15ml) were evaporated with a stream of nitrogen in darkness and replenished with 1 ml acetone for HPLC analysis.

Carotenoids were analyzed using a Perkin Elmer HPLC system (Shelton, CT) equipped with a Series 200 pump, autosampler, and diode array detector. An analytical Spheriosorb ODS2 column (Waters, 4.6 × 250 mm, 5 μm) was used with a guard cartridge. Solvents A and B were acetonitrile:water (9:1, *v*/*v*) and ethylacetate, respectively, both containing 1% triethylamine. The solvent was programmed as an isocratic condition (A:B, 7:3) for 40 min at a flow rate of 1 ml/min and flushed with

100% solvent A for 5 min. A 40 µl sample was injected. Chromatographic detection was set at 280 nm and 475 nm, and spectral data were simultaneously collected from 200 to 700 nm. Phytoene (CaroteNature, Lupsingen, Switzerland) and β-carotene (Sigma-Aldrich, St. Louis, USA) standards of predetermined concentration were used to quantify phytoene and β-carotene in samples.

PCR and Southern blots

Genomic DNA was isolated from leaf tissue of wild type and putative transformants by the method of Skroch and Nienhuis (1995). To determine if transgenes were integrated, primers were designed to amplify fragments from *PG* promoter and *PSY-C* genes under the following conditions. Primers PGF02 (forward) and PSYCR02 (reverse) were 5'-TGA GAC GGG AGA AGA CAA GCC AGA CAA A-3' and 5'-CCG TTT TAC CAA AGC CGC CTG TTT CAT-3', respectively. The PCR reaction mixture contained 50 ng of genomic DNA, 1x PCR buffer, 200 µM dNTP, 0.2 µM forward and reverse primers and 1 µl Advantage2 polymerase mix (Clontech Laboratories, Inc. Mountain View, CA, USA) in a total volume of 50 µl. The reaction consisted of an initial denaturation at 94 ºC for 3 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 68 ºC for 30 s, and elongation at 72 ºC for 2 min, and a final elongation at 72 ºC for 10 min. Amplified products were visualized on 1% (w/v) agarose gels.

Twenty micrograms of genomic DNA was digested with HindIII and AvaII at 37 ºC overnight and then electrophoresed on a 1% (w/v) agarose gel at 24 V overnight, respectively. DNA fragments were then transferred onto an $N^+$ Hybond nylon membrane

(Amersham Hybond™) by alkaline transfer. A probe covering partial segments of the PG promoter and PSY-C gene was labeled using PCR DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany). The membrane was then hybridized with the DIG-labeled probe in DIG Easy Hyb solution at 45 ºC overnight. DIG-labeled nucleic acids were then detected with CDP-Star (Roche Applied Science, Mannheim, Germany) by exposing the membrane under a LAS-4000 Chemiluminescent Image System (Fuji Film Life Science, USA). Probe labeling, hybridization, washing and detection were conducted according to the manufacturer's instruction.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from rind, flesh and placenta tissues of wild type, vector control and transgenic melon fruits with RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA, Catalog No. 74904) according to the manufacturer's instruction. Then RNA was immediately converted into cDNA by priming with oligo (dT) using Advantage™ RT-for-PCR Kit (BD Biosciences Clontech, Palo Alto, CA, USA, Catalog No. 639505).

To detect *PSY-C* transcript, primers were designed to amplify the *PSY-C* under the following conditions. Forward and reverse primers were 5'-ACT GAG AAC CGC CGG AGA ATT GGA TGT-3' and 5'-TGA AGG GCC AAG GAG AGA CCT TGC ATA-3', respectively. The RT-PCR reaction mixture contained 50 ng of cDNA, 1x PCR buffer, 200 μM dNTP, 0.2 μM forward and reverse primers and 1 μl Advantage2 polymerase mix (Clontech Laboratories, Inc. Mountain View, CA, USA) in a total volume of 50 μl. The reaction consisted of an initial denaturation at 94 ºC for 3 min,

followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 68 ºC for 30 s, and elongation at 72 ºC for 2 min, and a final elongation at 72 ºC for 10 min. Amplified products were visualized on 1% (w/v) agarose gels.

Segregation analysis

Segregation of the *PSY-C* transgene and its inheritance were analyzed by germinating the seeds of $T_1$ progenies of three transgenic lines, 042201, 071506_3 and 071506_4, on the seed germination medium containing 200 mg $l^{-1}$ kanamycin. DNA was extracted from the kanamycin-survived seedlings, and then examined by PCR with primers PGF02/PSYCR02 under the conditions described before. Amplified products were visualized on 1% (w/v) agarose gels. Chi-square test was conducted to estimate the segregation ratio.

**Results and Discussion**

Generation of transgenic plants

Cotyledonary explants were inoculated with *A. tumefaciens* strain EHA105 carrying PSYC/pRD12. All regenerated plants that rooted on media containing 50 mg $l^{-1}$ kanamycin were selected for PCR analysis to screen for the presence of the *PSY-C*. Genomic DNA from all rooted kanamycin-resistant plants yielded a 500-bp fragment identical to the expected fragment amplified from the *PSY-C* gene (Fig. 22a).

Genomic DNA from wild type and all PCR-positive plants (Fig. 22a) were digested by restriction endonucleases HindIII and AvaII, respectively, and then subjected to Southern blot analysis to determine the number of copies of the *PSY-C* gene and identify where the transferred genes were incorporated into the plant's genome (Fig. 22b-c). Three sites of HindIII and two sites of AvaII were found on the plasmid (Fig. 22d). A 1.8-kb band appeared only in transgenic plants and plasmid control PSYC/pRD12 digested with HindIII, and a 9.4-kb fragment showed only in transgenic plants and plasmid control PSYC/pRD12 digested with AvaII. These two bands confirmed that *PSY-C* has been integrated into melon genomic DNA (Fig. 22b-c). Interestingly, an approximate 6.5-kb fragment was identified in transgenic plants (line 071506) digested with AvaII. It suggested that there may be an internal deletion occurred in the promoter region because they were kanamycin resistant indicating the region of *nptII* may be intact. Further investigations are needed to determine if there may be any other reasons to result in unexpected size of T-DNA insertion. The largest band appeared in line 042201 may indicate a different location of *PSY-C* integration in the melon genome (Fig. 22c).

