TRANSFORMATION OF ‘GALIA’ MELON TO IMPROVE FRUIT QUALITY

By

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TRANSFORMATION OF ‘GALIA’ MELON TO IMPROVE FRUIT QUALITY

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December 2005

Chair: Daniel J. Cantliffe
Major Department: Horticultural Science

Stable genetic transformation in ‘Galia’ muskmelon has not been achieved to date. The objectives of this study were to obtain an efficient and reliable *in vitro* regeneration system for ‘Galia’ muskmelon (*Cucumis melo* L. var. *reticulatus* Ser.) parental lines, and to attain a dependable transformation protocol as depicted through reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP). Once this was accomplished the ACC oxidase gene (CMACO-1) in antisense orientation was inserted into both ‘Galia’ parental lines using an *Agrobacterium*-mediated transformation system. By reducing ACC oxidase, it was hoped that ripening would be delayed thus allowing a longer time-frame to ship fruit in a firm condition. Three protocols using different melon explants, i.e. cotyledon, hypocotyl and true-leaf, and several plant hormonal balances were used on ‘Galia’ male and female parental lines with the aim to induce *de novo* shoot *in vitro* regeneration. The best explant to induce *in vitro* regeneration was cotyledon using 1 mg L\(^{-1}\) benzyladenine (BA) and 0.001 mg L\(^{-1}\) α-Naphthaleneacetic acid (NAA) as
plant hormones. Efficient transformation rates as high as 13% were achieved when hypocotyl and the GFP reporter gene were used, however, more than 27 weeks were needed to attain seedlings. Using cotyledon as explants and either the GUS or GFP gene, transformation rates of 8-10% were observed, and transgenic seedlings were obtained in fewer than 11 weeks. The ACC oxidase gene from melon (CMACO-1) in antisense orientation was used to transform ‘Galia’ male and female parental lines. Two (TGM-AS-1 and TGM-AS-2) independent ‘Galia’ male transgenic diploid plants were obtained using the ACC oxidase gene. A postharvest evaluation was carried out on ACC oxidase transgenic and wild type fruits. Antisense fruits had reduced ACC oxidase enzyme activity, as well as lower ethylene production compared to wild type fruits. Moreover, fruit softening was delayed in CMACO-1. The results of this research indicated that the insertion of an ACC oxidase gene in antisense orientation in the ‘Galia’ male parental inbred reduced ethylene synthesis in transgenic (TGM-AS) T₀ fruits. As a result of low ethylene production by TGM-AS fruits, several parameters such as yellowing of the rind, ripening index, and fruit softening were delayed by as much as 10 days. Other non-ethylene dependent traits, such as fruit size, seed development, and mesocarpic total soluble solids, titratable acidity and pH were not affected by the transgene presence. TGM-AS-1 and TGM-AS-2 T₁ seeds, obtained from these T₀ evaluated fruits, will be used to obtain and select future improved lines with extended shelf-life.
CHAPTER 1
INTRODUCTION

Melon (Cucumis melo L.) belongs to the Cucurbitaceae family, and is an important worldwide commodity (Food and Agriculture Organization [FAO], 2005). Within Cucumis melo species several important horticultural groups can be identified: cantalupensis, inodorus, flexuosus, conomon, dudaim, and momordica (Robinson and Decker-Walters 1999). Melons belonging to these horticultural groups vary in their field performance and postharvest characteristics.

Developed from a breeding program initiated in the mid-1960s ‘Galia’ muskmelon was the first Israeli melon hybrid. This commodity was released in 1973, and has exceptional characteristics such as fruit quality with 13-15% total soluble solids (TSS), bold flavor and a distinct musky aroma. Galia’s high-quality characteristics increased local market popularity within a short time. In fewer than 10 years, ‘Galia’ melons were distributed almost all over Western Europe, and became a unique and special market category of muskmelon (Karchi, 2000). The main disadvantage of ‘Galia’ is its short storage life, since it is harvested near peak maturity for optimum flavor. Storage is limited to two or three weeks, even when it is maintained in low temperature (6-8°C). Different strategies have been used in order to delay fruit softening in ‘Galia’ including the use of controlled atmospheres plus adding an ethylene absorbent (Aharoni et al., 1993a), reducing the storage time during transportation by using airfreight systems, instead of seafreight shipping (Bigalke and Huyskens-Keil, 2000), and applying inhibitors of ethylene action, such as 1-methylcyclopropene (Ergun et al. 2005).
Using traditional breeding methods and long-shelf life melon cultivars, breeders have been able to develop a strategy to insert gene(s) which have increased ‘Galia’ muskmelon shelf-life. However, this approach has resulted in a loss of favorable fruit quality characteristics (Karchi, personal communication). Using a plant biotechnology approach by means of plant transformation is a feasible alternative to introduce novel or native genes, which likely will increase ‘Galia’ muskmelon shelf life, without affecting its unique characteristics. This approach was used to delay fruit ripening in Cantaloupe melon (cv. Védrantais), by inserting the ACC oxidase gene in antisense orientation with promising results (Ayub et al. 1996; Guis et al. 1997b; Silva et al. 2004). However, in order to attain a consistent plant transformation protocol, it is imperative to have a reliable and efficient in vitro plant regeneration system. Nevertheless, ‘Galia’ muskmelon has not been easily in vitro cultivated and full regenerated plants are especially difficult or are impossible to obtain (Leshem, 1989; Leshem et al. 1994a; 1994b; Gaba et al. 1994; Gaba et al. 1996; Edriss et al. 1996; Kintzios and Taravira, 1997; Galperin et al. 2003a). Moreover, Gaba et al. (1999) reported ‘Galia’ muskmelon to be recalcitrant to transformation by Agrobacterium tumefaciens. Therefore, the research on plant in vitro parameters to induce de novo shoot regeneration in ‘Galia’ muskmelon will provide valuable information to attain a consistent and practical in vitro protocol, in order to obtain fully regenerated ‘Galia’ muskmelon plants. Moreover, the research to find favorable conditions for successful Agrobacterium-mediated transformation will enable us to insert any gene of interest into this recalcitrant muskmelon cultivar, specifically with the final aim to increase its shelf-life.
The objective of this research was to obtain an efficient and reliable *in vitro* protocol to induce *de novo* plant regeneration in ‘Galia’ muskmelon parental lines, as well as to attain a consistent and practical transformation methodology aimed to insert the ACC oxidase gene in antisense orientation, and delay the fruit ripening process. In order to achieve the objective of this research, several experimental approaches were taken. These were to 1) examine the effect of several plant hormones and their balances, as well as different melon explant sources on *de novo* shoot and root regeneration in ‘Galia’ male and female parental lines; 2) establish the favorable conditions for *Agrobacterium tumefaciens*-mediated transformation using two reporter genes, i.e., GUS and GFP in ‘Galia’ male and female parental lines; 3) transform ‘Galia’ male parental line with the ACC oxidase gene in antisense orientation (CMACO-1); and 4) evaluate transgenic CMACO-1 ‘Galia’ male fruit since a postharvest approach.
CHAPTER 2
LITERATURE REVIEW

2.1. Importance of Melon

2.1.1 Introduction

The Cucurbitaceae family consists of mostly frost sensitive, principally tendril-bearing vine plants which are found in sub-tropical and tropical regions around the world (Robinson and Decker-Walters, 1999). Nevertheless, some species within this family are well adapted to low temperatures and xerophytic conditions (Wien, 1997). Plants belonging to the Cucurbitaceae family are commonly well known as Cucurbits, and, according to their geographic origin they can be classified as new world and old world species. Two well-defined subfamilies, eight tribes, about 120 genera and more than 800 species are found in this family (Jeffrey, 1990). They are largely cultivated as vegetables and several parts of the plants are utilized for foodstuff. Fruits are the most commonly eaten part of the plant, but seeds, flowers, tendrils, very young shoots and roots are also used for food. In addition, Cucurbits are exploited as medicines, such as Cucurbita andreana, which has chemical compounds (cucurbitacins) with anticancer and antiinflamatory activities (Jayaprakasam et al. 2003). In addition, in China, fruits and roots of Cucumis melo are taken as emetic, leaves and seeds for hemATOMA, and stems to reduce hypertension. Cucurbits are utilized for unusual purposes as well, such as to store food (Gourd) or to be used as a sponge (Loofah) (Robinson and Decker-Walters, 1999).

The most important cultivated cucurbits –based on total production and harvestable area– around the globe are watermelon (Citrullus lanatus Thunb.), cucumber (Cucumis
Among the major cucurbit vegetables, *Cucumis melo* has one of the highest polymorphic fruit types and botanical varieties. This is as a consequence of genetic diversity in this species (Mliki et al. 2001). Therefore, some melon fruits can have excellent aroma, variety of flesh colors, deeper flavor and more juice compared to other cucurbits (Goldman, 2002).

### 2.1.2 Botany and Origin of *Cucumis melo*

Most melons are trailing indeterminate length vines and up to 15 m long; nevertheless some modern cultivars with shortened internodes, bushy appearance and concentrated-yield have been bred (Paris et al. 1982, Paris et al. 1985, McCollum et al. 1987, Paris et al. 1988). All melons are frost-sensitive, but many differ in their ability to survive cold and hot environments (Wien, 1997).

The main stem is almost round in shape. Stems may have some pubescence or not, but when present it is not so pronounced as in other cucurbits (Zitter et al. 1996). Leaves are simple, either three- or five-lobed, and borne singly at the nodes and commonly they may have a great variation in size, color, and shape (Kirkbride, 1993). Tendrils are borne in leaf axis and are simple (unbranched).
Sex expression in *Cucumis melo* is controlled by genetic factors, as well as by environment (Wien, 1997; Robinson and Decker-Walters, 1999). According to Wien (1997), at least four environmental factors, such as light energy, photoperiod, water supply, and temperature, have a strong influence on sex expression. Normally, physiological conditions which favor the increase of carbohydrates within the plant, such as low temperature, low nitrogen availability, short photoperiod and high moisture accessibility, promote female sex expression (Robinson and Decker-Walters, 1999). These environmental factors affect plant hormonal balance, which in turn determines sex expression. In general, gibberellins promote male flower development, whereas auxins and ethylene induce female flower production (Karchi, 1970). Melon plants bear perfect or imperfect flowers in several combinations: perfect (hermaphroditic) flowers are capable of self-pollination, and imperfect flowers are either pistillate (female) or staminate (male) (Zitter et al. 1996). Most melon cultivars are andromonoecious (hermaphroditic and staminate flowers present at the same plant), although monoecious (pistillate and staminate flowers) forms are found as well (Seshadri and More, 2002).

Melon fruits are generally classified as an indehiscent ‘pepo’, with three ovary sections or locules. According to Robinson and Decker-Walters (1999), a ‘pepo’ is a fleshy fruit with a leathery, non-septate rind derived from an inferior ovary. The edible flesh is derived from the placentae or mesocarpic tissue (Seymour and McGlasson, 1993). Among the different parts of a melon plant, fruit has the highest diversity in size, form, external ornamentation, and internal and external color (Kirkbride, 1993). For instance, Périn et al. (2002b) reported that fruits as short as 4 cm long (*Cucumis melo* L. var. *agrestis*) and as long as 200 cm (*Cucumis melo* L. var. *flexuosus*), attaining weights of
between 50 g and more than 15 kg (a 300-fold variation size), are known (Naudin, 1859). Variation is also expressed in flesh color (orange, orange light or pink, green, white or even mixture of these colors), rind color (green, yellow, white, orange, red, gray or blend of these colors), rind texture (smooth, warty, striped, netted, rough or combination of these textures), form (round, flatten or elongated), and size (from 4 up to 200 cm) (Kirkbride, 1993; Goldman, 2002). Some melon fruits (depending of the cultivar) when ripe have an abscission layer at the attachment zone between the fruit and stem, whereas others remain attached to the stem even after they are ripe (Kirkbride, 1993).

Africa and Asia have been suggested as possible sites of origin (Robinson and Decker-Walters, 1999). Nevertheless, Kerje and Grum (2000) reported, based on genetic studies, crossing attempts with other *Cucumis* species, and world distribution of melon varieties, that the origin of melon appears to be Africa. The domestication process of melon started in Egypt over 3,000 years ago (Pangalo, 1929). From this area, melon species dispersed throughout the Middle East and Asia, where a secondary domestication and diversification development may have occurred (Kerje and Grum, 2000).

### 2.1.3 Classification and Cultivars

According to Jeffrey (1990), melon classification may be listed as follows:

**Class:** *Dicotyledoneae*

**Subclass:** *Dilleniidae*

**Superorder:** *Violanae*

**Order:** *Cucurbitales*

**Family:** *Cucurbitaceae*

**Tribe:** *Melothrieae*

**Subtribe:** *Cucumerinae*
Genus: *Cucumis*

Subgenus: *Melo*

Species: *Cucumis melo*

The high polymorphism of fruits in cultivated melons has lent botanists to propose different infraspecific classifications. An excellent, updated and complete study on *Cucumis* genus was undertaken by Dr. Joseph H. Kirkbride, Jr. (1993) from USDA. His book titled “Biosystematic Monograph of the Genus *Cucumis* (Cucurbitaceae)” is a cornerstone in melon classification.

In 1859 the french botanist Charles Naudin, using mostly living plants grown in the gardens of the Natural History Museum of Paris, proposed the first useful system of infraspecific categorization for *Cucumis melo* L. Naudin (1859) subdivided this species into ten groups, which were later revised by Munger and Robinson (1991), proposing trinomial names. However, several classifications have been reported (Alefeld, 1866; Cogniaux and Harms, 1924; Whitaker and Davis, 1962; Pangalo, 1958; Filov, 1960; Grebenščikov, 1986; Kirkbride, 1993; Pyzhenkov and Malinina, 1994; Robinson and Decker-Walters, 1999). Recently, Pitrat et al. (2000) proposed a complete synthesis of infraspecific classification of melon. They identified the synonymous epithets used in several publications in order to propose their classification. These authors recognized 16 groups and denominated them as *varietas* or *variety*.

On the other hand, Smith and Welch (1964) and Robinson and Decker-Walters (1999) considered that Naudin's categories are horticultural groups and not botanical varieties based in phylogeny.
2.1.3.1 Naudin’s categories for *Cucumis melo* L. are listed below:

1. Cantalupensis group. Cantalupe and muskmelon. Medium size fruits with netted, warty or scaly surface, flesh usually orange but sometimes green, flavor aromatic or musky. Fruits with abscission layer at maturity. Usually andromonoecious plants.

2. Inodorus group. Winter melons: honeydew, canary, casaba and crenshaw. Fruits usually larger, later in maturity and longer keeping than those of the Cantalupensis group. Rind surface smooth or wrinkled, but not netted, flesh typically white or green and lacking a musky odor. Fruits do not detach from the peduncle when mature. Typically andromonoecious plants.

3. Flexuosus group. Snake melon or Armenian cucumber. Fruits are very long, slender and often ribbed. They are used when immature as an alternative to cucumber. Monoecious plants.

4. Conomon group. Makura uri and Tsuke uri (pickling melons). Small fruits with smooth, tender skin, white flesh, early maturity and usually with little sweetness or odor. They are used as a pickling, but also eaten fresh or cooked. Andromonoecious plants.

5. Dudaim group. Pomegranate melon, chito melon, Queen Anne’s pocket melon and mango melon. Small, round to oval fruits with white flesh and thin rind.

6. Momordica group. Phoot and snap melon. Small fruits with oval to cylindrical shape. Flesh is white or pale orange, low in sugar content. Smooth surface. Most of the cultivars are monoecious.

Alternatively, Guis et al. (1998) reported a new categorization of horticulturally important melons. These authors based their classification on a previous biosystematic monograph of the genus *Cucumis* (Cucurbitaceae) reported by Kirkbride in 1993, who used morphological, cytological and macro-distributional data to systematize that genus.

2.1.3.2 Guis’s categories:

7. *C. melo* var. *cantaloupensis* Naud. Medium size fruits, rounded in shape, smooth surface or warty, and often have prominent ribs and sutures, if there is netting, is sparse. Orange flesh, aromatic flavor and high in sugars.

8. *C. melo* var. *reticulatus* Ser. Medium size fruits, and netted surface. If ribs are present, they are not-well marked, flesh color from green, white to red orange. Most are sweet and have a musky odor.

9. *C. melo* var. *saccharinus* Naud. Medium size fruits, round or oblong shape, smooth with grey tone sometimes with green spots, very sweet flesh.
10. *C. melo* var. *inodorus* Naud. Smooth or netted surface, flesh commonly white or green, lacking the tipical musky flavor. These fruits are usually later in maturity and longer keeping than *cantaloupensis* or *reticulatus*.

11. *C. melo* var. *flexuosus* Naud. Long and slender fruit, green rind and finely wrinkled or ribbed. Green flesh and usually eaten as an alternative to cucumber. Low level of sugars.

12. *C. melo* var. *conomon* Mak. Small fruits, smooth surface, crisp white flesh. These melons ripe very rapidly, develop high sugar content but little aroma.

13. *C. melo* var. *dudaim* Naud. Small fruits, yellow rind with red streak, white to pink flesh.

On the other hand, Stepansky et al. (1999) proposed an intraspecific classification of melons based on phenotypic and molecular variation. They studied a collection of 54 accessions representing diverse melon genotypes (*cantaloupensis, inodorus, conomon, chito, dudaim, momordica, flexuosus, agrestis* and some non-defined varieties) from more than 20 countries, building with their data a “botanical-morphological” dendogram. Likewise, DNA polymorphism among the accessions was assessed using inter-SSR-PCR and RAPD techniques. They concluded that the molecular phylogeny agreed, broadly, with the classification of melon into two subspecies, and it did not contradict the division into “horticultural varieties.”