A total of 2804 explants of '150' (from 11 experiments) were used to regenerate eight plants from four explants, becoming four transgenic lines 042201, 071503, 071506 and 071519. Lines 071503, 071506 and 071519 revealed a single copy of the *PSY-C* integrated into the genome of each plant. Two copies of the *PSY-C* exhibited in the genome of line 042201. A 6-kb band in all samples digested with HindIII and a 3-kb band in all samples digested with AvaII were detected. Based on the sequence

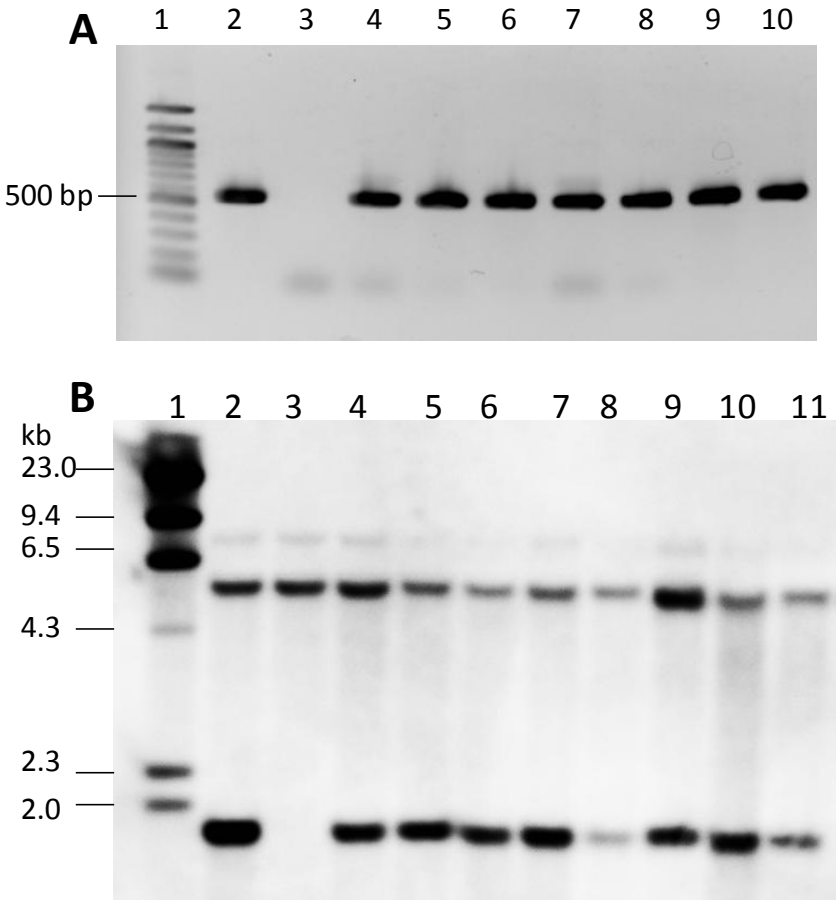


**Fig. 22** PCR and southern blot analyses of putative transformants. **a** Preliminary screen for detecting the presence of the *PSY-C* gene in putative transformants using PCR analysis. Lane 1: 100-bp ladder; lane 2: PSYC/pRD12 (positive control); lane 3: wild type honeydew '150' (negative control); lanes 4-10: kanamycin-resistant plants of honeydew '150', **b and c** integration of the *PSY-C* gene as detected by Southern blot analysis of HindIII-digested (**b**) and AvaII-digested (**c**) genomic DNA from PCR-positive plants. Lane 1: digoxigenin-labeled DNA molecular weight marker II; lane 2: PSYC/pRD12 in a wild type honeydew '150' (positive control); lane 3: wild type honeydew '150' (negative control); lanes 4: line 042201; lane 5-7: line 071503 (071503_2, -71503_3 and 071503_4); lane 8-10: line 071506 (071506_2, 071506_3 and 071506_4); lane 11: line 071519, d restriction map of the T-DNA

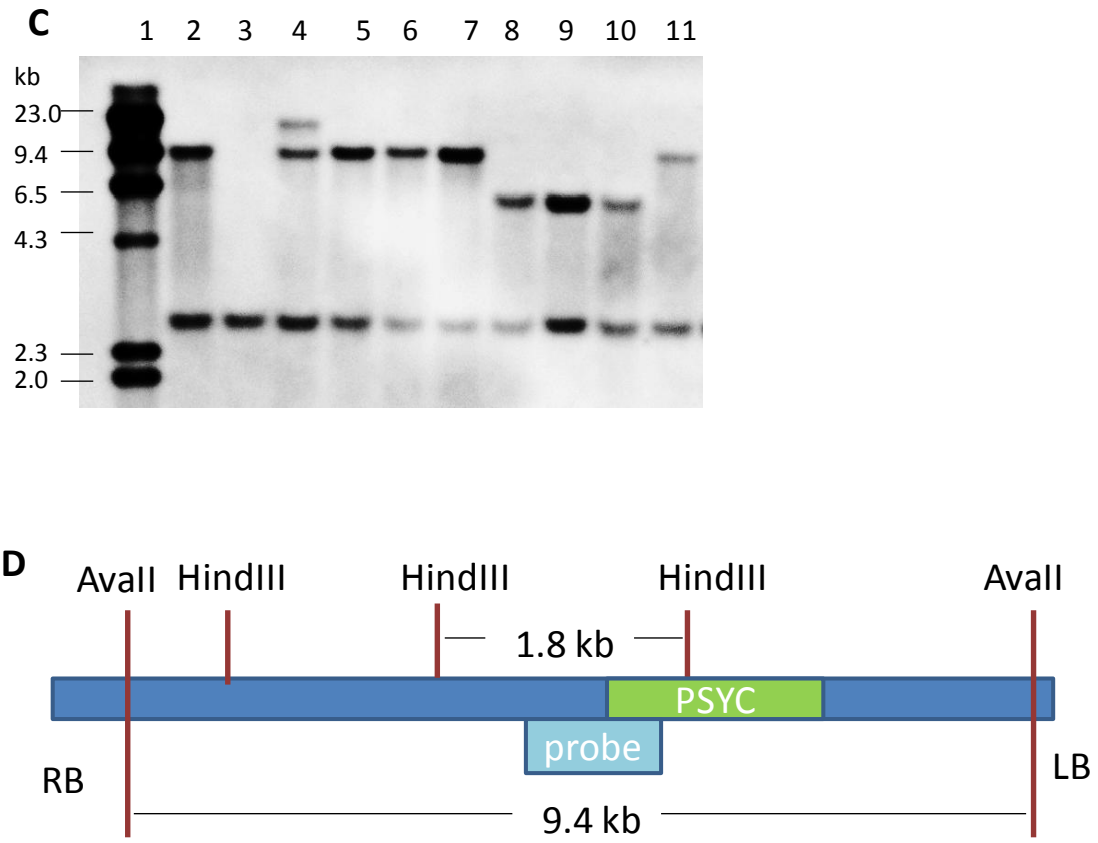


**Fig. 22** Continued

information and similarity, it was assumed to be melon *PSY* (GenBank: Z37543) which was 94% homologous to watermelon *PSY-C*.

Ploidy and morphology of transgenic plants and their fruit harvest

Flow cytometry was conducted to analyze ploidy level of transgenic melons using a broccoli leaf tissue as an internal control. In the histograms (Fig. 23), peak 'broccoli-G1' between two 'melon' peaks represented diploid broccoli cells whose genome size is

similar to triploid melon genome size. All the transgenic plants had over 50% of tetraploid cells (peak "melon-2" on 400 PI-Area) while the diploid control plant had more than 50% of diploid cells (peak "melon-1" on 200 PI-Area). Thus, 100% of the transgenic plants are tetraploids (Fig. 23b-e). The peak 'melon-2' in diploid represented the dividing melon diploid cells; the peaks on 800 PI-Area in tetraploid were from dividing melon tetraploid cells; the peaks 'broccoli-G2' on 600 PI-Area indicated dividing diploid broccoli cells.

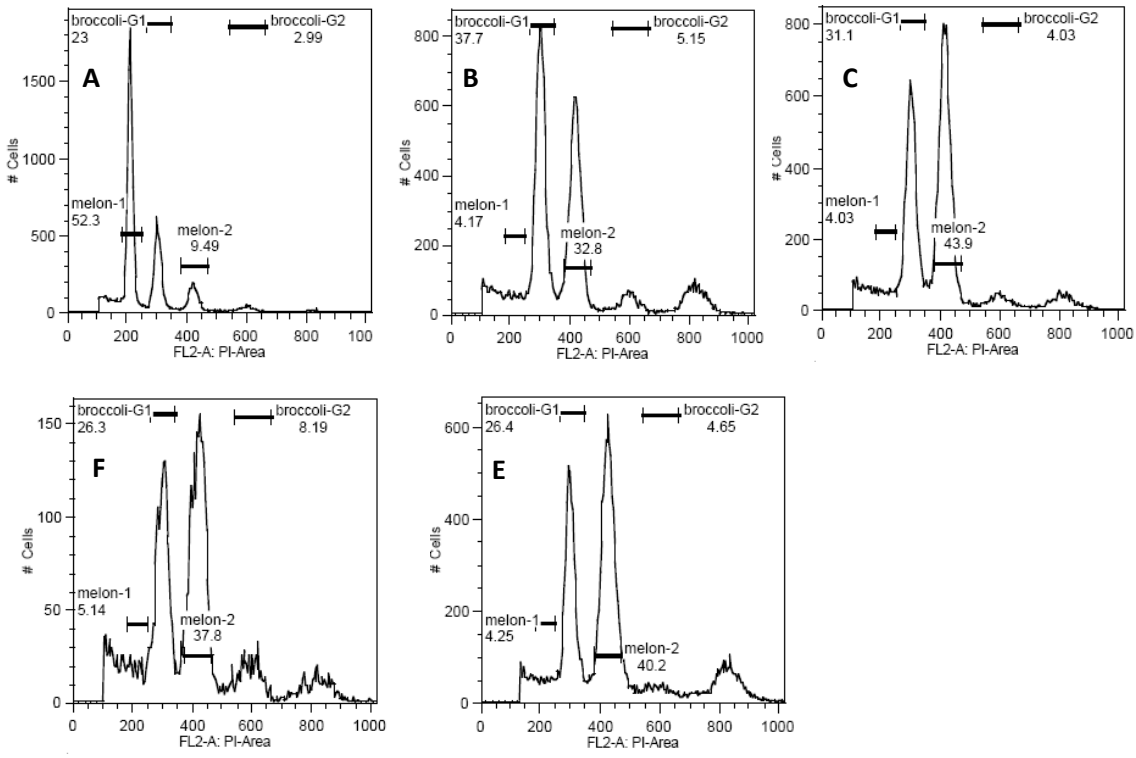


**Fig. 23** Flow cytometry histograms of leaf tissues from honeydew '150' transgenic lines. **a** A diploid from seed-grown plant, **b** 042201, **c** 071503, **d** 071506, **e** 071519

Our previous study showed the percentage of polyploids regenerated from cotyledonary explants of '150' through *in vitro* culture was 50-60% (see Chapter III). This higher frequency of tetraploids that occurred in transformants was unexpected; however, except line 071519 (Fig. 24c, left), other tetraploid transgenic honeydew plants can still grow and develop normally, which was in agreement with our early observation in non-transgenic honeydew '150' (Chapter III).