Recently, Liu et al. (2004) concluded after an extensive evaluation of 72 melon accessions belonging to six melon varieties - *cantaloupensis, reticulatus, inodorus, acidulus* and *saccharinus*- that accessions which were previously classified in the same variety by traditional taxonomy were also located closely to each other using Principal Component Analysis (PCA) approach in 35 different morphological and physiological plant characters.

In general, both Naudin’s and Guis’ categorizations have more common features than contrasting ones; therefore both are well accepted among scientists.
2.1.4 Climacteric and Non-climacteric Fruits

Fruit in general can be classified as either climacteric or non-climacteric on the basis of their respiration pattern and autocatalytic ethylene production peak during ripening (Tucker, 1993, Hadfield et al. 1995). Climacteric fruits, such as tomato, peach, avocado, apple and pear, have a respiratory burst and a pronounced autocatalytic ethylene production while the ripening process is proceeding. Non-climacteric fruits, such as bell pepper, watermelon, strawberry, grape, and citrus, do not show evidence of an increased ethylene evolution or respiratory rise coincident with ripening (Seymour and McGlasson, 1993). This fruit categorization might not be completely strict for all species. Within a species there could be both climateric and non-climateric fruits. As a general rule, melon fruits have been considered as a climateric type; usually reticulatus and cantaloupensis melon varieties belong to this group. However, non-climateric melon fruits are available as well, most of them fitting in inodorus variety (Seymour and McGlasson, 1993; Zheng and Wolf, 2000; Périn et al. 2002a).

Therefore, it is not easy to define a set of criteria that may be used to predict the ripening-related respiratory and ethylene evolution performance of specific fruit and then extrapolate that behavior for another fruit related-cultivar or species.

2.2 Postharvest Physiology of Melon

2.2.1 Physiological Changes During Ripening

In order to achieve a typical melon fruit growth pattern, pollination, satisfactory double fertilization and a normal development of the ovules have to take place (Wien, 1997). Fruit growth patterns among melon cultivars can be similar or quite diverse. In 1971 Pratt reported that both the ‘Honey Dew’ and ‘cantaloupe’ types reached half of their total fruit growth almost at the same time (15 and 20 days after anthesis); however,
the ‘Honey Dew’ melon attained four times as much size fruit than ‘cantaloupe’.
Likewise, McCollum et al. (1987) described a comparable fruit growth in two melon
genotypes (NY and D26) for the first 14 days after anthesis, but from 21 days after
anthesis to full slip, NY had greater fresh weight than did D26 fruits. McCollum et al.
(1988) measured fruit growth in ‘Galia’ and ‘Noy Yizre’el’ muskmelon cultivars, and
reported that both fruits had sigmoidal growth curves. However, differences between the
cultivars were apparent; i.e. ‘Galia’ fruits were larger than ‘Noy Yizre’el’ fruits at each
stage of development and continued to grow until the time of abscission.

Due to genetic diversity, melon fruits have a wide variation in ripening behavior.
Fruits belonging to the *reticulatus* and *cantaloupensis* varieties have a quick climacteric
at, or close to, the time of fruit maturity and abscission, although the abscission process is
absent in some muskmelon (*reticulatus*) varieties (Sakata and Sugiyama, 2002). On the
other hand, *inodorus* and *saccharinus* type fruits may have the climacteric process
extended up to several days or it may be absent (Miccolis and Saltveit, 1991; Aggelis, et

The moment of fruit maturation as well as the beginning of fruit ripening depends
upon the melon variety (Liu et al. 2004). In *reticulatus* and *cantaloupensis* varieties the
abscission characteristic is one of the most practical standards to estimate the harvest
maturity (Pratt et al. 1977; Larrigaudiere et al. 1995). Other indexes of muskmelon
harvest maturity include fruit color and appearance of the netted pattern. A muskmelon
fruit color chart has been prepared for ‘Galia’, which categorized six different levels of
maturity: 1, very dark green; 2, green; 3, light yellow with some green areas; 4, light
yellow; 5, yellow; and 6, dark yellow to orange peel (Fallik et al. 2001). On the other
hand, in those melon varieties, such as *inodorus*, *flexuosus* and *saccharinus*, where an abscission layer is not formed, other characteristics are used to assess harvest maturity. For example according to Portela and Cantwell (1998), at commercial melon production level a variety of subtle changes in external color (green to white), peel texture (hairy to smooth), aroma at the blossom end (none to detectable), and fruit density (low to high) are used in order to assess the harvest maturity point.

Fruit ripening is a genetically determined event that involves a series of changes in color, texture, and flavor (Hashinaga et al. 1984, Keren-Keiserman et al. 2004). Flavor is a multifaceted human perception, which involves taste and aroma (Shewfelt, 1993). According to Tucker (1993), fruit flavor depends on the complex interaction of sugars, organic acids, phenolics and a wide variety of volatile compounds. In general, the quality of melon fruit is mostly associated to both elevated sugar level and excellent flavor in mesocarpic tissue (McCollum et al. 1988; Shewfelt 1993; Wyllie et al. 1995). In netted melons, final fruit quality is also influenced by shading of the melon plant (Nishizawa et al. 2000). It was reported by Pratt et al. (1977) that in the State of California, for ‘Honey Dew’ melon a minimum of 10% soluble solids is legally required for market, but high quality melons can even reach soluble solids content as high as 17% (Pratt et al. 1977; Bianco and Pratt, 1977). Regarding the melon fruit volatile compounds, a comprehensive study on the key aroma compounds in melon as well as their development and cultivar dependence has been described previously (Wyllie et al. 1995).

Fruit storage shelf life is dissimilar among melon varieties. *Cantaloupensis* and *reticulatus* fruits have a shorter shelf life compared to fruits belonging to *inodorus* and *saccharinus* varieties (Liu et al. 2004). Muskmelon and netted melons have a storage life
of approximately 10-14 days at cool temperatures (6-9°C) and proper humidity (90-95%) conditions (Gull, 1988). Muskmelon and netted melons can be kept at storage conditions of 2-5°C and a relative humidity of the air of 90-95%. Netted melon fruits are more prone to lose moisture. This may be a result of the presence of fissured epidermal tissue (netted), which is an elaborated system of lenticels, therefore allowing a more rapidly water loss as a result of evaporation (Webster and Craig, 1976). ‘Honey Dew’ type melons should be stored at 7 to 10°C and 85-90% relative humidity up to three or four weeks, but lower temperatures of 6°C can cause chilling injury (Gull, 1988; Suslow et al. 2001, Lester et al. 2001).

2.2.2 Ethylene Production

Ethylene is a plant gas hormone, which is involved in the melon fruit ripening process (Giovannoni, 2001). A burst of ethylene production coincides with ripening in climateric melon fruits (Pratt et al. 1977). Kendall and Ng (1988) measured ethylene from two netted (reticulatus variety) and three non-netted (Casaba type-inodorus variety) muskmelon cultigens and their hybrids immediately after harvest and found that the netted muskmelon fruits synthesized considerable quantities of ethylene at or close harvest. Conversely, the non-netted fruits did not produce ethylene until as late as 20 days after harvest. Hybrids were generally intermediate to the parental lines in rate and time of ethylene production. These results suggested that ethylene production in Cucumis melo fruit is regulated by both genetic and developmental factors.

Because netted fruit melons produce ethylene during ripening, they do not require exogenous ethylene application after harvest (Pratt, 1971). However, inodorus fruit types may require exogenous ethylene application after harvest, in order to obtain a more uniform and rapid ripening, as well as better development of color, wax, and aroma (Gull,
1988, Suslow et al. 2001). Likewise, *inodorus* fruit types must be harvested when they have already acceptable soluble solids content, because melon fruit generally do not increase their sugar content after harvest (Bianco and Pratt, 1977).

In general, orange- or green-fleshed and netted rind fruit melons produce higher amounts of ethylene than green- or white-fleshed and smooth rind fruits (Zheng and Wolff, 2000; Liu et al. 2004). However, exceptions to this generalization may be found in netted melons. Shiomi et al. (1999) measured the ethylene biosynthetic capacity in two netted cultivars, ‘Earl’s Favourite’ and ‘Andes’, and found that ethylene production in ‘Earl’s Favourite’ fruit remained low even at their commercial harvest maturity stage, whereas ‘Andes’ fruit exhibited a typical climacteric pattern with a high ethylene production. They concluded that the ‘Earl’s Favourite’ fruit used in that experiment behaved like a non-climacteric fruit.

2.2.3 Biochemical Changes During Ripening

2.2.3.1 Introduction

Major biochemical changes take place in fruit during maturation and ripening (Jiang and Fu, 2000; Giovannoni, 2001, Lelievre et al. 2000, Pech et al. 2002). The melon fruit ripening process requires a high metabolic activity, i.e. synthesis and/or degradation of new structural, soluble and enzymatic proteins, novel mRNAs, changes in plant hormones levels, and DNA transcription, as well as accumulation of original pigments, organic acids and sugars, and the release of volatile compounds (Bianco and Pratt 1977; Miccolis and Saltveit 1995; Larrigaudiere et al. 1995; Dunlap et al. 1996; Guillén et al. 1998; Aggelis et al. 1997a; Sato-Nara et al. 1999; Flores et al. 2001a; 2001b; Villanueva et al. 2004). All these anabolic and catabolic events need both energy and a carbon-nitrogen-framework for building blocks, which are supplied via respiration. The two most
important respiratory substrates found in melon fruit are sugars and organic acids (Seymour and McGlasson, 1993). Likewise, ethylene is the major plant hormone involved in the melon fruit ripening process (Bianco and Pratt, 1977; Lelièvre et al. 1997; Sato-Nara, 1999).

2.2.3.2 Carbohydrate Metabolism

Sweetness is the most important edible quality attribute of ripe melon fruits (Yamaguchi, et al. 1977; Lester and Shellie, 1992; Artes et al. 1993). Sucrose, glucose and fructose are the major sugars found in the mesocarp of ripe melon fruits. High levels of sucrose attribute fruit sweetness in melon (McCollum et al. 1988; Hubbard et al. 1990; Burger et al. 2003; Villanueva et al. 2004).

Muskmelon fruit do not store starch as some other fruits do (i.e. apple, and banana), therefore the fruit requires a constant supply of translocated photoassimilate from the leaf canopy for sugar use and accumulation during development and ripening (Pratt, 1971; Hubbard et al. 1989; Hubbard et al. 1990). Consequently, any factor which has an effect on photoassimilate translocation during fruit development will reduce sucrose content (Hubbard et al. 1990). For example, presence of viral infections, such as cucumber mosaic virus, in melon plants causes an alteration in carbon metabolism in source leaves, and in resource partitioning among the various plant organs because there is an increased in respiration, and a decrease in net photosynthetic rate in infected leaves (Shalitin and Wolf, 2000; Shalitin et al. 2002).

In *Cucumis melo*, sucrose is not the only translocated photoassimilate carbohydrate, since galactosyl-sucrose oligosaccharides raffinose and stachyose can be found in the phloem (Mitchell et al. 1992; Gao and Schaffer, 1999; Gao et al. 1999; Volk, et al. 2003).
Mitchell et al (1992) reported maximal amounts of sucrose (60 mM), stachyose (50 mM), and raffinose (10 mM) in sap phloem measurements.

Sugar continues to accumulate during fruit development (Pratt, 1971; Pratt et al. 1977). Beginning at early fruit enlargement and reaching its maximum at full maturity (McCollum et al. 1988; Seymour and McGlasson, 1993; Burger et al. 2002; Burger et al. 2003). The trait for sugar accumulation is controlled by a single recessive gene, called \textit{suc} (Burger et al. 2002). Therefore, sucrose accumulation is controlled through several hormones and enzymes, as well as compartmentation processes (McCollum et al. 1988; Hubbard et al. 1989; Ofosu-Anim and Yamaki, 1994; Lee et al. 1997; Ofosu-Anim et al. 1998; Gao et al. 1999; Gao and Schaffer, 1999; Feusi et al. 1999; Carmi et al. 2003; Volk et al. 2003). Likewise, sugar accumulation is affected quantitatively by environmental and physiological factors as well, such as salinity, nutrient availability, shading, cellular size in the fruit, and available foliar area (Hubbard et al. 1990; del Amor et al. 1999; Nishizawa et al. 2000; Nishizawa et al. 2002; Kano, 2002; Kano, 2004).

Netted, muskmelon, and Honeydew fruits have similar, but not identical, patterns of sugar accumulation (Seymour and McGlasson, 1993). For instance, Bianco and Pratt (1977) reported that both ‘Honey Dew’ and ‘PMR-45’ fruits have a parallel pattern for sugar accumulation including total sugars, sucrose, glucose and fructose. Likewise, McCollum et al. (1988) reported that ‘Galia’ and ‘Noy yizre’el’ fruits accumulated glucose and fructose, in nearly equal amounts, during the first 24 days after anthesis. Sucrose accumulation built up 24 days after anthesis and it was the predominant sugar at the ripe stage. Similar results were obtained one year later by Hubbard et al. (1989), who measured the concentrations of sucrose, raffinose saccharides, glucose, fructose, and
starch in one orange-fleshed netted melon and three green-fleshed muskmelons, two of them categorized as sweet melons and one as a non-sweet type (‘Birds Nest’).

In an extensive study, Stepansky et al. (1999) found considerable variation in sugar content and composition in mature flesh of melon fruits from 56 different genotypes belonging to *cantaloupensis*, *inodorus*, *conomon*, *chito*, *dudaim*, *momordica*, *flexuosus*, *agrestis*, and some non-defined varieties. Among the 14 genotypes classified as *cantaloupensis*, total sugars ranged between 40-100 mg/gfw, and sucrose was 50-70% of the total sugar, although a few accessions had lower levels. Within the *inodorus* group, both low and high sucrose-accumulating genotypes were observed. Some genotypes reached only ~30mg/gfw total sugar, mostly glucose and fructose, whereas others had a high sucrose accumulation (~50 mg/gfw). Among the six *conomon* genotypes analyzed, there were fruits with almost no sucrose (line 85-893) accumulation as well as genotypes with intermediate and high sucrose levels. In the *chito* and *dudaim* varieties five genotypes were evaluated, four out of five genotypes accumulated less than 10 mg/gfw sucrose, but interestingly, the last one (PI 164320) had an unusual sugar pattern profile as it accumulated high levels of total sugar, due mostly to elevated glucose and fructose levels. Most members of the *agrestis* group accumulated extremely low levels of sugars, however, two accessions (PI 164493 and PI 436532) had high total sugars (41 and 58 mg/gfw respectively). The *momordica* and *flexuosus* genotypes did not accumulate significant amounts of sucrose or hexose. These authors also indicated that in the sweeter melon varieties, sucrose was generally the most significant component that contributed to variation in total sugars.
The physiological and biochemical aspects of sucrose accumulation in melon fruit has been investigated extensively (McCollum et al. 1988; Hubbard et al. 1989; Hubbard et al. 1990; Gao et al. 1999; Gao and Schaffer, 1999; Feusi et al. 1999; Carmi et al. 2003; Volk et al. 2003; Burger et al. 2003; Villanueva et al. 2004). As previously mentioned, melon plants translocate sucrose, stachyose and raffinose as main soluble sugars, which are used as the carbon supply for sucrose synthesis in the fruit. Two enzymes, acid invertase (EC 3.2.1.26) and sucrose phosphate synthase (SPS) (EC 2.4.1.14) have been implicated as the determinants of sucrose accumulation in melon fruit (Hubbard et al. 1989; Stepansky et al. 1999). Both enzymes are inversely related in melon sink tissues, such as fruits (Hubbard et al. 1989; Hubbard et al. 1990; Gao et al. 1999). During sucrose accumulation, acid invertase activity decreases, as a result less sucrose degradation. At the same time, SPS activity begins to increase significantly (Hubbard et al. 1989; Hubbard et al. 1990; Gao et al. 1999). In addition, SPS activity is higher in sweet melon fruit compared with non-sweet genotype fruits, suggesting its function in sucrose accumulation (Hubbard et al. 1990).

Stepansky et al. (1999) stated “the final content of sucrose in the fruit mesocarp of sweet melon is a function of two factors: the rate of sucrose accumulation, coupled with the duration of the accumulation period until abscission or harvest.”

2.2.3.3 Organic Acids

Organic acids are compounds regularly found at low amounts in sweet ripe melon fruit types, such as \textit{inodorus}, \textit{cantaloupensis} and \textit{reticulatus} varieties (Yamaguchi et al. 1977; Seymour and McGlasson, 1993). On the other hand, non-sweet ripe melon fruits, \textit{(flexuosus} variety) are able to accumulate higher amounts of organic acids (Stepansky et al. 1999; Pitrat et al. 1999). For instance, Burger et al. (2003) reported that the high-
organic acid fruit content characteristic is conferred by a single dominant gene, called $So$, which is found only in melon varieties that do not accumulate high levels of sugars and which are used for non-dessert purposes. In the recessive condition ($so$), melon fruits have a low-organic acid attribute. Furthermore, these authors stated that the evolution of horticultural sweet melon varieties required the sequential selection of three recessive mutations: first a recessive mutation that allowed for non-bitter fruit ($bif$), then a recessive mutation for low-acid fruit ($so$), followed by a recessive mutation for high sucrose fruit ($suc$) (Burger et al. 2003). Despite the fact that low-organic acid level is a genetically regulated feature, several environmental factors, such as salinity can affect quantitatively, the organic acid level in melon fruit (del Amor et al. 1999).