Irregular leaf shape happened in the early stage of line 042201 and 071503 (Fig. 24a), but it did not affect plant growth, flowering, fruiting and seeding. Plant size of line 071519 was smaller than those of other lines. This line grew slower on the elongation/ rooting medium containing 50 mg $l^{-1}$ kanamycin and cannot produce normal healthy roots compared to other transgenic plants. It had smaller but longer leaves and shorter internodes (Fig. 24b-c) during all the life stages and did not produce fruit. Seeds of the transgenic fruits were bigger and round compared to those of wild type, but they can normally germinate and grow to plants (Fig. 24d-e). The different shape of the seeds was also observed in non-transgenic tetraploid honeydew '150' regenerated from *in vitro* culture (see in Chapter III). No abnormal morphologies have been found in the progeny of line 042201 (Fig. 24b-c, right; Fig. 24e).

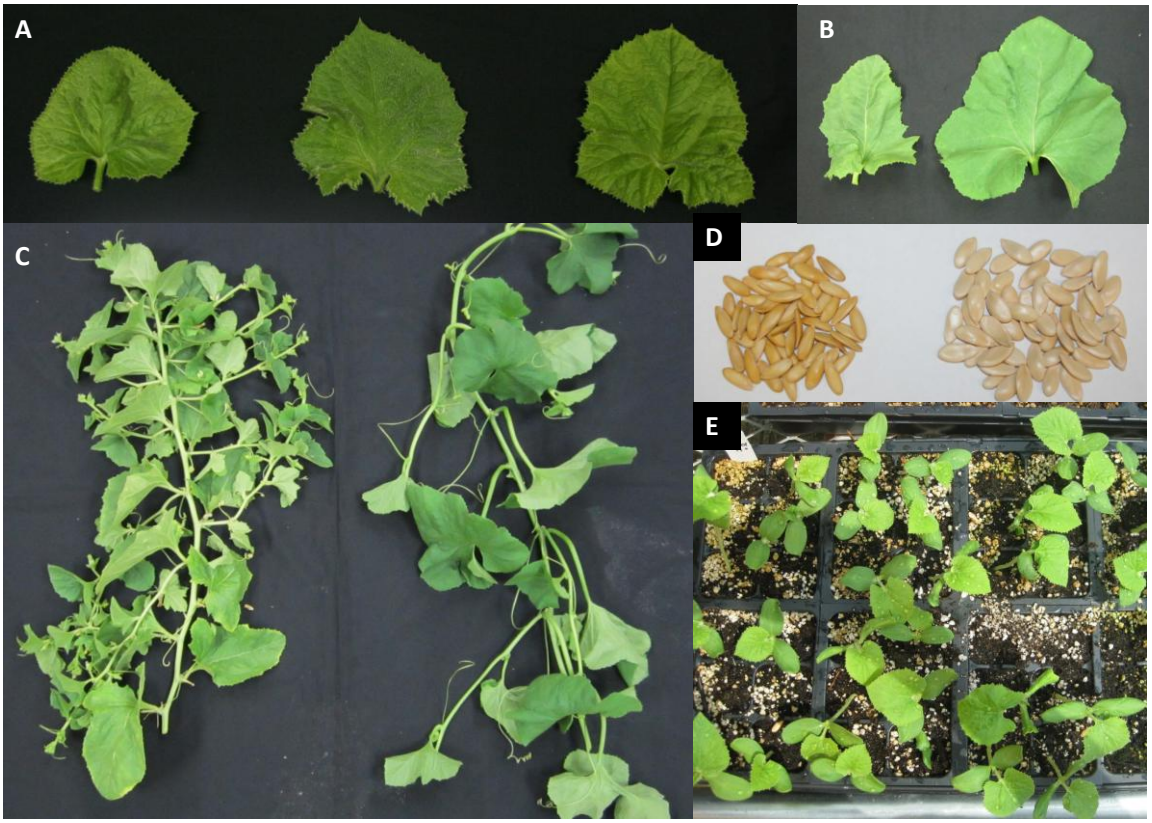


**Fig. 24** Morphologies of transgenic plants and their $T_1$ progenies. **a** Leaves of wild type '150' (left) and transgenic line 042201(middle) and 071503 (right), **b** leaves of line 071519 (left) and line 042201's progeny (right) , **c** line 071519 (left) and line 042201's progeny (right), **d** seeds of wild type '150' (left) and transgenic line 071506 (right), **e** progeny seedlings of line 042201

Fully mature fruits were harvested from non-transgenic and transgenic plants during 34-41 days after pollination (DAP); however, the fruit harvested from transgenic 071506_4 was 42 DAP but had to be cut from vine prior to color break due to an unhealthy condition. When harvested, the fruit rind was still creamy color but the area around the blossom end began to turn orange (Fig. 25). To let it reach the same ripening stage as other normally harvested fruits, the fruit was stored in a warm room (25±2 ºC under cool white fluorescent lights with 16 h light/8 h dark photoperiod and 60-80 µmol

$m^{-2}$ $s^{-1}$ light intensity) for 2 weeks before being processed for HPLC analysis and mRNA extraction.

Fruit colors and their related carotenoids

Fruit 042201 (34 DAP) had a orange-color rind and green-color flesh; fruit 071506_3 (35 DAP) had a pale orange-color rind and green-color flesh. After storing for two weeks, the rind color of fruit 071506_4 still remained creamy but the orange area around the blossom end extended a little. Wild type and vector control fruits exhibited cream-color rinds and green-color flesh (Fig. 25).

The concentrations of phytoene and β-carotene in all the tissue samples extracted from transgenic and non-transgenic melons were analyzed by HPLC (Figs. 26-28). The peaks of both phytoene and β-carotene were detected in the rind tissue of transgenic fruits; however, only trace volumes were found in the rind tissues of non-transgenic fruits and the flesh tissues of all melons. Similar peaks of β-carotene were shown in the placental tissues of transgenic and non-transgenic fruits.

Based on the calculation of HPLC data (Table 10), phytoene and β-carotene levels were similar in the flesh and placental tissues between the transgenic and non-transgenic fruits. However, the rind tissues of the transgenic fruits had remarkably higher concentrations of phytoene and β-carotene than those of wild type and vector control fruits. The highest concentrations existed in fruit 042201 rind tissue, which is in accordance with the bright orange color. In this fruit rind tissue, the phytoene content was approximately 2-fold higher than that of other transgenic fruits, and no phytoene

was detected in wild type and vector control fruits; the β-carotene dramatically increased approximately 32 fold compared to wild type, It was also 2- to 3-fold higher than other transgenic lines. Fruit 071506_3 and 071506_4 rind β-carotene contents were approximately 11- and 9-time higher than wild type, respectively.


**Fig. 25** Fruits from wild type (**a**), vector control (**b**) and transgenic plants (**c**-**e**) of honeydew '150'. **c** 042201, d 071506_3, **e** 071506_4

D E

**Fig. 25** Continued

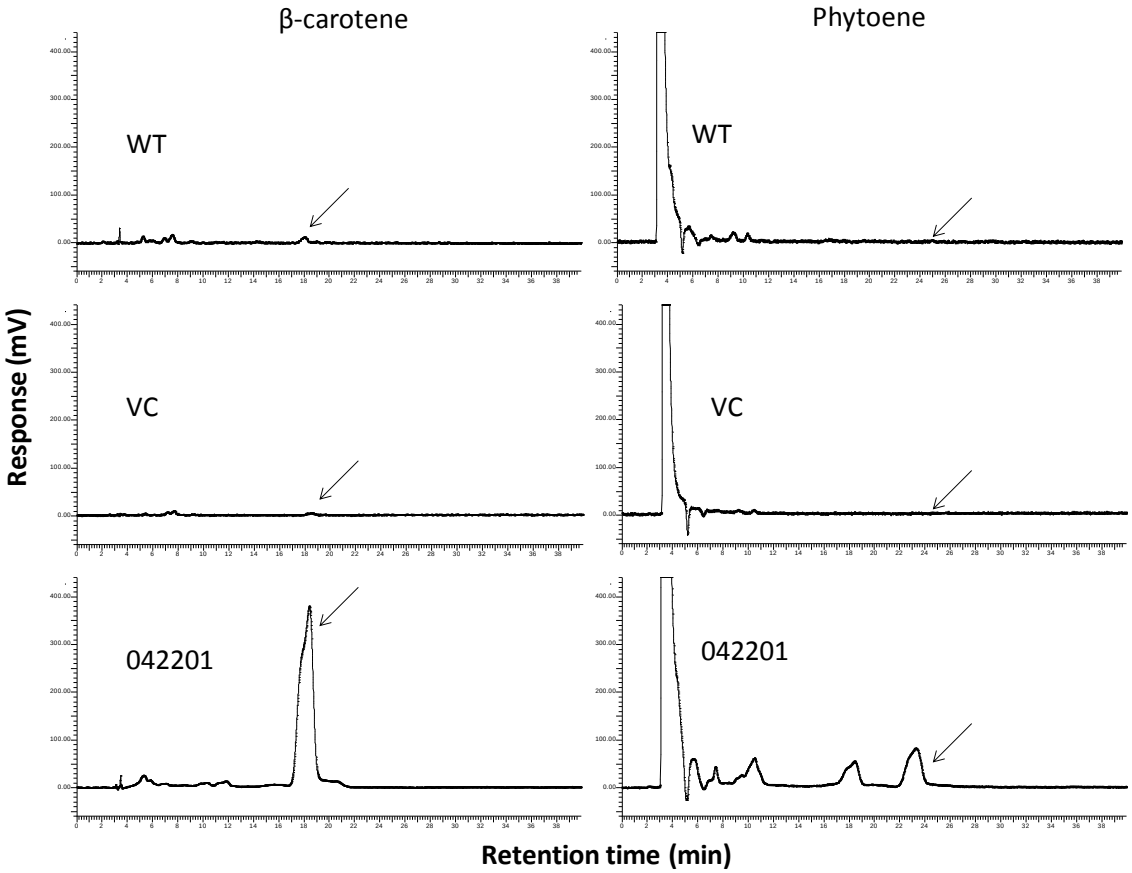


**Fig. 26** HPLC separation of carotenoids in rind tissue extractions from wild type (WT), vector control (VC) and transgenic line 042201 of honeydew '150'