Citric and malic acids are the most important organic acids found in the flesh of different melon varieties (Leach et al. 1989; Flores et al. 2001b; Burger et al. 2003). In 1989, Leach et al. studied the organic acid fractions from 12 melon cultivars, and reported that citric acid was the major component in all melon cultivars that they analyzed. Similarly, Flores et al. (2001b) found that the major organic acids found in wild type and transgenic cantaloupe melon fruit were citric and malic acids. Artes et al. (1993) found that titratable acidity in four melon varieties varied from 0.14% in ‘Tendral’ up to 0.50% in ‘Galia’ melon fruits.

2.2.3.4 Volatiles

The aroma or fragrance of melon fruits are essential quality factors, for consumer quality (Yamaguchi, 1977), and they are strongly linked to the ripening process (Wang et al. 1996; Beaulieu and Grim, 2001). Unlike sugar accumulation in the ripe fruit, the aroma fruit caused by volatile production continues after harvest (Wyllie et al. 1995). The volatile profile, as well the identification of the main ‘melon odor’ substances in melon
fruits have been the subject of a considerable amount of research (Kemp et al. 1972; Yabumoto et al. 1977; Yabumoto et al. 1978; Buttery et al. 1982; Horvart and Senter, 1987; Leach et al. 1989; Wyllie and Leach, 1990; Homatidou et al. 1992; Wang et al. 1996; Ueda et al. 1997; Bauchot et al. 1998; Yahyaoui et al. 2002; Aubert and Bourger, 2004).

Early studies reported that the volatile ester pattern of ripe muskmelon (*reticulatus* varieties) and Honey Dew (*inodorus* varieties) type fruit were extremely similar, except for ethyl butyrate, which was more abundant in muskmelon (Kemp et al. 1972; Yabumoto et al. 1977; Yabumoto et al. 1978). The volatile profile of melon fruit was made of around 35-50 volatile compounds (Kemp et al. 1972; Yabumoto et al. 1977; Yabumoto et al. 1978; Buttery et al. 1982). With the advance and improvement of extraction methods (Beaulieu and Grimm, 2001), such as Solid Phase Microextraction (SPME), as well as the analytical and detection techniques (Aubert and Bourger, 2004), such as sniffing port analysis, have shown that the volatile compound content responsible for ‘melon aroma’ is diverse and cultivar dependent. Indeed, Aubert and Bourger (2004) were able to differentiate statistically long-shelf life cultivars from wild and mid-shelf life melon cultivars, based merely upon volatile compound profiles. Moreover, Beaulieu and Grimm (2001) affirmed that roughly 240 volatile compounds have been reported from muskmelon fruit.

In the particular case of ‘Arava’ melon, which is a ‘Galia’-type melon, various volatile acetates were identified in the ripening fruit, including nine aliphatic, four aromatic, and one compound containing a sulfur moiety (Shalit et al. 2000). Benzyl acetate was the most abundant volatile compound in the headspace of this cultivar,
however, hexyl acetate and 2-methyl butyl acetate were also found in considerable amounts.

The aroma and taste of most melon fruits are influenced considerably by ester compounds, as well as to a certain extent by sulphur compounds (Yabumoto et al. 1977; Wyllie and Leach, 1990; Homatidou et al. 1992). Even though, Kemp et al. (1972) suggested that four unsaturated esters found in muskmelon fruit did not contribute significantly to the ‘melon aroma’. Yabumoto et al. (1977) using three different extraction methods for melon fruit volatiles stated that it was probable that the large quantities of volatile esters also play a critical role in the integrated flavor of melons, and that they are necessary for the strong and characteristic fruity aroma. According to Yabumoto et al. (1978), the volatile ester profile of ripe reticulatus variety (‘PMR-45’ and ‘Top Mark’) and inodorus variety (‘Honey Dew’ and ‘Crenshaw’) fruit were similar, and they fit into two groups, depending on the pattern exhibited by the production of that compound. One group had a continuously accelerating rate of production (ethyl esters) and another increased rapidly and then plateaued (acetate esters).

The major compounds responsible for ‘Honey Dew’ melon aroma are ethyl 2-methylbutyrate, ethyl butyrate, ethyl hexanoate, hexyl acetate, 3-methylbutyl acetate, benzyl acetate, (Z)-6-nonenyl acetate, and possibly (E)-6-nonenol and (Z,Z)-3,6-nonadienol (Buttery et al. 1982). Horvat and Senter (1987) identified eight novel volatile compounds from ‘Saticoy’ melon (reticulatus). In ‘Galia’-type melon cultivars C8 and 5080, six important aroma volatiles during ripening were found: ethyl acetate, isobutyl acetate, butyl acetate, 2-methylbutyl acetate, hexyl acetate, and 3-hexenyl acetate (Fallik et al. 2001).
The biochemical connection between the development of aroma volatiles and free amino acid content in melon ripening fruit is well established (Yabumoto et al. 1977; Wyllie and Leach, 1992; Wyllie et al. 1995). Wang et al. (1996) proposed that several amino acids, such as valine, isoleucine, methionine, and alanine may be the precursors of the majority of the esters found in melon fruit, providing the branched alkyl chain moiety, which is present in a significant proportion of volatile ester compounds.

The aroma volatile profile in each type of melon is a genetically controlled characteristic (Ueda et al. 1997; Yahyaoui et al. 2002). The presence or absence of seeds in the fruit cavity can modify the final aroma volatile profile (Li et al. 2002). Likewise, the developmental fruit stage may have a strong influence on aroma characteristics (Beaulieu and Grimm, 2001).

In summary, similar to other fruits the melon fragrance is made of complex mixtures of volatiles compounds. Both their production and profile in melon fruits is a genetically controlled attribute, which is associated with the ripening process (Wang et al. 1996), and which is regulated by ethylene (Bauchot et al. 1998; 1999). Therefore, the volatile compound profile is a cultivar-dependent characteristic (Aubert and Bourger, 2004).

2.2.3.5 Cell Wall Degradation

The plant cell wall is a dynamic structure, which determines cell shape and contributes to the functional specialization of cell types (Carpita and McCann, 2000). The plant cell wall is a highly organized structure composed of several polysaccharides, proteins, and aromatic compounds. Likewise, the new primary cell wall comes from the cell plate during cell division, and after differentiation many cells are able to develop within the primary wall, a secondary cell wall (Carpita and McCann, 2000). According to Bennett (2002), a simplest form of the structural model of the plant cell wall can be
 pictured as a core structure of cellulose microfibrils embedded in two coextensive networks of pectin and hemicellulose.

Fruit softening observed during ripening is associated with textural changes that are believed to result from modification and disassembly of the primary cell wall (Fischer and Bennett, 1991). Fruit softening and the underlying cell wall structural changes are complex. Softening or loss of firmness of the edible mesocarp of melon fruit starts in the middle (around 30-45 days after anthesis, depending on cultivar) of the development cycle, along with other typical changes connected with the ripening process (Lester and Dunlap, 1985). Some general events during melon fruit softening are (Lester and Dunlap, 1985; Lester and Bruton, 1986; McCollum et al. 1989; Ranwala et al. 1992; Fils-Lycaon and Buret, 1991; Simandjuntak et al. 1996; Rose et al. 1998; Hadfield et al. 1998; Rojas et al. 2001; Bennett, 2002):

1. There is no a significant change in total pectins (measured as total polyuronides) as a percentage of cell wall material, rather a substantial change in the relative solubility and depolimerazation of pectin levels is observed as fruit softening proceed, as well as a decrease in pectin molecular size. Quoting McCollum et al. (1989) “Quantitative changes in pectin content are apparently less important than are qualitative changes in the softening process.”

2. It seems likely that polygalacturonase (PG) enzyme(s) might not be involved in that solubilization process during the early ripening stages, however some PG-dependent developments may contribute to overall pectin disassembly at later stages.

3. Other enzymes could be involved in that early pectin solubilization process, for instance β-galactosidases and / or β-galactanases.

4. Hemicellulose polymers undergo important modifications, such as changes in the degree of solubility and modifications from large molecular size to smaller size, and loss of specific sugars.

5. Most of the non-cellulosic neutral sugars decrease significantly in the mesocarp of ripening fruits, regularly Galactose, Mannose and Arabinose, whereas other neutral sugars such as Xylose might or not increase during fruit softening.
6. Other enzymatic activities, such as pectin methylesterase, which have been associated with pectin metabolism in other fruits (Harriman et al. 1991; Tieman et al. 1992), and/or other proteins, such as expansins, which have been proposed to disrupt hydrogen bonds within the plant cell wall polymer matrix (Rose et al. 1997; Civello et al. 1999), could also be involved in melon fruit softening. More evidence has appeared regarding the expansins’ role in early fruit softening. Brummell et al. (1999) obtained two types of transgenic tomato plants, some were suppressed and another were overexpressed in the LeExp1 protein. Tomato fruit in which Exp1 protein accumulation was inhibited by 3% were firmer than control fruit throughout the ripening process. Conversely, fruit overexpressing high amounts of LeExp1 protein were much softer than control fruit, even in mature green stage before the ripening event had commenced.

Rose et al. (1998) and Bennett (2002) proposed a complete model of the temporal sequence of cell wall changes, pectinase activity, and PG-mRNA expression in ripening ‘Charentais’ melon fruit at defined developmental stages, unfortunately they did not include the role of expansins in cell wall degradation in their model.

Among the different plant hormones which are involved in fruit development, ethylene has the main role during melon fruit softening (Rose et al. 1998). Internal ethylene concentrations inside ‘Charentais’ melon fruit cavity increases, concomitant with a loss of flesh firmness during ripening. Furthermore, Guis et al. (1999) using antisense ACC-oxidase transgenic melon plants to reduce ethylene production, reported that plants did not have substantial changes in pectin molecular mass observed in the wild type fruit. Moreover, exogenous ethylene application to those transgenic fruits resumed both accelerated fruit softening and a downshift in the size of cell wall polymers. Additionally, transgenic melon plants (antisense ACC oxidase) were also used to study the role of ethylene in regulating cell wall-degrading enzyme activities (Botondi et al. 2000). In transgenic ‘Charentais’ melon, cell-wall degradation process is regulated by both ethylene-dependent and ethylene-independent mechanisms. In support of a fruit softening-ethylene involvement, it was established that ripening-regulated expansin
gene(s) in tomato were influenced directly by ethylene, and the expression of that gene parallels the pattern of xyloglucan disassembly, and early fruit softening (Rose et al. 1997).

The exact hormonal, molecular and enzymatic mechanisms by which all these processes take place in melon ripening fruit, and finally develop to the fruit softening event are not well understood. Maybe by using updated molecular and genetic techniques, such as cDNA microarrays (Fonseca et al. 2004), in order to monitor the gene expression during fruit development and ripening, there will be more evidence to understand and manipulate the melon fruit softening process. What's more, as Bennett (2002) proposed: ‘future research should focus on using genetic strategies to assess the potential for synergistic interactions by suppression of both hemicelluloses and pectin disassembly in ripening fruit.’

2.2.3.6 Pigments

Flesh color of melons is another important quality attributes for a consumer appeal (Yamaguchi, 1977). In general, four basic and distinctive flesh colors can be observed in melon fruits: orange, light-orange or pink, green, and white (Watanabe et al. 1991; Goldman, 2002).

According to Seymour and McGlasson (1993) the principal pigments in orange-fleshed melons are: β-carotene (84.7%), δ-carotene (6.8%), α-carotene (1.2%), phytofluene (2.4%), phytoene (1.5%), lutein (1.0%), violaxanthin (0.9%) and traces of other carotenoids. Likewise, Watanabe et al. (1991) evaluated nine different melon cultivars belonging to the four basic and distinctive flesh colors. They found that the orange-fleshed colored melon cultivars 'Iroquois', 'Blenheim Orange', 'Birdie Red',
'Quincy' and 'Tiffany' contained about 9.2 to 18.0-µg/g β-carotene as the major pigment, as well as a small amount of phytofluene, α-carotene, ζ-carotene and xanthophylls. They also measured pigments in light-orange-fleshed ‘Hale’s Best’ melon, which contained about 4.0-µg/g β-carotene, and phytofluene; α-carotene, ζ-carotene and xanthophylls were also present but in small amounts. Finally, in the green-fleshed melon 'Earl's Favourite' and 'Fukunoka', and white-fleshed colored melon 'Barharman', their main components were β-carotene and xanthophylls.

Chlorophyll and carotenoid changes in developing fruit muskmelon were studied earlier by Reid et al. (1970). They evaluated three melon cultivars: ‘Crenshaw’, ‘Persian’, and ‘PMR 45’. In all the fruits, chlorophyll content decreased to an intermediate level five weeks after anthesis, and they suggested that chlorophyll loss was probably due to dilution through growth, because there were no more chlorophyll synthesis, but an enlargement of the fruit occurred. In ‘PMR 45’ and ‘Chenshaw’ fruits, however, there was a successive rapid decrease, which was concurrent with the ripening process. Carotenoids content increase steadily three weeks after anthesis to high levels at full maturity. The development of orange pigmentation was a gradual event, starting at the placentae and progressing outward through the mesocarp, until the flesh was uniformly orange at full maturity.

Forbus et al. (1992) used Delayed Light Emission (DLE), a nondestructive method, to study physical and chemical properties related to fruit maturity in Canary melons. They found that chlorophyll and yellow pigments decreased with fruit development, having a high correlation with maturity index (IM). Flügel and Gross (1982) studied pigment and plastid changes during ripening of the green-fleshed ‘Galia’ muskmelon.
fruit. They observed that the carotenoid profile in the exocarp and mesocarp did not change during development. Also, relatively low levels of chlorophyll and carotenoids were found in the flesh. Yellowing of the exocarp was due to increased chlorophyll degradation during ripening, as well as a partial decrease in total carotenoids took place.

In conclusion, it seems that pigment profile accumulation and degradation in melon fruit is a cultivar-dependent characteristic, which is expressed during fruit maturity.

2.2.4 Ethylene and Molecular Changes During Ripening

2.2.4.1 Introduction

There are ethylene-dependent and ethylene-independent biochemical and physiological pathways throughout melon fruit ripening (Pech et al. 1999; Hadfield et al. 2000; Srivastava, 2002; Silva et al. 2004), which both coexist at the same time in the climacteric fruit. Likewise, besides ethylene several plant hormones, such as indole-3-acetic acid (IAA) and abscisic acid (ABA), are involved in melon fruit ripening (Larrigaudiere et al. 1995; Dunlap et al. 1996; Guillén et al. 1998; Martínez-Madrid et al. 1999).

2.2.4.2 Biosynthesis, Perception and Effects of Ethylene

Ethylene biosynthesis goes from methionine, through S-adenosylmethionine (SAM), then to 1-aminocyclopropane-1-carboxylic acid (ACC), and finally to ethylene (Yang and Baur, 1969; Adams and Yang, 1979; Yang, 1980; Yang, 1982; Yang and Hoffman, 1984). Two regulatory enzymes in this pathway are ACC synthase (ACS) (EC 4.4.1.14) and ACC oxidase (ACO) (EC 1.14.17.4). The latter enzyme was formerly known as ethylene-forming enzyme (EFE) by Adams and Yang (1979) because the reaction mechanism was not known at that time. ACC synthase is generally considered as the rate-limiting step in ethylene biosynthesis (Yang and Hoffman, 1984).
Both ACS and ACO melon enzymes are coded by a multigene family (Miki et al. 1995; Yamamoto et al. 1995; Lasserre et al. 1996; Lasserre et al. 1997), therefore several isoenzymes are recognized in melon tissues. For instance, Miki et al. (1995) and Ishiki et al. (2000) isolated three cDNAs for ACC synthase from wounded mesocarp tissue of melon fruits. Lasserre et al. (1996) reported the isolation and categorization of three genomic clones, identified by screening a melon genomic DNA library with the cDNA pMEL1, corresponding to three putative members of the ACC oxidase gene family in *cantaloupensis* melon. These authors in addition determined the entire sequence of these genes and found that they were all transcriptionally active. One genomic clone, named *CM-ACO1*, presented a coding region with four exons and interrupted by three introns. The other two genes, *CM-ACO2* and *CM-ACO3*, were only interrupted by two introns, at same positions as *CM-ACO1*. The degree of DNA homology in the coding regions of *CM-ACO3* relative to *CM-ACO1* was 75%. In contrast, the degree of DNA homology of *CM-ACO2* relative to both *CM-ACO1* and *CM-ACO3* were 59% in their coding region.

ACS and ACO melon multigenes are differentially activated and expressed by several environmental and developmental factors (Yamamoto et al. 1995; Lasserre et al. 1996; Shiomi et al. 1999; Zheng et al. 2002). Yamamoto et al. (1995) using tissue printing and immunoblot analysis with antibodies specific for ACO, were able to identify in which part of the fruit the ethylene synthesis started at the early stages of ripening. They reported that the rate of ethylene production (accumulation of ACO protein) in melon fruits increased initially in the placental tissue, then in mesocarp tissue and finally at the rind. They also concluded that levels of ACO mRNA and protein were low in unripe fruit stage, but became detectable in placental tissue at the pre-climacteric period,
and their levels increased in the mesocarp at the climacteric stage. All these results suggested that the central region of melon fruit (placental tissue and seeds) plays a major role in the production of ethylene during the early stage of fruit ripening.