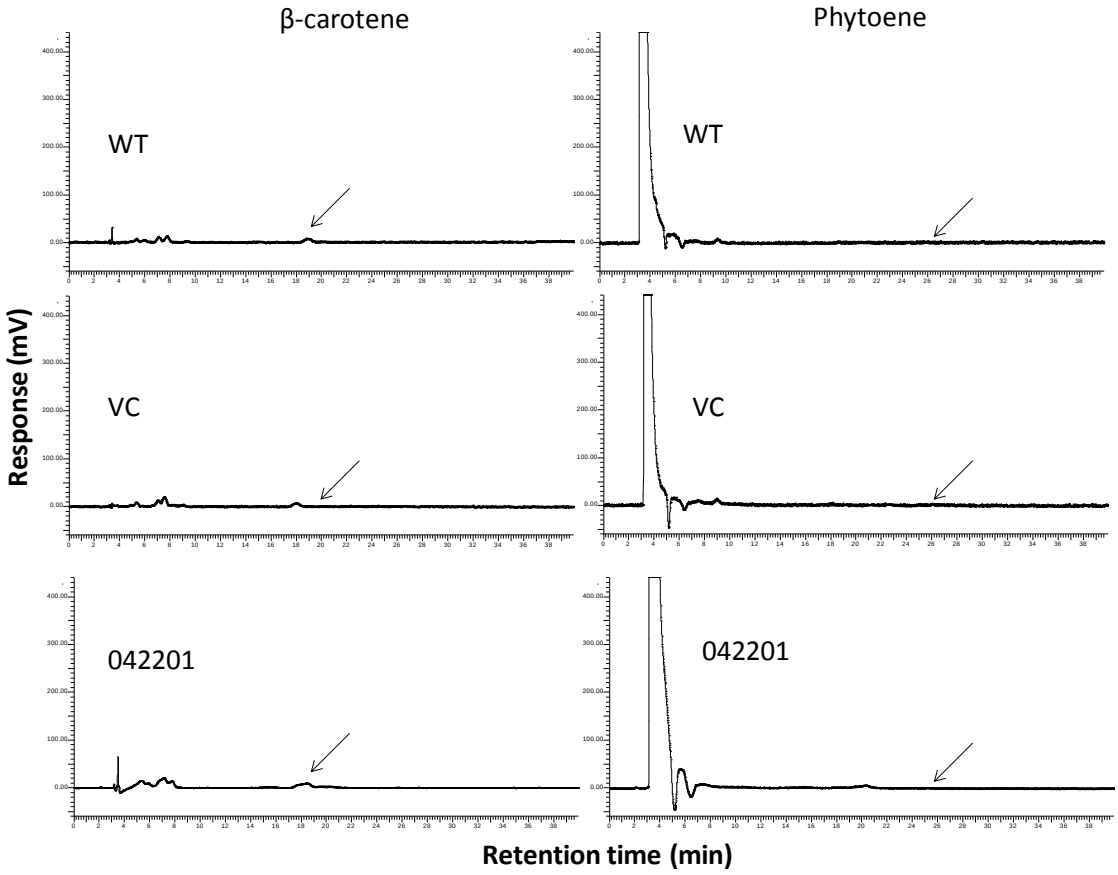


**Fig. 27** HPLC separation of carotenoids in flesh tissue extractions from wild type (WT), vector control (VC) and transgenic line 042201 of honeydew '150'

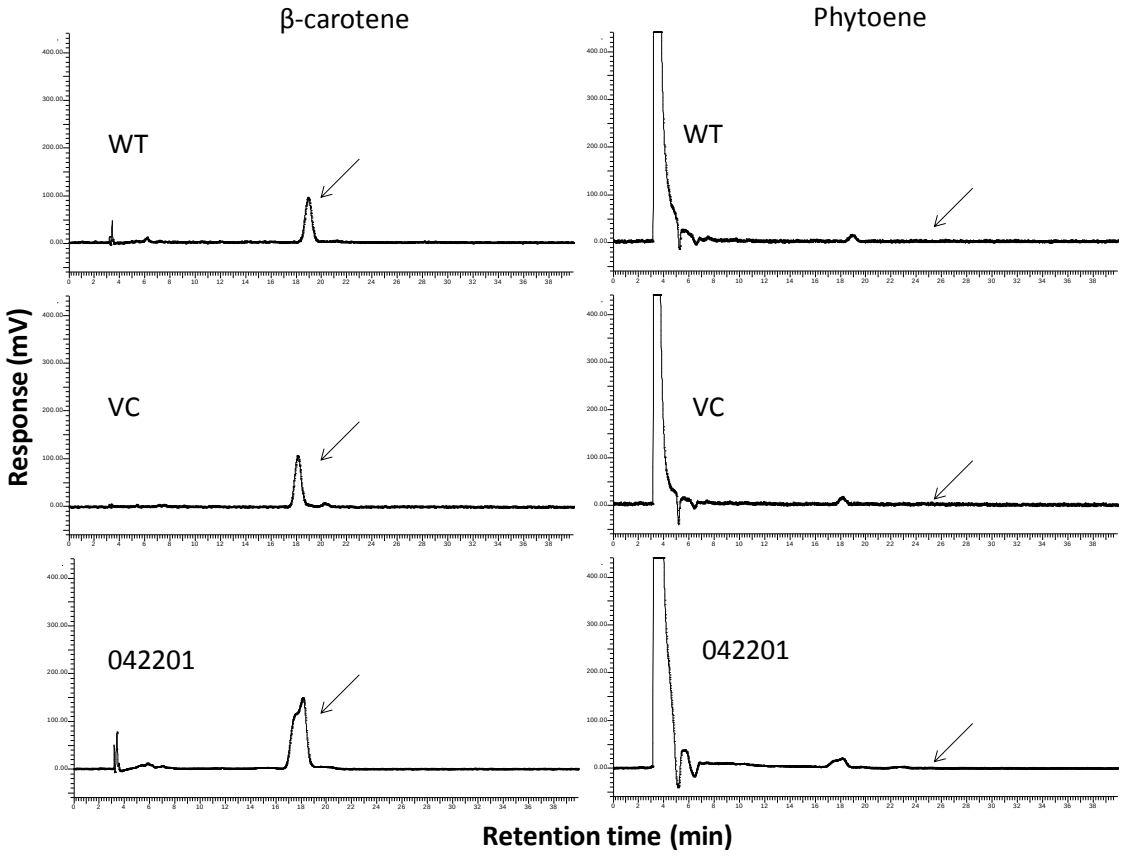


**Fig. 28** HPLC separation of carotenoids in placenta tissue extractions from wild type (WT), vector control (VC) and transgenic line 042201 of honeydew '150'

**Table 10** HPLC analysis of phytoene and β-carotene concentrations in rind, flesh and placenta tissues and the corresponding colors of transgenic honeydew '150'

| Tissue | Genotype | Phytoene ($\mu g\ g^{-1}$ FW) | β-carotene ($\mu g\ g^{-1}$ FW) | Color |
|---|---|---|---|---|
| Rind | Wide type | - | 0.480 | Creamy |
| | Vector control | - | trace | Creamy |
| | 042201 | 10.985 | 15.124 | Orange |
| | 071506_3 | 4.364 | 5.614 | Pale orange |
| | 071506_4 (whole) | 4.005 | 4.389 | Mixed color |
| | 071506_4 (around stem end) | - | 1.908 | Creamy |
| | 071506_4 (around blossom end) | 5.567 | 7.740 | Pale orange |
| Flesh | Wide type | - | 0.393 | Green |
| | Vector control | - | 0.257 | Green |
| | 042201 | - | trace | Green |
| | 071506_3 | - | 0.729 | Green |
| | 071506_4 | - | 0.891 | Pale orange |
| Placenta | Wide type | - | 6.762 | Orange |
| | Vector control | - | 5.547 | Orange |
| | 042201 | - | 2.855 | Orange |
| | 071506_3 | - | 3.598 | Orange |
| | 071506_4 | - | 2.698 | Orange |

Expression of *PSY-C* transgene

Total RNA was extracted from rind, flesh and placental tissues of wild type, vector control and transgenic fruits, and then cDNA was immediately synthesized from the RNA. *PSY-C* transgene transcription was examined using RT-PCR (Fig. 29). Transcript was not detected in wild type and vector control but was detected in transgenic fruits. Fruit 042201had stronger expression of *PSY-C* than others in the rind tissue, which is in accordance with the dramatic increase of β-carotene level (Table 10). *PSY-C* expression levels in the flesh and placental tissues were lower than those in the rind tissues. No correlation was found between *PSY-C* expression level and carotenoid contents.

Phytoene is a direct product of phytoene synthase catalyzation in the carotenoid biosynthesis pathway (Cunningham and Gantt 1998). Therefore, change of phytoene levels in transgenic melons is an important indicator of *PSY-C* transgene functioning in this study. Phytoene was detected only in the rind tissues (Fig. 26) but not flesh and placenta of the transgenic fruits (Figs. 27-28 and Table 10); however, the RT-PCR results indicated that the *PSY-C* transgene expressed in all the tissues. Thus, we assume that post-transcriptional mechanisms of the *PSY-C* in the flesh and placental tissues were inhibited. The 4.8-kb *PG* promoter used in this study contains four regulatory regions which control temporal and spatial transcription of its ligated functional gene described by Montgomery et al. (1993), Nicholass et al. (1995) and Lau et al. (2009). According to these reports, the interaction of these regulatory regions directed ripening-specific expression in outer pericarp only in ripe tomato fruit. In contrast, our results showed *PG* promoter induced *PSY-C* expression in all the tissues (outer and inner pericarps). Further studies will be needed to explain whether the PG promoter caused exclusive change of β-carotene in the rind tissues. Another possibility of inhibited accumulation of β-carotene in the flesh and placental tissues might be related to *PSY-C* tissue-specific function. The accumulation of phytoene only happened in the rind but not the flesh and placental tissues while *PSY-C* expression was detected in all the tissues. This inexplicable result indicates that different mechanisms of carotenoid accumulation may exist in different tissues of honeydew due to different metabolite precursor pools in different tissues.

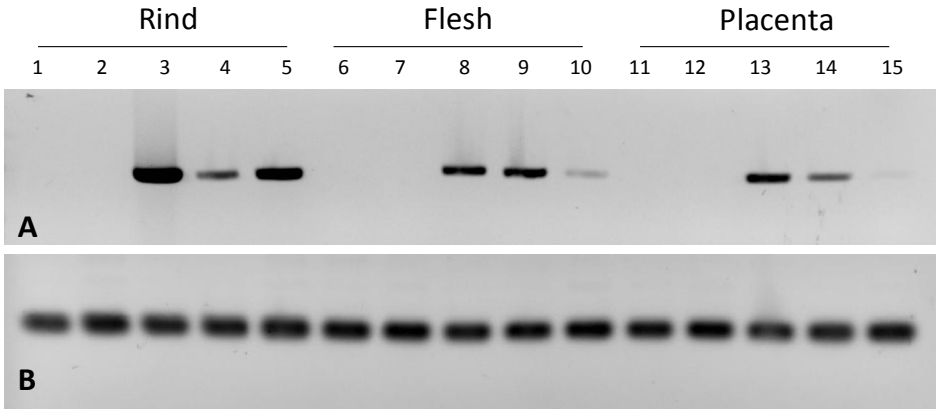


**Fig. 29** RT-PCR analysis of *PSY-C* transgene expression in transgenic honeydew '150'. **a** *PSY-C* transcript in different tissues of wild type, vector control and transgenic fruits (042201, 071506_3 and 071506_4), **b** 18S rRNA used as an internal control. Lane 1-5: rind tissue; lane 6-10: flesh tissue, lane 11-15: placental tissue

Segregation and inheritance in the $T_1$ progenies of the transformants

PCR analysis was used to detect the presence of *PSY-C* in the $T_1$ progeny of each transgenic fruit (Fig. 30). PCR results revealed single locus segregation in genotypes 042201 and 071506_3 (Table 11). However, transgenic genotype 071506_4 showed a low possibility of Mendelian segregation probably due to the early harvest and abnormal seed development.

To confirm the PCR results, we compared PCR-positive/negative individuals with their corresponding kanamycin sensitive/resistant seedlings growing on the selection medium containing 200 mg $l^{-1}$ kanamycin. Like wild type (Fig. 31a), kanamycin sensitive seedlings' root development was inhibited while kanaymcin resistant seedlings developed normal healthy roots on the medium (Fig. 31b). Although kanamycin sensitivitive seedlings elongated and cotyledons opened, their true leaves

were smaller than those on kanamycin resistant seedlings (Fig. 31c-d), and further development was restrained. Eventually the true leaves failed to expand and were bleached out resulting in the plants' death. Based on the comparison of results, most of the kanamycin resistant seedlings were PCR positive and most of the kanamycin sensitive seedlings were PCR negative. For the seedlings having controversial results, we re-extracted DNA and re-did PCR analysis to assure the *PSY-C* presence.