A RT-PCR assay was used by Lasserre et al. (1996) to detect the differential expression of ACO melon genes (CM-ACO1, CM-ACO2, and CM-ACO3). They found that these three genes were differentially expressed during development, ethylene treatment and wounding. CM-ACO1 was induced during fruit ripening, and also in response to wounding and ethylene treatment in leaves. CM-ACO2 was detectable at low levels in etiolated hypocotyls, whereas CM-ACO3 was expressed in flowers and it was not induced by any treatment tested.

Lasserre et al. (1997) found that the regulation of the CM-ACO1 gene was connected preferentially to stress responses, while the CM-ACO3 gene seemed to be associated with developmental routes. Moreover, Bouquin et al. (1997) using the 5’-untranslated region of the CM-ACO1 gene fused to the β-glucuronidase (GUS) reporter gene were capable of measuring the transcriptional activation in leaves of the CM-ACO1 gene after wounding and ethylene stimulation. Their results suggested that induction of CM-ACO1 gene expression occurs via two direct and independent signal transduction pathways in response to both stimuli. Zheng et al. (2002) studied some genetic aspects of ethylene production and its relationship to the RFLPs of the ACC oxidase and ACC synthase genes in two melon cultivars. One cultivar had high ethylene production during fruit ripening (‘TAM Uvalde’) and another had low levels of ethylene production (‘TAM Yellow Canary’). Their results of single-copy-reconstruction assays suggested that the
CMACO-1 gene was present as a single copy, whereas the CMACS-1 gene was a component of a multigene family in both melon cultivars.

It has been suggested that differences in ethylene production among melon fruits might be the result of transcriptional changes in ACS and ACO genes (Shiomi et al. 1999). These authors measured the *ACS-1, ACO-1* and *ACO-2* mRNA expression pattern in exocarp, mesocarp and placental tissues of ‘Earl’s Favorit’ (recognized as non-climacteric) and ‘Andes’ (known as climacteric) fruit cultivars at different stages of maturity, finding that mRNA *CMACS-1* transcripts accumulated only in the mesocarp and placentae of ‘Andes’ fruit at 50 DAP (commercial harvest maturity stage). This accumulation was coincident with increases in ACS activity, ACC content and maximum ethylene production. In contrast, *CMACO-1* mRNA accumulated in elevated levels in the mesocarp and placentae of both cultivars at 50 DAP, but in ‘Andes’ cultivar those transcripts were more abundant than in ‘Earl’s Favorit’ fruit. In the exocarp, the *CMACO-1* mRNA level was low for both cultivars. *CMACO-2* mRNA was constitutively expressed in placentae and mesocarp at low levels, and non-detectable in the exocarp. These results suggested that the difference in ethylene-forming ability between these two cultivars may result from the expression of *CMACS-1* mRNA and *CMACO-1* mRNA during the fruit ripening process.

Ethylene perception is mediated by specific receptors, which have been cloned and completely described for several plants, such as *Arabidopsis* (Fluhr and Mattoo, 1996; Johnson and Ecker, 1998), tomato (Tieman et al. 1999; Tieman et al. 2000), tobacco (Terajima et al. 2001), and carnation (Reid and Wu, 1992; Shibuya et al. 2002). In *Cucumis melo*, ethylene-receptor-like homolog genes have been reported as well (Sato-
Nara et al. 1999, Takahashi et al. 2002; Nukui et al. 2004; Cui et al. 2004). Sato-Nara et al. (1999) isolated and characterized two cDNAs, which were described as putative ethylene receptors, from muskmelon using the Arabidopsis ethylene receptor genes \textit{ETR1} and \textit{ERS1} sequences. These authors measured the expression pattern of these cDNAs during fruit enlargement and ripening by means of Northen blot assay, finding that both clones were expressed in a stage- and tissue-specific manner. They named their cDNAs as \textit{Cm-ETR1} (Accession No. AF054806) and \textit{Cm-ERS1} (Accession No. AF037368).

Three years later (Takahashi et al. 2002), polyclonal antibodies against melon receptor, \textit{Cm-ERS1}, were prepared in order to determine the temporal and spatial expression pattern of Cm-ERS1 protein during melon fruit development. They reported that Cm-ERS1 protein formed a disulphide-linked homodimer and it was present in microsomal membranes but not in soluble fractions. In addition, their results revealed that a post-transcriptional regulation of Cm-ERS1 expression affects stage- and tissue-specific accumulation of this protein. That transition pattern was not cultivar-dependent because it was observed in two different melon cultivars, i.e. ‘Fuyu A’ and ‘Natsu 4’.

The cloning and characterization of two melon ethylene receptor genes has allowed their use as molecular genetic tools in heterologous systems with promising results (Nukui et al. 2004; Cui et al. 2004). The overexpression of a missense mutated melon ethylene receptor gene, \textit{Cm-ETR1/H69A}, in a heterologous plant, \textit{Nemesia strumosa}, conferred reduced ethylene sensitivity (Cui et al. 2004), making transgenic plants that had a significantly extended flower longevity compared with the wild type counterpart. On the other hand, because ethylene inhibits the establishment of symbiosis between rhizobia and legumes, a point mutated \textit{Cm-ERS1/H70A} gene was used to transform \textit{Lotus}
*japonicus* plants in order to examine how and when endogenous ethylene inhibits that rhizobial infection and nodulation (Nukui et al. 2004). Endogenous ethylene in *L. japonicus* roots inhibits rhizobial infection at the early stages (primary nodulation), and suggested that ethylene perception also assists negative feedback regulation of secondary nodule initiation.

Ethylene has a profound influence on climacteric fruit ripening, and in some cases it induces biochemical and physiological changes in non-climacteric fruit (Leliévre et al. 1997; Giovannoni, 2001; Périn et al. 2002a). In research with 63 different cultivars from eight market types of melon, belonging to *Cantaloupensis* and *Inodorus* varieties, Zheng and Wolff (2000) were able to demonstrate a significant correlation between RFLP polymorphisms and ethylene production in the fruit. These RFLPs were associated with flesh color, rind texture and postharvest decay characteristics in the melon genotypes examined. Low ethylene production and green- and white-flesh color were associated with the presence of a putative RFLP-MEL1 allele *Ao* (15kb), whereas high ethylene production and orange-flesh color were associated with allele *Bo* (8.5 kb) in the homozygous condition. Some melon cultivars, such as ‘Honeybrew’ and ‘HD Green flesh’, did not accumulate any detectable ethylene. Likewise, Périn et al. (2002a) reported that in the non-abscission melon fruit PI 161375, exogenous ethylene failed to stimulate abscission, loss of firmness, ethylene production and expression of ethylene-inducible genes. These authors obtained a recombinant population of Charentais X PI 161375 inbred lines segregating for fruit abscission and ethylene production. Genetic analysis showed that both characters are controlled by two independent loci. They concluded that
the non-climacteric phenotype in fruit tissues was attributable to ethylene insensitivity conferred by the recessive allelic forms from PI 161375.

As it was previously pointed out, ethylene-dependent and ethylene-independent biochemical and physiological pathways take place and coexist during melon fruit ripening. Hadfield et al. (2000) made a differential screening of a ripe melon fruit cDNA library and identified 16 unique cDNAs corresponding to mRNAs whose accumulation was estimated by ripening. Expression of fifteen, out of sixteen cDNAs, was ripening regulated, and twelve of them were fruit specific. Three patterns of gene expression were observed when the expression of cDNA clones was examined in transgenic ACC oxidase melon fruit. One group of cDNAs corresponded to mRNAs whose abundance was reduced in transgenic fruit but still inducible by ethylene treatment. The second group of mRNAs was not significantly altered in the transgenic fruit and it was not affected by ethylene treatment, indicating that these genes are regulated by ethylene-independent factors. The third group of cDNAs had an unexpected pattern of expression, low levels of mRNA in transgenic fruit and even remaining low after ethylene treatment. Obviously, the regulation of this third group of genes appears to be ethylene-independent.

Pech et al. (1999) described some ethylene-dependent events in cantaloupe Charentais melon fruits and divided them into two main groups. One group was considered as general metabolism, such as yellowing of the rind, fruit softening, volatile production, presence of climacteric respiration, abscission of the fruit, and susceptibility to chilling injury. The other group included enzyme activities, such as galactanase, α-arabinosidase, β-galactosidase, endo-polygalacturonase, ACC synthase (negative feedback) and ACC N-malonyltransferase. Likewise, these authors categorized ethylene-
independent characteristics into the same two groups, one group included in general metabolism, such as coloration of the flesh, accumulation of sugars and organic acids, accumulation of ACC, and loss of acidity. Within enzyme activities non-induced by ethylene were pectin methyl esterase, exo-polygalacturonase and ACC synthase (induction at onset of ripening). This classification of ethylene-dependent and ethylene-independent events provides valuable basic information, which might be used to design biochemical and/or molecular strategies with the aim to control melon fruit ripening.

Ethylene regulates fruit ripening by coordinating the expression of several genes (Lelièvre et al. 1997; Aggelis et al. 1997a; Aggelis et al. 1997b; Yang and Oetiker, 1998; Jiang and Fu, 2000; Périn et al. 2002a). The most common genes which are frequently regulated by ethylene during fruit ripening embrace some members of the ACS and ACO multigene family, phytoene synthase, endo-polygalacturonase, galactanase, one homologue of S-adenosyl-L-homocysteine hydrolase (SAHH) and even a mRNA, which is ripening-specific, named MEL2 of unidentified function (Karvouni et al. 1995; Aggelis et al. 1997a; Aggelis et al. 1997b; Hadfield et al. 1998; Pech et al. 1999; Guis et al. 1999; Périn et al. 2002a).

New insight knowledge will be available on ethylene role(s) in melon fruit development and ripening, when the molecular techniques which have produced information from model systems, such as Arabidopsis and tomato, might some day be applied to different melon genotypes. Moreover, that information could also be retrieved from wild and landraces melons, in order to find ripening genes to be transferred to commercial varieties.
2.3 Melon Biotechnology

2.3.1 Genetic Improvement

2.3.1.1 Traditional Breeding

For several thousands of years, human beings have altered the genetic background of plants, which have been used as crops (Fehr et al. 1987; Snustad et al. 1997). This genetic alteration was achieved by first selecting one type of plant or seed in preference to another, instead of randomly taking what nature provided (Fehr et al. 1987; Snustad et al. 1997). Subsequently, human selection for specific traits such as faster plant growth, larger and more nutritious seeds or sweeter fruits dramatically changed the domesticated plant species compared to their wild type counterparts (Fehr et al. 1987). The improved plant characteristics selected by those early agriculturalists were transmitted genetically to the succeeding plant generations, and later on, plant breeding methods as an art and science discipline was born (Suslow et al. 2002).

_Cucumis melo_ plants were not the exception to that human selection and plant breeding effort. Traditional breeding methods in melon have led to a considerable varietal improvement. Strong sexual incompatibility barriers at the interspecific and intergeneric levels have restricted the use of that genetic potential to develop new and enhanced melon cultivars (Niemirovicz-Szczt and Kubicki 1979; Robinson and Decker-Walters, 1999). Melon plant improvement by traditional hybridization is slow and limited to a restricted gene pool (Pitrat et al. 1999). It is possible to produce viable intraspecific melon hybrids between wild type melons and commercial melon varieties, with the aim to transfer some particular melon genetic traits, such as disease resistance to fungi, bacteria, virus and insects, or tolerance to environmental factors, such as salinity, flooding, drought, and high or low temperature, to commercial melon varieties (Dane,
1991). Commercial melon varieties, with sweet, non-bitter and low-acidic fruits, carry three genes (suc/suc, so/so, bif/bif), which control high-quality-fruit traits, in recessive form (Burger et al. 2003). Therefore, any intraspecific crosses, using traditional breeding methods, between melon land races (Seshadri and More, 2002) and commercial melon cultivars will produce hybrid fruit with low quality characteristics, because of the effect of dominant genes controlling low-sweetness, high-acid, and high-bitterness level in the melon land race fruit.

In conclusion, it is highly desirable to have other genetic breeding tools, besides traditional hand crossing, in order to obtain improved melon cultivars. The same results obtained from conventional breeding methods can be developed using biotechnology strategies (Table 2.2). It is well documented that using genetic engineering strategies it is feasible to overcome most of the genetic barriers among plants, which are unsurpassable by traditional breeding techniques (Vasil, 1990; 1996; 1998; 2003).

2.3.1.2 Improvement Through Genetic Engineering

According to the ‘FAO statement on biotechnology’, The Convention on Biological Diversity (CBD) defines biotechnology as: “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use” (FAO, 2004). This scientific discipline in its broad sense covers many of the tools and techniques that are commonplace in agriculture and food production, whereas in its narrow or molecular sense it considers only the new DNA techniques, molecular biology and reproductive technological applications, including differente technologies such as gene manipulation, gene transfer, DNA typing and cloning of genes, plants and animals (FAO, 2004).
Table 2.2 Similarities between conventional and biotechnological methods for melon plant improvement.

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</table>

Modified from Ezura (1999).

During the 1970s, molecular biology and genetic engineering research laid the foundation for the development of transgenic plants in 1983 using the Ti plasmid from the soil bacterium Agrobacterium tumefaciens (Herrera-Estrella et al. 1983). This bacterium transfers a specific fragment of the Ti plasmid (T-DNA) which can be engineered to contain a selectable marker and/or genes of interest, into the plant nuclear genome under in vitro conditions. Once inserted, nontransformed plants can be killed in culture by the toxic substance the marker gene codes resistance to. Within the plant biotechnology discipline, plant tissue culture methods has had an essential role, allowing the development of transgenic plants with a number of desirable agronomic, pest resistance and food traits. It is commonly accepted that the term “plant tissue culture” refers to in vitro cultivation on nutrient media of any plant part, a single cell, tissue or an
organ under a sterile environment, leading to a whole *de novo* regenerated plant (Nunez-Palenius et al. 2005a).

After the milestone plant transformation achievement by Herrera-Estrella et al. (1983), a number of technological difficulties were surpassed, allowing the cloning and insertion of different genes and engineering of transgenic plants with: a) resistance to plant viruses, fungi and insects, tolerance to herbicides, salinity, drought, heavy metals and low/high temperatures, b) improved nutritional quality (proteins, oils, vitamins, and minerals among others), shelf life of fruits and vegetables, flavor and fragrance, c) novel production of vaccines, pharmaceuticals, and therapeutic and prophylactic proteins, d) reduced production of allergens, and e) phytoremediation activity (James and Krattiger, 1996; Vasil, 2002; 2003; Nuñez-Palenius et al. 2005). The first transgenic commercial plant variety in the USA was released in May 1994, when Calgene marketed its Flavr-Savr™ delayed ripening tomato (James and Krattiger, 1996). At the present time, more than 50 transgenic crops have been approved for commercial planting, and at least 100 more are under field trials and/or regulatory review (Vasil, 2003).

The influence of plant genetic engineering on commercial crop production is evident by the global increase of cultivated land with transgenic crops, also known as Genetically Modified Organisms (GMOs). All this increase has happened in a relatively short time span, i.e. less than a decade. According to James (2003), the global land area of transgenic crops continued to grow for the seventh consecutive year in 2003, 15%, 9 million hectares, compared with 12% in 2002. In 2003, 25% of the aggregate area of four main crops, i.e. soybean, maize, cotton and canola, totaling over one quarter billion hectares was GMOs. The market value of transgenic crops is expected to increase from
$450 million in 1995 to over $7 billion by 2005 (James and Krattiger, 1996; James, 2003), for instance, the total market for transgenic seed now exceeds $3 billion (Vasil, 2003).

Throughout an eight-year period, from 1996-2003, the land area of transgenic crops in the world increased 40 times, from 1.7 million hectares in 1996 up to 67.7 million hectares in 2003 (Figure 2.1). Plants containing herbicide (Roundup) tolerance was the dominant trait used, followed by insect resistance conferred by *Bacillus thuringiensis* (Bt) toxin.

![Global Land Area of Transgenic Crops](image)

The highest yearly increase of global land area cultivated with transgenic crops was observed during the period 1997-1999 (Figure 2.2). Afterward, in 2000, a substantial decline in the rate of yearly increase took place. Nevertheless, it seems from most recent data (2001 to 2003) that global land area cultivated with GMOs will be increased and it might be able to attain again similar levels as previous years (James, 2001; 2002; 2003).
USA cultivated more land area (42.8 million hectares) of transgenic crops than any other nation (Figure 2.3). In the same year a growth rate of 10% in planted area of transgenic crops was reflected a strong increase in both Bt toxin maize and herbicide tolerant maize, and continued growth in herbicide tolerant soybean (James, 2003). These transgenic plants, for the period of 2003, were grown by 7 million farms in 18 different countries, two million more of farms and two more countries than in 2002. Globally in 2003, the most common and commercialized transgenic crops were: soybean (41.4 million hectares), maize (15.5 million hectares), cotton (7.2 million hectares), and canola (3.5 million hectares). Similarly in 2003, herbicide tolerance installed in soybean, maize, canola and cotton occupied 49.7 million hectares (73%) of the global 67.7 million hectares.
Thus, world land area planted with GMOs is increasing substantially every year. According to FAO (2004), global population reached 6 billion on October 12, 1999, and in view of the current human population growth rates (1.5%), it is expected that human beings in this world will be approximately 11 billion for the year 2050 (Swaminathan, 1995). Doubling or tripling of the world food and fiber production by 2050 cannot be achieved using existing crop technology (James and Krattiger, 1996). Therefore, more research with plant molecular tools and transgenic crops must be accomplished in order to reach the needs to maintain such high human populations.

2.3.1.3 Melon Biotechnology

Since the first report of successful transformation of melon (*Cucumis melo*) (Fang and Grumet, 1990), several valuable plant features, such as virus resistance (Fang and
Grumet 1993; Yoshioka et al. 1993; Gonsalves et al. 1994; Huttner et al. 2001), tolerance to salinity (Bordas et al. 1997) and fruit quality improvement (Ayub et al. 1996; Ezura et al. 1997; Shellie, 2001; Silva et al. 2004; Nuñez-Palenius et al. 2003; 2004) have been incorporated to wild type germplasm. The first US trial of field-tested virus resistance in a commercial transgenic melon crop was done in 1993 and 1994 (Clough and Hamm, 1995). Although, a transgenic melon application was first registered in 1998 by the USDA-APHIS service (2004), there is still no commercial transgenic melon cultivars approved, despite of more than 140 transgenic melon field trials in the United States during the period 1987-2001 (Grumet and Gifford, 1998; Grumet, 2002; Gaba et al. 2004; Grumet, 2005. Personal communication). The lack of a commercial GMO melon is due to unstable expression or partial loss of the transgene of interest (due somaclonal variation and/or chromosomal rearrangements), and because the transgenic melon plant did not have any commercial advantage compared to the wild type counter part.

2.3.2 In vitro Regeneration

In order to achieve a successful commercial application from biotechnology in melon a competent de novo regeneration system from in vitro cultures is requiered (Guis et al. 1998). In the last 25 years, more than 40 in vitro melon regeneration protocols have been described, some using either organogenesis, somatic embryogenesis or both regeneration pathways (Table 3.2, 3.3 and 3.4). Melon plant regeneration has been reported from adventitious buds, somatic embryos, shoot primordia, protoplasts, and axillary buds. Several biological and physical factors influence in vitro regeneration efficiency rate, and all have to be considered in order to develop a reproducible and reliable melon regeneration protocol.
2.3.2.1 Genetic Control

Because genetic variability in melon is highly diverse (Monforte et al. 2003), genotype is the most important factor, determining regeneration potential. Melon varieties (*reticulatus, cantaloupensis, inodorus, flexuosus*, etc.) and commercial cultivars have differences on their regeneration ability under the same *in vitro* protocol and environmental conditions (Orts et al. 1987; Oridate et al. 1992; Gray et al. 1993; Ficcadenti and Rotino, 1995; Molina and Nuez, 1995a; Kintzios and Taravira, 1997; Galperin et al. 2003a; 2003b). Likewise, organogenesis and somatic embryogenesis responses in melon cultures are also genotype-dependent. For instance, Oridate et al. (1992) and Gray et al. (1993) reported that *reticulatus* varieties were more prone to produce *in vitro* somatic embryos than *inodorus* varieties.

Oridate et al. (1992) found significant differences in somatic embryogenic response from melon seeds among 18 commercial cultivars. They made reciprocal crosses between those cultivars, in order to obtain the F₁ seeds and evaluate their embryogeny response. Some lines produced a large number of somatic embryos whereas others did not produce any somatic embryos or the response was very low. Moreover, these authors were able to transfer, by sexual crosses, the embryogenic regeneration response from superior to inferior responding cultivars, demonstrating that the capacity to *de novo* regenerate through somatic embryogenesis by different melon cultivars was under genetic control. These researchers were unable to determine the specific mode of inheritance of the somatic embryogenic capacity due to variation in the range of somatic embryogenesis from F₂ seeds.

Gray et al. (1993) developed an improved protocol for high-frequency somatic embryogenesis from melon seeds. Using the cultivar ‘Male Sterile A 147’, the authors
tested several factors, such as, changes in plant hormone levels and combinations, type of culture media, and incubation time of explants in those media. This protocol was tested on 51 commercial melon cultivars, where all underwent somatic embryogenesis, but exhibited from 5% to 100% explant response and 0.1 to 20.2 embryos per explant, indicating again a genetic factor in melon embryogenesis response.

Melon regeneration through organogenesis is also affected by melon genotype (Orts et al. 1987; Ficcadenti and Rotino, 1995; Molina and Nuez, 1995a; Kintzios and Taravira, 1997; Galperin et al. 2003a; 2003b). Orts et al. (1987) found significant differences in the morphogenetic response of a diverse group of melon cultivars. The percentage of calli with developed shoots ranged from 0 to 44.3 among cultivars. Variability in morphogenetic responses was found between seed lots of the same cultivar. Comparable results were obtained by Ficcadenti and Rotino (1995), who evaluated the morphogenetic response of 11 melon cultivars belonging to the *reticulatus* and *inodorus* genotypes. These authors found that melon morphogenetic response was affected by genetic background, i.e. *C. melo* var. *inodorus* genotypes exhibited high narrow shoot regeneration rates whereas wide differences were noted among the *reticulatus* types. The number of shoots per explant ranged from 6.0 to 17.3 for *reticulatus* varieties and from 12.2 to 14.2 for *inodorus* genotypes.

A complete statistical approach was used by Molina and Nuez (1995a) to detect genotypic variability of the *in vitro* organogenetic response (shoot regeneration) among individual melon seeds. Their results clearly evidenced the presence of highly significant differences for organogenetic response, among plants from a specific seed population. These authors used data from stochastic simulation to study the accuracy of different
analysis to detect the presence of genotypic heterogeneity within a population. These analyses, together with their experimental results, allowed the separation of seed genotypes differing up to 5% in their regeneration ability. Afterward, Molina and Nuez (1996) reported the inheritance of organogenesis response in melon cv. ‘Charentais’, by studying the distribution of the shoot regeneration frequency in F1 and F2 generations from parents representing extreme values for that in vitro organogenesis response. Their results suggested a genetic model with two genes, partial dominance, independent segregation and similar effects for both genes. Recently, Galperin et al. (2003b) claimed that the high competence for adventitious regeneration in the BU-21/3 melon genotype was controlled by a single dominant locus, without cytoplasmic interactions.

On the other hand, Kintzios and Taravira (1997) evaluated 14 commercial melon cultivars for plant regeneration capability. Only six cultivars responded positively to a shoot induction treatment. Similarly, Galperin et al. (2003a) screened 30 different commercial melon cultivars for shoot de novo regeneration. In 24 out of 30 melon genotypes, no detectable normal shoot growth was observed. Five of those which were able to regenerate, exhibited very low regeneration efficiency. Only the genotype BU-21, an inbred line, had profuse regeneration of multiple shoots.

In summary, melon in vitro response is under genetic control, however, other factors should be taken into account for melon regeneration as well. Among them, plant hormones have a paramount importance upon the melon in vitro response.

### 2.3.2.2 Polyploidization and Somaclonal Variation

Diploid melon plants have 24 chromosomes (haploid stage n=x=12), and a genome size of 0.94 pg (454 Mbp/1C) (Arumuganathan and Earle, 1991). A natural and spontaneous increase in the ploidy level has been observed in field-growing melon plants
(Nugent and Ray, 1992), nevertheless, this ploidy increase can also been induced in muskmelon plants using chemical compounds, such as colchicine (Batra, 1952; Kubicki, 1962). In fact, polyploidy as a method of plant breeding received an increase amount of attention in the late 1930s, when it was discovered that polyploidy could be induced with colchicine treatment and the earlier demonstration that heat treatment in early embryogeny stages could also induce a chromosome doubling (Batra, 1952).

Numerous tetraploid and triploid muskmelon plants have been obtained since the 1930s (Batra, 1952; Kubicki, 1962; Nugent and Ray, 1992; Fassuliotis and Nelson, 1992; Ezura, et al. 1992a; Adelberg, 1993; Nugent, 1994a; Nugent, 1994b; Adelberg et al. 1995; Adelberg et al. 1999). According to Ezura et al. (1992b), Fassuliotis and Nelson (1992) and Nugent (1994b), tetraploid melon plants are characterized by having large male and hermaphrodite flowers, protrudent stigmas, low fertility, thickened and leathery leaves, rounded cotyledons, highly hairy leaves and stems, short internodes, flat fruits, large fruit blossom-end scar, increased number of vein tracts on the fruit, and round seeds. Nonetheless, Shifriss (1941) had previously been reported that tetraploid melon plants were highly fertile and no later in maturity than the ordinary diploids. Moreover, Batra (1952) reported that the quality of melon tetraploids was superior to diploids in certain varieties and that tetraploids were sufficiently fertile to be propagated readily from seeds.

Regarding the fruit quality of tetraploid melon plants, Batra (1952) and Nugent (1994a, 1994b) reported that tetraploid fruits were superior in sugar level, firmness, and had better color than diploid fruits. However, tetraploid melon plants were less productive, because they had smaller and flatter fruits than diploids, most cultivars had
low fertility, and the fruits had an increased tendency to suffer of easy cracking, therefore reducing considerably their marketable properties.

As it was mentioned before, triploid melon plants have also been produced (Ezura et al. 1993; Adelberg et al. 1995; Adelberg et al. 1999). Despite triploids plants grew more vigorously than diploids, and their fruits were not as flat as tetraploids. These triploids melon plants did not have any marketable advantage over diploids ones, because still the percentage of cracking in those triploid fruits was greater than diploid fruits, and their sugar content was lower. In addition, triploid plants required adjacent diploid pollinators as a result that they did not set fruit when self-pollinated (Ezura et al. 1993; Adelberg et al. 1995).

When modern biotechnology, specifically plant tissue culture, was applied to Cucumis melo in order to obtain reliable regeneration protocols, somaclonal variation was a common observable fact, therefore tetraploid, octaploid, mixoploid, and aneuploid melon plants were easily recovered from in vitro cultures (Bouabdallah and Branchard, 1986; Fassuliotis and Nelson, 1992; Ezura et al. 1992a; 1992b; Debeaujon and Branchard, 1992; Kathal et al. 1992; Ezura and Oosawa, 1994a; 1994b; Ezura et al. 1994). According to Ezura et al. (1995), somaclonal variation could be used to obtain variants lines with low-temperature germinability in melon. Changes in fatty acid patterns have been found in melon callus tissue (Halder and Gadgil, 1984), as well changes in a repetitive DNA sequences during callus culture have been detected (Grisvard et al. 1990). However, somaclonal variation has to be avoided in research, where genetic transformation is involved because genomic stability in transgenic plants has to be maintained in order to express the inserted transgene. In addition, regeneration of melon
plants has never been obtained from long-term calli tissue cultures of *Cucumis melo* (Grisvard et al. 1990).

The production of tetraploid regenerated melon plants has been observed from somatic embryogenesis (Ezura et al. 1992a; 1992b), organogenesis (Bouabdallah and Branchard, 1986; Fassuliotis and Nelson, 1992; Ezura et al. 1992a), and protoplast regeneration (Debeaujon and Branchard, 1992). Nevertheless, each morphogenetic pathway has a different effect on the frequency of recovered tetraploid plants, i.e. somatic embryogenesis (31%), adventitious shoots (30%), shoot primordia (4%), and axillary buds (0%) (Ezura et al. 1992a). Therefore, when a callus stage is involved in the regeneration process the likelihood to augment the ploidy level in the regenerated plant is increased as well. In addition, explant origin affects the frequency of tetraploid plants from melon tissue cultures (Adelberg et al. 1994). Immature cotyledons produced more tetraploid regenerants than mature cotyledons, while explants from apical meristems produced fewer or no tetraploid plants (Adelberg et al. 1994).

Ezura and Oosawa (1994a; 1994b) reported that the capacity of diploid melon cells to generate *in vitro* shoots was greater than tetraploid cells. The ability of tetraploid cells to differentiate into somatic embryos was greater than diploid cells. These same authors reported that the ability of somatic embryos to develop into plantlets decreased in the following order: diploid > tetraploid > octaploid. Ezura and Oosawa (1994a) and Kathal et al. (1992) reported the longer melon cells are kept under *in vitro* conditions, the greater the possibility to increase the ploidy levels in those cells. The frequency of chromosomal variation leading to aneuploid (hyperploid and hypoploid) plants at diploid, tetraploid and octaploid levels also increases.
Melon plant ploidy levels can be determined by cytological methods, such as counting the chromosome number using root squash tips (Ezura et al. 1992a; 1992b; Kathal et al. 1992; Ezura et al. 1993; Ezura et al. 1994; Adelberg et al. 1995) or young tendrils (Yadav and Grumet, 1994). These methods are very laborious and time consuming, and are not completely reliable because melon chromosomes are smaller in comparison with other plants, which complicated the easy chromosome observation. Unconventional and indirect techniques have been developed in order to determine the ploidy level of regenerated melon plants (Fassuliotis and Nelson, 1992; Adelberg et al. 1994; Adelberg et al. 1995). Among them, pollen grain shape and stomate length, as well as the chloroplast number in guard cells from stomata have been commonly used. Diploid plants have pollen grains with typical triangular-appearing shape and are tripolar, whereas tetraploid plants produce many square tetraporous, round-monoporous or oval biporous pollen grains (Fassuliotis and Nelson, 1992; Adelberg et al. 1994; Adelberg et al. 1995). Likewise, Fassuliotis and Nelson (1992) reported that in diploid plants the average stomate length was $22.1 \pm 2.15 \mu m$ and the average number of chloroplast inside the guard cell was $9.4 \pm 1.5$, while in tetraploid plants the average stomate length was $29.1 \pm 2.15 \mu m$ and the average number of chloroplast inside the guard cell was twice the diploid plant. All these data were measured in the epidermal layer from the third or fourth expanded leaf from the apex.

Flow cytometry is the only absolute, reliable and precise technique to determine the exact level of ploidy, and even it is useful to detect any chromosomal change or polysomaty state in melon tissues (Brown, 1984; Delaat et al. 1987; Dolezel et al. 1998;
Gilissen et al. 1993; Dolezel et al. 2004). Nevertheless, the economic constraint to purchase and operate this equipment restricts its wide usage.

In order to avoid somaclonal variation and regenerate mostly diploid plants from melon in vitro cultures, several strategies have been proposed. Among them, the induction of shoot primordium aggregates from shoot-tips then cultivating them in liquid medium shaken at low speed (Ezura et al. 1997b) was reported. Using this protocol, the frequency of tetraploids and mixoploids regenerated plants was less than 8% after 4 years of culture. The cryopreservation of shoot primordia cultures at low temperatures (liquid nitrogen) using a slow prefreezing procedure has given excellent results as well (Niwata et al. 1991; Ogawa et al. 1997). A reliable system for transformation of a cantaloupe Charentais type melon leading to a majority of diploid regenerants was developed (Guis et al. 2000). Unfortunately, this regeneration system did not generate completely developed transgenic shoots for other commercial melon cultivars (Nuñez-Palenius et al. 2002; Gaba, 2002, personal communication), again, a genetic factor is involved at some stage in melon culture in vitro response.

In summary, it is particularly important to avoid in vitro-conditions which produce polyploid melon plants or other induced somaclonal variation, in order to maintain commercial value of the new genotype.

2.3.2.3 Vitrification

Woody and herbaceous explants are prone to suffer anatomical, morphological and physiological abnormalities when they are cultivated in vitro. Several terms have been used to describe those abnormalities, such as vitrification, translucency, hyperhydration, succulency and glassiness (Paques and Boxus, 1987; Ziv, 1991).
Vitrification term is the most often used term to describe physical changes in leaves and roots of cultured explants (Paques et al. 1987).

Melon *in vitro* cultures are very sensitive to undergo spontaneous vitrification, even if explants are cultured on non-inductive media or conditions (Leshem et al. 1988a; 1988b). Different factors have been proposed to induce and maintain an explant in a vitrificated state, such as, high relative humidity inside the *in vitro* container, high water potential of the media, low agar level, deficiency in Ca$^{2+}$ level, high NH$_4$ concentration, presence of ethylene within the flask, and high a level of cytokinins (mostly BA) (Leshem et al. 1988a; 1988b; Paques and Boxus, 1987; Paques et al. 1987; Ziv, 1991). Leshem et al. (1988a) studied the development of vitrification in melon shoot tips cultured in solid and liquid media. These authors found that on solid medium the vitrification process gradually increased with time, whereas on liquid medium it was an ‘all-or nothing’ effect. Cytokinins had the major effect on vitrification induction on melon buds as well (Leshem et al. 1988b). Paques et al. (1987) and Kathal et al. (1994) reported that vitrification process was an inducible and reversible physiological event. If the tissues are frequently subcultured, vitrification may be avoided, however this may induces somaclonal variation.

The following modifications in the culture media have been suggested to avoid vitrification: increasing the agar concentration, diminishing chloride ions, reducing potassium, increasing calcium, adding cobalt, modifying the plant hormone balance by reducing the amount or type of cytokinins, suppressing the use of casein hydrolysate and adenine sulphate, and adding pectin, phoroglucinol, or phloridzine (Paques and Boxus, 1987; Paques et al. 1987; Ziv, 1991). *In vitro* environmental conditions can also be
altered including: several treatments have been proposed: a cold treatment to the plants before *in vitro* culture, reducing culture room temperature, increasing the daily dark period, increasing the container-environment gas exchange, and reducing the relative humidity within the flask (Paques and Boxus, 1987; Paques et al. 1987; Ziv, 1991).

### 2.3.3 Regeneration by Organogenesis

In addition to genotype, explant source or type has a main role on melon *in vitro* regeneration (Adelberg et al. 1994; Ficcadenti and Rotino, 1995; Molina and Nuez, 1995b; Curuk et al. 2002b). Shoots, roots, and complete melon plants have been *de novo* regenerated through organogenesis using diverse explants, among them: cotyledons (from immature and quiescent seeds, and/or seedlings), hypocotyls, roots, leaves, protoplasts, and shoot meristems have been reported (Table 3.2. and Table 3.3). This *in vitro* organogenetic pathway may produce plants by direct regeneration (non-callus growth between explant culture and *de novo* shoot induction) (Table 3.2) or indirect regeneration (involving callus growth before *de novo* shoot induction) (Table 3.3).