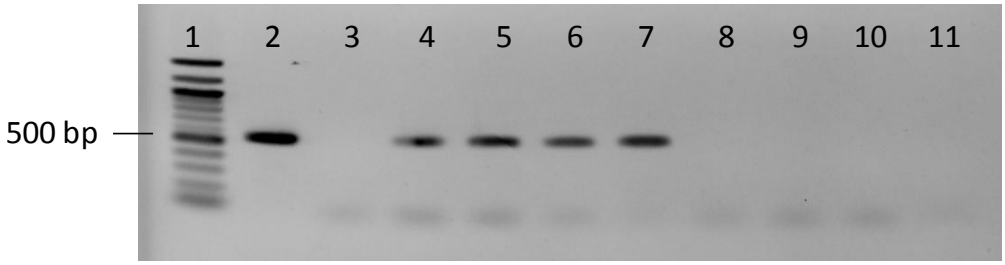


**Fig. 30** PCR analysis to determine the segregation of *PSY-C* transgene in the $T_1$ progeny of transgenic honeydew '150'. Lane 1, 100-bp ladder; lane 2, PSYC/pRD12 (positive control); lane 3, wild type honeydew '150' (negative control); lanes 4-7, PCR-positive $T_1$ progeny; lanes 8-11, PCR-negative $T_1$ progeny

**Table 11** Segregation ratio of *PSY-C* transgene in $T_1$ progeny of transgenic honeydew '150'

| Genotype | Total number of seeds | PCR positive | PCR negative | Transgene segregation ratio | Probable *PSY-C* locus | $\chi^{2a}$ | P |
|---|---|---|---|---|---|---|---|
| 042201 | 58 | 42 | 16 | 3:1 | 1 | 0.207 | 0.6491 |
| 071506_3 | 35 | 27 | 8 | 3:1 | 1 | 0.086 | 0.7693 |
| 071506_4 | 51 | 29 | 22 | 3:1 | 1 | 8.948 | 0.0027 |

[a] Chi-square value is calculated based on the hypothesis of 3:1 segregation ratio

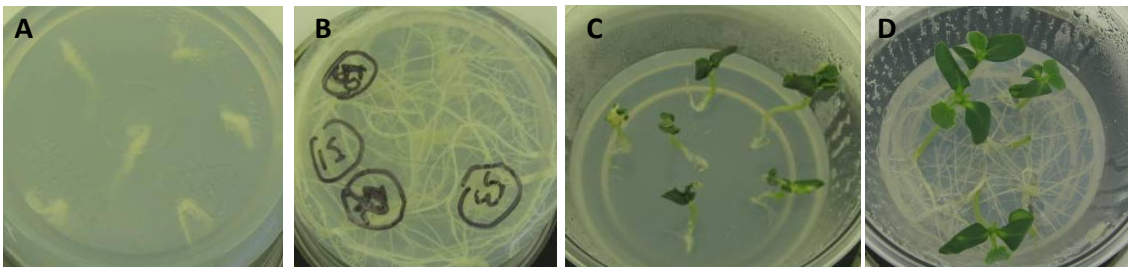


**Fig. 31** Seed germination of wild type and transgenic progeny of honeydew '150' on the selection medium containing 200 mg $l^{-1}$ kanamycin. **a-b** Root development of wild type (a) and transgenic progeny (b), **c-d** cotyledon and true leaf development of wild type (c) and transgenic progeny (d)

## Conclusion

We transferred a watermelon *PSY-C* gene with a fruit-specific PG promoter into our elite honeydew breeding line '150'. Two transgenic lines having normal morphologies have been generated with up to 32-fold higher β-carotene in fruit rind tissue than wild type honeydew. It was postulated that elevating β-carotene in the rind tissue may be related to the fact that PG promoter was found to express only in outer pericarp in tomato fruit depending on regulatory elements (Montgomery et al. 1993). This indicated that the watermelon *PSY-C* gene may have a potential to increase β-carotene level in fruit flesh tissue if a different promoter is used. Further studies will be needed to help understand the mechanism of β-carotene accumulation in different tissues of melons as an essential solution to improve carotenoid in melons.

# CHAPTER VI

# SUMMARY AND CONCLUSIONS

To genetically manipulate β-carotene level in melon fruits, two elite breeding lines, western shipper cantaloupe 'F39' and honeydew '150', were used to establish regeneration and transformation systems. Six media were examined to evaluate the capability of shoot regeneration from different explants types. Each explant type showed a great potential to produce shoots. MS basal medium supplemented with 1 mg $l^{-1}$ BA, 0.26 mg $l^{-1}$ ABA and 0.8 mg $l^{-1}$ IAA induced highest frequency of shoot regeneration from cotyledonary explants in both 'F39' and '150'. Removal of IAA from this medium solved a curving-up problem of cotyledonary explants in '150'.

Our study then proceeded to establish an *Agrobacterium*-mediated transformation protocol for both lines based on the optimal regeneration system. Several factors have been tested to optimize the protocol, such as *A. tumefaciens* strains, inoculation time, co-cultivation conditions, kanamycin concentrations and antibiotics. *A. tumefaciens* strain EHA105, carrying pCNL56 containing *nptII* and *gusA* genes, was selected to develop the protocol. Putative transformants were evaluated using GUS histochemical assay, PCR and Southern blot analyses. Based on these parameters, 0.3±0.1% of the cotyledonary explants regenerated transgenic plants in 'F39'; and 0.5±0.3% of the cotyleondary explants produced transgenic plants in '150'.

Whereafter, a watermelon *PSY-C* gene, under the control of a fruit-specific promoter of PG gene, was transferred into honeydew '150'. Putative transformants were

evaluated using PCR and Southern blot analyses. *PSY-C* transgene was expressed in all the tissues of the transgenic fruits according to RT-PCR results. Changes of phytoene and β-carotene concentrations were detected in the transgenic fruits using HPLC analysis. Transgenic lines produced up to 32-fold higher β-carotene in their rind tissues than the wild type.

In this study, we observed several problems in regeneration and transformation of melons: 1) high percentage (50-60%) of polyploid shoots regenerated from cotyledons; 2) chimeras and abnormal plants produced through transformation; 3) low transformation efficiency; and 4) tissue-specific accumulation of β-carotene in honeydew melons. Further studies are needed to reduce or solve these problems, and to elevate β-carotene level in flesh tissue of melons.

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