Cotyledons and true leaves have commonly a higher regeneration frequency (above 80%) of *de novo* shoots using direct organogenesis compared to other melon explants (Moreno et al. 1985a; 1985b; Kathal et al. 1986; Orts et al. 1987; Tabei et al. 1991; Ficcadenti and Rotino, 1995; Yadav et al. 1996; Nuñez-Palenius et al. 2002, Galperin et al. 2003a; 2003b). Likewise, Molina and Nuez (1995b) studied the variation in regeneration ability among and within several populations of leaf, cotyledon and hypocotyl melon explants. These authors reported that *in vitro* clonal selection to improve the regeneration frequency from leaf explants also raises the organogenetic response in other explant types. These results suggest the presence of a partial common genetic system controlling the regeneration frequency of all type of explants.
In addition to explant type, other non-genotype *in vitro* culture components may have some influence upon the efficiency of melon regeneration through organogenesis, among them, environmental factors and media composition.

### 2.3.3.1 Medium Composition

It is generally accepted that medium composition has a greater effect upon organogenic regeneration than environmental factors. This is due to the fact that plant growth regulators have an enormous effect on *in vitro* cell development. In general, a cytokinin/auxin ratio greater than 1 is used in order to induce *de novo* bud formation, however, auxins are not always a prerequisite to achieve that goal (Table 2.3 and Table 2.4), and cytokinins alone are able to induce bud formation. Among cytokinins, 6-benzylaminopurine (N<sup>6</sup>-benzyladenine, BA) is the most frequently used in high levels (1 mg/l or higher) to induce bud formation. BA concentration is lowered (0.5 mg/l or lower) to allow shoot elongation. Elongated shoots are then transferred to a plant growth regulator-free medium or with low-auxin level (NAA or IAA) to induce the rooting process. If indirect regeneration is used, two- or even a three-step method has to be utilized. First, an induction callus growth is stimulated on the explant by applying strong cytokinins (TDZ) and auxins (2,4-D) to the medium culture. Second, those calli, which have green nodules are transferred to a low-level plant hormone-medium to induce shoot differentiation, and third, differentiated shoots are cultured in a low-cytokinin medium to induce shoot elongation (Table 2.3). In general, indirect regeneration is longer in time than direct regeneration to recover a whole regenerated plant. This is due to the several subcultures are required for shoot elongation and two or three months are needed for rooting using the indirect method (Moreno et al. 1985a; 1985b; Kathal et al. 1986; 1994).
Table 2.3 Melon regeneration (shoots, roots and/or complete plants) through direct organogenesis.

<table>
<thead>
<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Induction Medium (Plant Growth Regulators)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Cotyledon and primary leaf              | ‘Bananna’, ‘Dixie Jumbo’, Planters Jumbo’, ‘Morgan’, ‘Cavaillon Red Flesh’, and ‘Saticoy Hybrid’ | BR-1 medium: 0.1mg/l NOA, 20 mg/l 2iP, and 0.1 mg/l CCC  
HC medium: 0.05 mg/l NOA, 10 mg/l 2iP, and 0.1 mg/l CCC | 1         |
<p>| Hypocotyl from 11 to 13-day-old seedlings | ‘Amarillo Oro’                                                           | 4.5 mg/l IAA                                                                      | 2         |
| Leaf (0.3-0.5 cm) from 14-day-old seedlings | ‘Pusa Sharbati’                                                         | 0.22 mg/l BA and 0.2 mg/l 2iP                                                    | 3         |
| Cotyledons                              | ‘Halest Best’, ‘Iroquois’, and ‘Perlita’                                 | NAA and BA                                                                        | 4         |
| Cotyledons from mature seeds, and cotyledons and leaves from 5 to 7-day-old seedlings | ‘Accent’, ‘Galia’, ‘4215’, ‘Preco’, and ‘Viva’                            | 1 mg/l BA                                                                         | 5         |
| Cotyledons from 4-day-old seedlings     | ‘Superstart’, ‘Hearts of Gold’, ‘Hale’s Best Jumbo’ and ‘Goldstart’       | 0.88 mg/l IAA and 1.13 mg/l BA                                                   | 6         |
| Cotyledons from 4-day-old seedlings     | Not reported                                                             | 0.2 mg/l BA                                                                        | 7         |
| Cotyledons from 9 to 10-day-old seedlings | ‘Topmark’                                                               | 1 mg/l                                                                             | 8         |</p>
<table>
<thead>
<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Induction Medium (Plant Growth Regulators)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Cotyledons from mature seed, cotyledons and hypocotyl from 10-day-old seedlings, and leaf segment and petioles from 3-weeks-old seedlings</td>
<td>‘Earl’s Favorite Harukei No.3’</td>
<td>0.01 mg/l 2,4-D or 1 mg/l IAA and 0.1 mg/l BA</td>
<td>9</td>
</tr>
<tr>
<td>Cotyledons from 8-day-old seedlings</td>
<td>‘Charentais’ and ‘Gulfstream’</td>
<td>1.12 mg/l BA for ‘Charentais’ and 1.12 mg/l BA and 1.75 mg/l IAA for ‘Gulfstream’</td>
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<td>Cotyledons from 2-day-old seedlings</td>
<td>‘Sunday Aki’</td>
<td>1 mg/l BA, 50-200 µM salicylic acid and 10 mM proline</td>
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<tr>
<td>Cotyledons from 7-day-old seedlings</td>
<td>Five Inbred lines from Teziers</td>
<td>0.1 mg/l NAA and 0.5 mg/l BA</td>
<td>12</td>
</tr>
<tr>
<td>Cotyledons from immature seeds</td>
<td>‘Miniloup’, ‘L-14’, and ‘B-Line’</td>
<td>2.25 mg/l BA</td>
<td>13</td>
</tr>
<tr>
<td>Cotyledons from mature seeds</td>
<td>‘Prince’ and ‘Andes’</td>
<td>1 mg/l BA</td>
<td>14</td>
</tr>
<tr>
<td>Cotyledons from 4-day-old seedlings</td>
<td>11 genotypes</td>
<td>0.63 mg/l BA and 0.26 mg/l ABA</td>
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<td>3-4 cm diam expanded leaves</td>
<td>‘Hale’s Best Jumbo’ and ‘Ananas El Dokki’</td>
<td>0.87 mg/l IAA, 1.13 mg/l BA and 0.026 ABA</td>
<td>16</td>
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<td>Cotyledons from 7-day-old seedlings</td>
<td>‘Pusa Madhuras’</td>
<td>0.22 mg/l BA</td>
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<td>Explant Source</td>
<td>Cultivar</td>
<td>Induction Medium (Plant Growth Regulators)</td>
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<tr>
<td>Cotyledons from 2-week-old seedlings</td>
<td>14 cultivars</td>
<td>Embryogenesis: 1.98 mg/l 2,4-D and 4.99 mg/l Organogenesis: 0.01 mg/l 2,4-D and 0.059 mg/l BA 0.22 mg/l BA and 0.33 mg/l 2iP</td>
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<td>Leaves from 10 day-old seedlings</td>
<td>‘Védrantais’</td>
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<tr>
<td>Cotyledons from 2-day-old seedlings</td>
<td>‘Galia’ male and female parental lines</td>
<td>0.001 mg/l NAA and 1 mg/l BA</td>
<td>20</td>
</tr>
<tr>
<td>Cotyledons from mature seeds</td>
<td>‘Yellow Queen’, ‘Yellow King’, and ‘Hybrid AF-222’</td>
<td>1 mg/l BA</td>
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<tr>
<td>Proximal zone of the Hypocotyl from 4-day-old seedlings</td>
<td>‘Revigal’</td>
<td>1 mg/l BA</td>
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<td>Cotyledons from 4 to 5-day-old seedlings</td>
<td>Some Turkish cultivars: ‘Hasanbey’ 1, ‘Yuva’, ‘Kirkagac 637’, ‘Topatan’, ‘Kuscular’ and ‘Ananas’</td>
<td>Medium and plant growth regulators from reference 6</td>
<td>23</td>
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<tr>
<td>Cotyledons from mature seeds</td>
<td>Thirty melon genotypes</td>
<td>1 mg/l BA</td>
<td>24</td>
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</table>

<table>
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<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Callus Induction Medium (CIM) and Shoot Induction Medium (SIM) (Plant Growth Regulators)</th>
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</thead>
<tbody>
<tr>
<td>Cotyledon-callus culture from 11 to 13-day-old seedlings</td>
<td>‘Amarillo Oro’</td>
<td>CIM: 1.5 mg/l IAA and 6.0 mg/l KIN SIM: 0.01 mg/l NAA and 0.1 mg/l BA</td>
<td>1</td>
</tr>
<tr>
<td>Hypocotyl-callus culture from 7-day-old seedlings</td>
<td>‘Pusa Sharbati’</td>
<td>CIM: 1.0 mg/l IAA and 0.5 mg/l KIN SIM: 0.5 mg/l BA and 0.5 mg/l 2ip</td>
<td>2</td>
</tr>
<tr>
<td>Cotyledon-callus from 7 to 9-day-old seedlings</td>
<td>‘Charentais T’, ‘Doublon’, ‘CM 17 187’, and ‘Piboule’</td>
<td>CIM: 2 mg/l IAA and 2 mg/l KIN SIM: no plant growth regulators</td>
<td>3</td>
</tr>
<tr>
<td>Cotyledon-callus from 11 to 13-day-old seedlings</td>
<td>15 cultivars belonging to <em>cantaloupensis, inodorus</em> and <em>reticulatus</em> varieties</td>
<td>CIM: 6.0 mg/l KIN and 1.5 mg/l IAA SIM: Same as CIM</td>
<td>4</td>
</tr>
<tr>
<td>Cotyledon-protoplasts from 2-week-old seedlings</td>
<td>‘Hong-Xin-cui’</td>
<td>Protoplast Culture Medium and CIM: 0.5 mg/l 2,4-D, 0.5 mg/l Zeatin and 0.5 mg/l BA SIM: 0.3 mg/l 2,4-D, 1.0 mg/l Zeatin and 0.5 mg/l BA</td>
<td>5</td>
</tr>
<tr>
<td>Leaf segment (1.0 X 0.5 cm) from 8 to 10-day-old seedlings and petiole segment (0.4-0.8 cm) from 3 to 4-week-old seedlings</td>
<td>‘Cantaloupe PMR’</td>
<td>CIM: 5.0 mg/l NAA and 2.5 mg/l BA SIM: no plant growth regulators</td>
<td>6</td>
</tr>
<tr>
<td>Cotyledon-protoplasts from 2-week-old seedlings</td>
<td>‘Charentais’</td>
<td>Protoplast Culture Medium and CIM: 0.05 mg/l 2,4-D and 0.5 mg/l BA SIM: 2 mg/l BA</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 2.4 Continued

<table>
<thead>
<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Callus Induction Medium (CIM) and Shoot Induction Medium (SIM) (Plant Growth Regulators)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Cotyledon-protoplasts from 12-day-old seedlings and fully expanded leaves-protoplasts from 3-week-old seedlings</td>
<td>‘Charentais T’ and F1 hybrid cv. ‘Preco’</td>
<td>Protoplast Culture Medium and CIM: 0.75 mg/l BA SIM: 1.0 mg/l 2,4-D and 0.1 mg/l BA</td>
<td>8</td>
</tr>
<tr>
<td>Root-callus culture from 21-day-old seedlings</td>
<td>‘Pusa Sharbati’</td>
<td>CIM: 0.61 mg/l 2iP and 0.68 mg/l BA SIM: 0.22 mg/l BA</td>
<td>9</td>
</tr>
<tr>
<td>Cotyledon and Hypocotyl from 11-13 day-old-seedlings</td>
<td>‘Charentais’</td>
<td>CIM: 2.5 mg/l NAA and 1 mg/l BA SIM: 0.01 mg/l NAA and 6 mg/l Kin</td>
<td>10</td>
</tr>
</tbody>
</table>

Other plant hormones, such as gibberellins, and ABA (Kathal et al. 1986; Niedz et al. 1989; Ficcadenti and Rotino, 1995), and/or plant additives, such as proline, proline analogues, ornithine, salicylic acid, aspirin, and fish protein hydrolysates (Shetty et al. 1992; Milazzo et al. 1998; et al. 1999), Calcium antagonists (Leshem and Lurie, 1995), silver nitrate (Niedz et al. 1989; Roustan et al. 1992; Yadav et al. 1996), have been used to increase in vitro shoot regeneration frequency, nevertheless, the obtained results using both types of components (plant hormones and plant additives) are either melon genotype-dependent or are not consistent among reported results.

2.3.3.2 Environmental Factors

Environmental factors, such as light, temperature, nature of media gelling agent, and relative humidity within flask, influence the efficiency of regeneration method (Niedz et al. 1989; Ficcadenti and Rotino, 1995; Yadav et al. 1996; Kintzios and Taravira, 1997; Curuk et al. 2003). For instance, Niedz et al. (1989) studied the effect of temperature (22, 25 and 29°C) and light (0, 5, 10, 60 and 3,000 µmol m⁻² s⁻¹) on the percentage of bud initiation in cotyledonary explants of ‘Hale’s Best Jumbo’ melon. The greatest bud initiation was obtained when explants were cultured at 29°C under a range of light intensity of 5 to 30 µmol m⁻² s⁻¹. Conversely, lower temperatures (22 and 25°C), darkness, and higher light intensities (60 and 3,000 µmol m⁻² s⁻¹) reduced bud initiation. Similarly, Kintzios and Taravira (1997) tested two levels of light intensity, 50 and 250 µmol m⁻² s⁻¹ on shoot and root induction in 14 different melon cultivars. As expected, lower light intensities induced a greater root induction in many melon cultivars. Also, higher PPFD (250 µmol m⁻² s⁻¹) values adversely affected shoot induction from cotyledonary explants. Interestingly, Curuk et al. (2003) recently described that
regeneration from hypocotyls (proximal part to the cotyledons) of *Cucumis* species does not require light.

High relative humidity within the flask culture might induce ethylene accumulation, which affects shoot regeneration in melon cotyledons (Roustan et al. 1992). These authors added several levels of silver nitrate (60-120 µM AgNO₃) into culture media to inhibit ethylene action. They were able to obtain a two-fold increase in shoot regeneration by using silver. Furthermore, a transgenic antisense ACC oxidase line, which had little ethylene production, displayed a 3.5-fold increase in regeneration frequency compared to wild type line (Amor et al. 1998).

As it was pointed out previously, the nature of medium gelling agent also has an important role in melon regeneration (Ficcadenti and Rotino, 1995; Yadav et al. 1996). Ficcadenti and Rotino, (1995) described that using agar, instead of ‘gelrite’, they were able to attain a better cotyledon organogenetic response. Likewise, Yadav et al. (1996) preferred ‘phytagel’, as a substitute of agar for leaf organogenesis.

### 2.3.4 Regeneration by Somatic Embryogenesis

In addition to organogenesis, somatic embryogenesis is an alternative *de novo* morphological pathway that it can be induced in explants to form and recover whole dicots plants (Liu and Cantliffe, 1983). Somatic embryogenesis was described in melon explants before organogenesis and used to recover complete melon plants (Table 2.5) (Blackmond et al. 1981b). In general, cotyledonary tissue has been the most efficient explant for the induction of melon somatic embryogenesis (Table 2.5).

An embryogenic response in melon is affected by the nature of explant and genotype. Gray et al. (1993) reported significant differences in the frequency of
embryogenic muskmelon explants when they compared two commercial sources of the same melon cultivar. High regeneration frequency, up to 100%, may be attained from cotyledon explants with an average number of 20.2 embryos per explant (Gray et al. 1993). However, not all melon cultivars achieve that such a high regeneration frequency, some as low as 5% with 0.1 embryos per explant (Gray et al. 1993). The conversion rate of somatic embryos to plantlets might be a limiting step in some melon genotypes, for example, Trulson and Shahin (1986) were able to recover only 5 melon plants from hundreds of somatic embryos, Branchard and Chateau (1988) reported a 12% conversion rate, and Homma et al. (1991) reported a conversion rate from 7% up to 61%, depending on explant type.

Tabei et al. (1991) concluded that cotyledons were the best explant to induce somatic embryogenesis in the melon cultivar ‘Earl’s Favorite Harukei No.3’ using high concentrations of 2,4-D. Homma et al. (1991) tested the effects of explant shape on the production of melon somatic embryos, finding that the most reproducible result was obtained with explants consisted of radicle, hypocotyl and a proximal part of cotyledon. Oridate et al. (1992) found significant differences in somatic embryogenesis capability from 18 different melon cultivars from four genotypes. These authors concluded that genetic differences in somatic embryogenic formation capacity existed among cultivars rather than among genotypes.

Debeaujon and Branchard (1993) published a complete and extensive review on somatic embryogenesis in Cucurbitaceae, including Cucumis melo, where they concluded that even though somatic embryogenesis and plant recovery have been obtained from numerous plant sources including protoplasts,
<table>
<thead>
<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Induction Medium (IM) and Development Medium (DM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons from 5-day-old seedlings</td>
<td>‘Hale’s Best No.36’ and ‘Rocky Ford’</td>
<td>IM: 1.0 mg/l 2,4-D and 0.5 mg/l BA DM: 0.5 mg/l 2,4-D and 0.25 mg/l BA</td>
<td>1</td>
</tr>
<tr>
<td>Expanded cotyledons</td>
<td>‘Charentais T’</td>
<td>IM: 4.52 mg/l 2,4-D and 0.44 mg/l BA DM: no hormones</td>
<td>2</td>
</tr>
<tr>
<td>Cotyledons from mature seeds and cotyledons and hypocotyls from 10-day-old seedlings, leaves and petioles from 3-week-old plantlets</td>
<td>‘Earl’s Favorite Harukei No. 3’</td>
<td>IM: 2.0 mg/l 2,4-D or 25 mg/l IAA DM: no hormones</td>
<td>3</td>
</tr>
<tr>
<td>Cotyledons and hypocotyls from mature seeds</td>
<td>‘Green Pearl’ and ‘Earl’s Favourite’</td>
<td>IM: 4 mg/l 2,4-D, 2 mg/l NAA, and 0.1 mg/l BA DM: no hormones</td>
<td>4</td>
</tr>
<tr>
<td>Hypocotyls from mature seeds</td>
<td>‘Earl’s Favourite’</td>
<td>IM: 1 mg/l 2,4-D, 1 to 4 mg/l NAA, and 0.1 mg/l BA DM: no hormones</td>
<td>5</td>
</tr>
<tr>
<td>Cotyledons from 1-day-old seedlings</td>
<td>‘Earl’s Favourite Haru 1’</td>
<td>IM: 1 mg/l 2,4-D, 1 mg/l NAA, and 0.1 mg/l BA DM: no hormones</td>
<td>6</td>
</tr>
<tr>
<td>Protoplasts from 12-day-old cotyledons</td>
<td>‘Charentais T’ and F1 hybrid ‘Preco’</td>
<td>IM: 1 mg/l 2,4-D and 0.1 mg/l BA DM: no hormones</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 2.5 Continued

<table>
<thead>
<tr>
<th>Explant Source</th>
<th>Cultivar</th>
<th>Induction Medium (IM) and Development Medium (DM) (Plant Growth Regulators)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons and hypocotyls from mature seeds</td>
<td>18 cultivars belonging to reticulatus, inodorus, makuwa and intermediated type between reticulatus and cantaloupensis varieties</td>
<td><strong>IM:</strong> 3.0 mg/l 2,4-D and 0.1 mg/l BA <strong>DM:</strong> no hormones</td>
<td>8</td>
</tr>
<tr>
<td>Cotyledons from mature seeds</td>
<td>52 cultivars</td>
<td><strong>IM:</strong> 5 mg/l 2,4-D and 0.1 mg/l TDZ <strong>DM:</strong> no hormones</td>
<td>9</td>
</tr>
<tr>
<td>Mature seeds</td>
<td>‘Earl’s Favourite’</td>
<td><strong>IM:</strong> 4 µg/l 2,4-D and 0.1 µg/l BA <strong>DM:</strong> no hormones</td>
<td>10</td>
</tr>
<tr>
<td>Hypocotyls from imbibed seeds</td>
<td>‘Prince’ and ‘Sunday Aki’</td>
<td><strong>IM:</strong> 1 mg/l 2,4-D, 2 mg/l NAA and 0.1 mg/l BA <strong>DM:</strong> no hormones</td>
<td>11</td>
</tr>
<tr>
<td>Cotyledons from mature seeds</td>
<td>‘Védrantais’</td>
<td><strong>IM:</strong> 2.2 mg/l 2,4-D and 0.11 mg/l BA <strong>DM:</strong> no hormones</td>
<td>12</td>
</tr>
<tr>
<td>Cotyledons from mature seeds</td>
<td>‘Yellow Queen’ and ‘Yellow King’</td>
<td><strong>IM:</strong> 5.0 mg/l 2,4-D and 0.075 mg/l TDZ <strong>DM:</strong> no hormones</td>
<td>13</td>
</tr>
<tr>
<td>Mature seeds</td>
<td>‘Védrantais’ and ‘Earl’s Favourite Fuyu A’</td>
<td><strong>IM:</strong> 2.0 mg/l 2,4-D and 0.1 mg/l BA <strong>DM:</strong> no hormones</td>
<td>14</td>
</tr>
</tbody>
</table>

the best results were observed with explants coming from seedling parts, especially cotyledons and hypocotyls. These authors also reported that the genetic constitution of donor plants seems to play a key role in the success of somatic embryogenesis.

### 2.3.4.1 Medium Composition

Media composition (mostly plant growth regulators) has a great effect on melon somatic embryogenesis. The embryogenic pathway involves a two-stage methodology process: first, explants are cultured onto an ‘induction’ medium, to which auxins have been added, and second, ‘induced’ explants are transferred to development media, where full and complete normal embryo development takes place in the absence of the induction hormone. Auxins are prerequisite for induction of somatic embryogenesis (Tabei et al. 1991; Oridate et al. 1992; Debeaujon and Branchard, 1993; Gray et al. 1993; Guis et al. 1997a; Nakagawa et al. 2001).

In general, the most common and efficient auxin to induce somatic embryogenesis in melon explants is 2,4-D (Oridate and Oosawa, 1986; Debeaujon and Branchard, 1993). However, other auxins can be used, such as IAA and NAA, although NAA at high concentrations can induce abnormal embryo growth (Tabei et al. 1991). Likewise, Tabei et al. (1991) reported that IAA was the most efficient auxin to induce somatic embryogenesis in ‘Earl’s Favorite Harukei No.3’ melon. Auxins can be used in combination with cytokinins, such as BA and TDZ, and/or other hormones, such as ABA (Trulson and Shahin, 1986; Tabei et al. 1991; Homma et al. 1991; Debeaujon and Branchard, 1992; Gray et al. 1993; Guis et al. 1997a; Nakagawa et al. 2001). Hormones are removed to mature the embryos (Table 3.4), nevertheless gibberellins can be added to the culture medium (Tabei et al. 1991). ABA (10 mgL⁻¹) was supplemented into the
culture medium in order to control the desiccation process and to increase the survival rate before cryopreservation (Shimonishi et al. 1991).

The type and concentration of carbohydrate in the media plays a role in somatic embryogenesis in melon (Oridate and Yasawa, 1990; Debeaujon and Branchard, 1992; Gray et al. 1993, Guis et al. 1997a). Oridate and Yasawa, (1990) reported that a complex combination of different sugars, such as sucrose, glucose, fructose, and galactose, led to the highest rate of somatic embryogenesis. Similarly, Gray et al. (1993) concluded that the sucrose concentration in embryo induction and development media had a profound effect on somatic embryogenesis, i.e. 3 % sucrose produced a greater explant response than lower or higher levels of the carbohydrate. These authors found that sucrose concentration also exerted an effect on the relative percentage of somatic embryo stages recovered and on abnormal embryo development and precocious germination. Guis et al. (1997a) tested the effects of several levels of sucrose, glucose and maltose, on inducing melon somatic embryogenesis. Glucose enhanced the embryogenic response by almost two-fold, whereas maltose at any level totally somatic embryogenesis. Nakagawa et al. (2001) reported that the addition of mannitol to the initial media increased the frequency of somatic embryogenesis in ‘Prince’ melon.

2.3.4.2 Environmental Factors

Several physical factors can affect melon embryogenesis, among them, presence and quality of light, and physical state of media culture are the most important. In order to induce somatic embryogenesis, melon explants are cultured in light (Trulson and Shahin 1986; Branchard and Chateau 1988; Tabei et al. 1991; Homma et al. 1991; Shimonishi et al. 1991; Ezura et al. 1992a; Debeaujon and Branchard, 1992; Oridate et al. 1992, Debeaujon and Branchard, 1993; Hosoi et al. 1994; and Nakagawa et al. 2001).
Somatic embryo formation has been augmented by pretreatment in a dark period, usually one or two weeks before placing in light (Gray et al. 1993, Guis et al. 1997a). Culturing on solid medium (Branchard and Chateau, 1988) is better than using liquid medium. Different gelling agents have been used for this purpose, obtaining improved developed embryos by using ‘gelrite’ and/or ‘phytagel’ as a substitute for agar (Branchard and Chateau, 1988). Nevertheless, recently Akasaka-Kennedy et al. (2004) reported that somatic embryos from ‘Vedrantais’ and ‘Earl’s Favourite Fuyu A’ melon cultivars underwent development without vitrification, if agar was used instead of ‘gelrite’. Thus different melon cultivars give diverse responses under similar in vitro conditions.

Kageyama et al. (1991) and Moreno et al. (1985a) reported that the vitrification state of regenerated plants is increased if liquid cultures are used during the initial steps of somatic embryogenesis. Kageyama et al. (1991) reported that consecutive washing of somatic embryos with hormone-free MS medium with 0.5% activated charcoal increased the number of somatic embryos two-fold.

### 2.3.5 Haploid Plants and Embryo Culture

Hybrid cultivars represent the F₁ progeny of crosses that may involve inbred lines, clones, or populations (Fehr et al. 1987). The most common type of hybrid cultivar is produced by crossing two or more inbred lines, which have to be homozygous for certain important traits (Fehr et al. 1987). The production of inbred lines in *Cucumis melo* requires several generations, taking more than seven years of inbreeding in order to obtain homozygosity (Yashiro et al. 2002). Through using a plant biotechnology approach, such as production of haploid melon plants, is possible to reduce the amount of time to obtain melon inbred lines. Diploids can be induced by chromosome doubling agents, such as colchicine or oryzalin (Yetisir and Sari, 2003; Lotfi et al. 2003). This
approach has been used to obtain plants tolerant to diseases, such as virus or powdery mildew (Kuzuya et al. 2000; 2002; 2003; Lotfi et al. 2003).

According to Guis et al. (1998), using androgenesis and gynogenesis have not been successful to produce haploid melon plants. Using either gamma or soft X-ray irradiated pollen will induce in situ gynogenetic haploid parthenogenesis in melon (Sauton and Dumax de Vaulx 1987; Cuny et al. 1992; 1993; Yanmaz et al. 1999; Kuzuya et al. 2000). Sauton and Dumax de Vaulx (1987) developed an in vitro technique (commonly named embryo rescue or embryo culture) to recover muskmelon haploid plants. These authors obtained haploid plants after pollination of hermaphrodite flowers with irradiated (Co$^{60}$ $\gamma$-rays) pollen and in vitro culture of ovules or immature embryos. They also developed a new culture medium to allow further development of these embryos into plants, resulting with an average number of 2.5 haploid embryos per 100 seeds. Sauton and Dumax de Vaulx’s embryo culture technique has been applied not only to induce and rescue haploid melon plants, but also to culture diploid embryos as well in numerous melon cultivars with excellent results (Sauton, 1988; Kuzuya et al. 2000; Oliver et al. 2000; Kuzuya et al. 2002; Lotfi et al. 2003; Kuzuya et al. 2003; Yetisir et al. 2003).

The rate of melon haploid production is affected by genotypic factors and environmental growth conditions of donor plants (Sauton, 1988; Cuny et al. 1992; 1993; Yanmaz et al. 1999). Sauton (1988) studied haploid embryo production on seven melon cultivars, belonging to five melon types, i.e. reticulatus, cantaloupensis, inodorus, conomon and acidulus. Gynogenetic haploid embryo production among those melon genotypes ranged from 0 to 1.7%. Cuny et al. (1993) reported significant differences in haploid embryo production between two melon cultivars, ‘Vedrantais’ which produced
an average number of haploid embryos of 3.5%, whereas ‘F1.G1’ formed 1.7%. Sauton (1988) found that ‘Arizona’ muskmelon produced the highest number of haploid embryos (3%) if melon plants were grown during the summer season. Cuny et al. (1992) reported the effect of season planting on haploid embryo induction. These authors found that haploid embryo induction was greater in ‘Vedrantais’ melon fruits obtained from plants grown in summer than those harvested in autumn.

Unlike other plant systems, such as carrot and tobacco, melon pollen is able to tolerate high $\gamma$-irradiation doses (up to 3,600 Grays) and still germinate –in vitro as well as in vivo- and inducing parthenocarpic haploid plant production (Cuny et al. 1992; 1993; Yanmaz et al. 1999). However, a significant reduction in pollen tube length has been observed using high radiation doses. This reduction was proportional to the amount of $\gamma$-radiation used (Cuny et al. 1992; 1993; Yanmaz et al. 1999). High $\gamma$-irradiation doses can induce an increase in the percentage of necrotic haploid embryos. The most common gamma irradiation doses are between 250 and 350 Grays (Sauton and Dumax de Vaulx 1987; Cuny et al. 1992; 1993; Beharav and Cohen, 1995a; Yanmaz et al. 1999; Lotfi et al. 2003; Yetisir and Sari, 2003), and for soft-X-rays the doses are between 65 and 130 kR (Kuzuya et al. 2000; 2002; 2003).

Niemirovicz-Szczt and Kubicki (1979) demonstrated that strong incompatibility events occurred in the intergeneric and intraspecific crosses within the Cucurbitaceae family, avoiding sexual hybridization among Cucumis melo. Thus, in vitro embryo culture technique has been utilized to recover melon haploid plants, and to save valuable diploid plant material through zygotic embryo culture and somatic embryogenesis. Different diploid plant material has been recovered, such as hybrid plants obtained after
interspecific cross of *Cucumis melo* (PI140471) x *C. metuliferus* (PI 29190) (Norton, 1981), *Cucumis metuliferus* (PI 292190, PI 202681, 3503, and 701A) x *C. anguria* (PI 233646) (Fassuliotis and Nelson, 1988), *Cucumis melo* (‘Gylan’ gynoecious E6/10) x *C. metuliferus* (‘Italia’) (Beharav and Cohen, 1995a), *Cucumis melo* (‘Cantaloup Charentais’) x *Cucumis anguria* L. var. *longipes* (Dabauza et al. 1998), and *Cucumis melo* (‘Cantaloup Charentais albino mutant’) x *Cucumis myriocarpus* (Bordas et al. 1998).

In summary, the potential of *in vitro* embryo culture has lead to reduced time to obtain inbred melon lines. In addition, the improvement of this technique as well as the cointegration to marker-assisted selection (MAS) (Fukino et al. 2004) may eventually allow the transfer of disease resistance and/or other important horticultural traits from other cucurbits into *Cucumis melo* species.

### 2.3.6 Genetic Transformation

Two main natural and artificial genetic transformation processes have been used to obtain melon transgenic plants; *Agrobacterium tumefaciens* and particle gun bombardment (Table 2.6). Successful transformation of melon with *Agrobacterium rhizogenes* has not been reported. Gaba et al. (1992) and Gonsalves et al. (1994) used particle gun bombardment to transform melon explants and recover transgenic plants through organogenesis. Gray et al. (1995) used the same transformation protocol and recovered plants from embryogenic materials. Gonsalves et al. (1994) and Gray et al. (1995) reported both that *A. tumefaciens* and microprojectile gene transfer produced almost the same percentage of transgenic plants. Gray et al. (1995) produced stable normal plants via particle bombardment while embryos from *Agrobacterium*-mediated
transformation were abnormal. Mefoxine, was used to stop *Agrobacterium* growth may have caused this.

Transformation success via *Agrobacterium* or particle gun bombardement is genotype-, explant source-, and *in vitro* culture conditions-dependent (Fang and Grumet, 1990; Dong et al. 1991; Yoshioka et al. 1992; Valles and Lasa, 1994; Gonsalves et al. 1994; Gray et al. 1995; Bordas et al. 1997; Clendennen et al. 1999; Ezura et al. 2000; Nuñez-Palenius et al. 2002; Akasaka-Kennedy et al. 2004). The *Agrobacterium* strain, vector structure, and co-cultivation with acetylsyringone, have an influence on melon transformation efficiency (Dong et al. 1991; Yoshioka et al. 1992; Valles and Lasa, 1994; Bordas et al. 1997).

Fang and Grumet (1990) tested several factors, such as kanamycin concentration, *Agrobacterium* inoculum level, length of inoculation, period of co-cultivation, and the use of tobacco nurse cultures, on melon transformation efficiency rate. These authors found that 75 mg L$^{-1}$ kanamycin, fresh overnight grown bacteria at a concentration of $10^7$ – $10^8$ bacteria ml$^{-1}$ $(\text{OD}_{600} = 0.8)$, 10 min of inoculation, three days of co-cultivation, and not using tobacco nurse culture were the best conditions to attain an efficient transformation rate. Unfortunately, these transformation conditions were only tested in one melon cultivar, ‘Hale’s Best Jumbo’. A similar approach was described by Dong et al. (1991). These authors tested the sensitivity of melon cotyledons to kanamycin and methotrexate concentrations, co-cultivation time, and different selection schemes -1. no selection pressure, 2. explants were placed in selection medium immediately after co-cultivation, and 3. explants were placed under selection two weeks after co-cultivation-. The highest transformation frequency was obtained when 75 µg L$^{-1}$ methotrexate and 100
mg L\(^{-1}\) kanamycin, 5-6 days of co-cultivation period, and immediate selection pressure was routinely used.

These experiments were carried out on ‘Orient Sweet, F1 Hybrid’ melon cultivar. Vallés and Lasa (1994) reported that two days of co-cultivation with \textit{Agrobacterium tumefaciens} with cotyledons of ‘Amarillo Oro’ melon were necessary to reach an efficient transformation process. If they used a longer period of co-cultivation, no transgenic shoots were recovered.

Different bacterial and plant genes, which provide tolerance or resistance to several selectable chemical agents, have been used to delay or completely inhibit the growth of non-transformed buds and shoots of melon during the selection process. Among them, \textit{nptII}, which provides tolerance to aminoglycoside antibiotics (Fang and Grumet, 1990; Dong et al. 1991; Yoshioka et al. 1992; Gonsalves et al. 1994; Vallés and Lasa, 1994; Gray et al. 1995; Ayub et al. 1996; Bordas et al. 1997; Clendennen et al. 1999; Akasaka-Kennedy et al. 2004), \textit{dhfr}, which gives resistance to methotrexate (Dong et al. 1991), and \textit{CP4syn}, which offers tolerance to Glyphosate herbicide (Nuñez-Palenius et al. 2002; 2003) have been used. When typical concentrations (75-150 mg l\(^{-1}\)) of kanamycin are not able to inhibit the non-transgenic bud or shoot growth (Guis et al. 1998; Dong et al. 1991), other antibiotics, which are also detoxified by the neomycin phosphotransferase protein (NPTII), may be added to melon \textit{in vitro} cultures in order to improve the selection efficiency. Gentamycin, hygromycin, and paromomycin have been used in melon cultures as alternative to kanamycin with excellent results (Guis et al. 1998; Nuñez-Palenius et al. unpublished results, Ezura et al. personal communication).
<table>
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<tr>
<th>Transgene</th>
<th>Phenotypic Trait</th>
<th>Explant used and morphogenetic pathway</th>
<th>Cultivar</th>
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<td>Improved fruit quality</td>
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<td>GUS and GFP</td>
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<td>Reporter genes</td>
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Znot-reported.
Genetic transformation efficiency rate in most melon systems is normally lower than other plant species (Fang and Grumet, 1990; Dong et al. 1991; Gaba et al. 1992; Gonsalves et al. 1994; Bordas et al. 1997; Akasaka-Kennedy et al. 2004). Several transformation rate efficiency values have been reported according to transformation protocol and melon cultivar used, among them, average efficiencies such as 3-7% (Fang and Grumet, 1990), 4-6% (Dong et al. 1991), 1% (Gaba et al. 1992), 0.0%, 0.9%, and 1% (Gonsalves et al. 1994), 0.7-3% (Bordas et al. 1997), 2.4% (Guis et al. 2000), 10% (Nuñez-Paleniües et al, 2003), and 2.3% (Akasaka-Kennedy et al. 2004) has been depicted. Unfortunatly, in many cases most of the recovered transgenic plants had somaclonal variation especially ploidy changes [tetraploids (75%, Ayub et al. 1996), octaploids, mixoploids] or had morphogenetic altered characteristics, which were expressed in the T₀ and T₁ generation (Gonsalves et al. 1994).

2.3.6.1 Improvement of Disease Resistance

*Cucumis melo* is attacked by numerous viral, bacterial, mycoplasmal and fungal organisms, which cause severe diseases (Zitter et al. 1998). These diseases can affect melons at any plant developmental stage, causing enormous economic losses. According to Zitter et al. (1998), definitive disease control is reached by using genetically resistance melon cultivars. More than 30 viruses are able to induce disease symptoms in melon plants. Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV) are the most prevalent (Zitter et al. 1998; Gaba et al. 2004).

The first virus–resistant transgenic melon plants were obtained by Yoshioka et al. (1992). These authors transferred and over-expressed the gene for CMV coat protein via *Agrobacterium tumefaciens* using ‘Prince’, ‘EG360’ and ‘Sunday Aki’ melon cotyledons.
Those transgenic melon plants which over-expressed the CMV-CP gene, grown under greenhouse conditions, were found to be resistant to infection after inoculation with a low-dose of CMV (Yoshioka et al. 1993). Transgenic plants did not develop any symptoms of disease during 46-day observation period, while control plants had mosaic symptoms three days after inoculation. When the virus-dose was increased by ten fold, only a delayed appearance of symptoms was observed in transgenic plants (Yoshioka et al. 1993). After Yoshioka’s achievement, different authors were able to obtain transgenic melon plants over-expressing the CMV-CP for other melon cultivars, such as ‘Burpee Hybrid’, ‘Halest Best Jumbo’, and ‘Topmark’ (Gonsalves et al. 1994), and ‘Don Luis’, ‘Galleon’, ‘Hiline’, ‘Mission’, and a distinct ‘inbred line’ (Clough and Hamm, 1995). In addition, transgenic plants over-expressing either CMV-CP for specific viral strains - White Leaf strain- (Gonsalves et al. 1994) or multi-virus resistance –CMV, WMV, and ZYMV- (Clough and Hamm, 1995; Fuchs et al. 1997) were described. Gonsalves et al. (1994) found strong resistance to CMV-White Leaf strain in 5 out of 45 transgenic melon plants. Gaba et al. (2004) stressed that CP-protection gave effective field resistance, but not 100% protection.

Field trials were conducted to determine if transgenic plants would retard the spread of the aphid non-transmissible strain C of CMV (Tabei et al. 1994; Clough and Hamm, 1995; Fuchs et al. 1997; Fuchs et al. 1998). Clough and Hamm (1995) tested the level of resistance of five melon transgenic varieties to WMV and ZYMV. Transgenic melon plants had little or no virus infection, while more than 60% of the control plants developed virus-symptoms. Similar results were achieved by Fuchs et al. (1997), who evaluated transgenic melon resistance under high disease pressure, achieved by
mechanical inoculations, and/or natural challenge inoculations by indigenous aphid vectors. After five different trials, more than 90% of the homozygous and 75% of the hemizygous plants were not infected by two or three viruses whereas 99% of the wild type melon plants had mixed virus infections. Moreover, control plants were severely stunted (44% reduction in shoot length) and had poor fruit yield (62% loss), and most of their fruits were unmarketable (60%) compared to transgenic melon plants.

2.3.6.2 Improvement of Tolerance to Physical Factors

Several environmental factors, such as high or low temperature, salt accumulation, low-sun irradiance, drought, and flooding, seriously affect melon field cultivation and production (Robinson and Decker-Walters, 1999). Only one report on transgenic melon providing tolerance to one environmental factor has been published (Bordas et al. 1997). The \textit{HAL1} gene, which encodes a water soluble protein and provides halotolerance in yeast, was inserted using \textit{Agrobacterium tumefaciens}-transformation protocol to ‘Pharo’ and ‘Amarillo Canario’ melon cultivars. \textit{In vitro} shoots from transgenic and control plants were evaluated for salt tolerance after 16 days of incubation on medium containing 10 g L\(^{-1}\) sodium. The frequency and intensity of root formation were higher in \textit{HAL1}-positive plant populations compared to wild type plants. However, no differences in vegetative fresh weight and number of leaves between transgenic and control plants were scored. Moreover, greenhouse and field evaluation of transgenic plants was not reported.

2.3.6.3 Improvement of Postharvest Characteristics

According to Perishables Group Research, price, firmness and appearance are among the top criteria for consumers when deciding to purchase melons. Appearance, which includes color, texture, and look of any sign of damage or disease are the top criteria for consumers to purchase melons. Customers are interested in knowing
nutritional and ripening information in store displays (Anonymous, 2002). Extended shelf
life in melon fruit is an important quality attribute because increase the opportunity to
commercialize melon commodities.

The first transgenic melon plants carrying genes involved in fruit ripening process
were obtained by Ayub et al. (1996). Using the Agrobacterium-mediated transformation
system and cotyledons of the Charentais type Cantaloupe melon cv. ‘Védrantais’, these
authors were able to transfer the 1-aminocyclopropane-1-carboxilic-acid oxidase gene
(ACC oxidase from melon under the control of a constitutive promoter) in antisense
orientation to reduce the level of ethylene (Ayub et al. 1996). Different ripening
parameters were evaluated in transgenic ripening improved melon fruits, such as internal
and gas space ethylene production, total soluble solids, titratable acidity, flesh pigment
content, flesh firmness, rind and flesh color, harvest maturity (timing from anthesis to full
slip), and reversion to wild type behavior by exogenous ethylene treatment (Ayub et al.
1996; Guis et al. 1997b).

Ayub et al. (1996) and Guis et al. (1997b) found that in wild type fruit attached to
the vine, the internal ethylene concentration rose at 39 days after pollination and reached
maximum (120 ppm) production at 42 days. In antisense fruit the internal ethylene
concentration on the vine remained at low levels (0.6 ppm), even at late fruit
development stages (60 days after pollination). When wild type fruits were detached from
the plant there was a significant increase in the internal ethylene concentration, producing
180 ppm 48 hours later. Detached transgenic fruit also had an increase in ethylene
production, but reached only 10 ppm 12 days after harvest. Compared to wild-type,
antisense fruit did not undergo significant rind yellowing and flesh softening at maturity
period. Transgenic fruit remained attached to the vine for a longer period of time (65 days after pollination) compared to control plants (38 days after pollination). Only exogenous ethylene treatment (50 ppm) of transgenic fruits allowed the recovery of the wild type behavior and phenotype. There were no significant differences in carotenoid content (flesh color) and total soluble solids (°Brix) content in wild type and transgenic fruit at any stage of ripening.

Clendennen et al., (1999) utilized the product of the S-adenosylmethionine hydrolase (SAMase) gene (from T3 bacteriophage) to catalyze the degradation of SAM, the initial precursor of ethylene. Unlike the T-DNA construct used by Ayub et al. (1996), Clendennen et al. (1999) used a fruit specific promoter (chimeric ethylene-responsive E8/E4 promoter) aimed to overexpress the SAMase gene in two ‘American Cantalope’ lines, which were proprietary inbred lines from Harris Moran Seed Company, Inc. These authors evaluated several postharvest fruit quality parameters, such as fruit size and weight, firmness, mold susceptibility, external and internal color, soluble solids, harvest maturity (timing from anthesis to full slip, measured as Heat Units), and ethylene production, in wild type and transgenic melon fruits from plants grown under field conditions. These authors reported that transgenic melon fruit from both lines ‘A’ and ‘B’ did not differ in horticultural traits from wild type fruits, except for the intended goal of SAMase expression on ethylene biosynthesis and related events. In lab experiments, transgenic fruits produced half of the ethylene amount accumulated by wild type fruits. However, in field trials, the onset of maturity, measured on four different dates, was not significantly delayed in transgenic fruit compared to wild type, but transgenic fruits ripe more uniformly in the field. Firmness was also measured on transgenic and wild type
fruits from three different field trial locations. Significant differences were found in fruit internal firmness between transgenic and wild type melons, but only from one location. Clendennen et al. (1999) claimed that by expressing SAMase in a regulated manner by a fruit-specific promoter, transgenic fruits produced less ethylene than non-transgenic fruit resulting in a modified ripening and postharvest phenotype.

Silva et al. (2004) obtained transgenic Cantaloupe melon plants cv. Védrantais by inserting and overexpressing ACC oxidase from apple and not from melon as Ayub et al. (1996) protocol. These authors reported the characterization of ripening melon fruits, and their experimental comparison between transgenic and control fruit provided very similar results in almost all the evaluated parameters, such as harvest maturity, total soluble solids content, rind color, and internal ethylene production, to those previously reported by Ayub et al. (1996) and Guis et al. (1997b).

In Charentais type Cantaloupe melon (cv. Védrantais) climateric respiration, yellowing and carotenoid content of the rind, chilling injury, and formation of the peduncular abscission zone are events totally ethylene-dependent (Pech et al. 1999; Flores et al. 2001a; 2001b), whereas fruit softening, volatiles synthesis, and membrane deterioration are ethylene-partially dependent processes and display some ethylene-independent components (Bauchot et al. 1998; Bauchot et al. 1999; Guis et al. 1999; Flores et al. 2001a; 2001b; Flores et al. 2002). According to Guis et al. (1997b), Pech et al. (1999), Silva et al. (2004) sugar accumulation and the increase in carotenoid content in the flesh are ethylene-independent events. There is still some controversy about organic acid metabolism and loss of acidity during fruit melon ripening, i.e. Guis et al. (1997b) and Pech et al. (1999) suggested that the organic acid metabolism was ethylene-
independent, whereas Silva et al. (2004) recently implied that organic acid metabolism is an ethylene-dependent regulated process. It is likely that these differences in conclusions might be related to a different ethylene concentration reached by each transgenic fruit. In Silva’s report transgenic fruit attained a lower internal ethylene-level (<0.09 µL⁻¹) than Guis’s results (<0.5 µL⁻¹). This subtle difference in ethylene concentration could have obvious outcomes such as at the specific levels (Srivastava, 2002; Silva et al. 2004).

All fruit quality oriented transgenic melon plants have been obtained using just three melon cultivars, such as Védrantais and lines ‘A’ and ‘B’, belonging to one single variety, i.e. cantaloupensis (Ayub et al. 1996; Clendennen et al. 1999; Silva et al. 2004). Considering that there are seven commercial and horticultural important melon varieties (cantaloupensis, reticulatus, saccharinus, inodorus, flexuosus, conomon and dudaim) (Guis et al. 1998; Kirkbride, 1993), and hundreds of melon cultivars, much more work could be accomplished. There still remains a need to improve *in vitro* regeneration and transformation protocols (Guis et al. 1997a; Gaba et al. 2004; Akasa-Kennedy et al. 2004).

### 2.4 ‘Galia’ melon

#### 2.4.1 Introduction

‘Galia’ melon is an exotic, green-fleshed, and yellow skinned -finely-netted rind specialty hybrid muskmelon bred in Israel. ‘Galia’ is also easily recognized by the volatile-musky aroma that the fruit is able to release (Shalit et al. 2000; Fallik et al. 2001). This muskmelon was developed for semi-arid, dry-land, and open-field and summer season cultivation in Mediterranean areas. Turkey, Morocco and Spain are major producers of ‘Galia’ (Karchi, Personal communication). Due its high demand within
European market ‘Galia’ muskmelons have recently started being cultivated in Central American countries such as Guatemala, Honduras, Costa Rica, and Panama, and exported to the U.S. and Europe (Table 2.7). Open-field, ‘Galia’ muskmelon yields between 35-50 t/ha of excellent-quality fruit (Karchi, 2000). Using passive ventilated greenhouses and soilless culture in North Florida yields as high as 165 t/ha have been reported (Shaw et al. 2004). The ‘Galia’ type muskmelon world production is around 300,000-500,000 tons per year (Reho, 2004).

‘Galia’ muskmelon is a commodity reaching dissimilar prices, from $1.97 to $2.17 per fruit, mostly depending on quality characteristics and origin country (Table 2.8) (Produce1, INC. 2005, United States Department of Agriculture [USDA] Department of Commerce, U.S. Census Bureau, Foreign Trade Statistics, 2005). However, according to the USDA, ‘Galia’ high-quality muskmelons can reach prices in a range from $3.0 up to $5.0 per fruit (USDA, Department of Commerce, U.S. Census Bureau, Foreign Trade Statistics, 2005).

2.4.2 Botany and Origin

According to Karchi (2000), ‘Galia’ muskmelon was the first Israeli melon hybrid produced and was obtained through an extensive breeding program at the Newe Ya’ar Research Center of the Agricultural Research Organization (A.R.O – Israel) during the mid-1960s. ‘Galia’ has green-fleshed characteristics of ‘Ha’ Ogen’ type, which is a smooth-skinned, sutured melon and was introduced from Hungary to Israel during the 1950’s, and used as the female parental line. ‘Galia’ has also a golden-yellow netted rind from ‘Krymka’, Russian melon cultivar,
Table 2.7 ‘Galia’ Type Muskmelon Imports to U.S.

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<tr>
<th>ORIGIN</th>
<th>1999 Mt ($1,000)</th>
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which was used as the male parental line (Karchi, personal communication, 2004). ‘Galia’ was named after Dr. Karchi’s daughter and released as a Hybrid F1 for production in 1973-74 (Karchi, 2000).

‘Ha’ Ogen’ type melons are considered as members of *cantaloupensis* variety, whereas ‘Krymka’ cultivar belongs to the *reticulatus* variety (Kirkbride, 1993; Goldman, 2002). Since ‘Galia’ is a F1 Hybrid muskmelon obtained from crossing ‘Ha’ Ogen’ and ‘Krimka’, disagreement to whether ‘Galia’ F1 is a *cantaloupensis* variety (Zheng and Wolff, 2000; Staub et al. 2000; Lopez-Sese et al. 2003) or a *reticulatus* variety (Aharoni et al. 1992; Aharoni et al. 1993a; 1993b; Garcia et al. 1998; Fallik et al. 2000) has emerged.

### 2.4.3 Postharvest Physiology

‘Galia’ muskmelon has exceptional characteristics such as fruit quality with 13-15% total soluble solids (TSS), bold flavor and a distinct musky aroma. The main disadvantage of ‘Galia’ is its short storage life, since it is harvested near peak maturity for optimum flavor. Storage is limited to two or three weeks, even when it is maintained in low-temperature (6°C). Several pathogenic agents, such as *Alternaria alternata* (Fr.) Keissler and *Fusarium* spp. can severely reduce the ‘Galia’ fruit storage life, causing a rapid decay (Barkai-Golan et al. 1981; Aharoni et al. 1992; Aharoni et al. 1993a). However, using specific fungicides, such as imazalil (2,000 µL⁻¹), combined with wax coating, ‘Galia’ fruits can be protected from decay (Aharoni et al. 1992; 1993a).

Unfortunately, the residue level of imazalil in Israeli ‘Galia’ wax coated fruits is 3-5 µL⁻¹, more than the maximum tolerance limit (0.5 mL⁻¹) imposed by most of European...
countries (Anonymous, 1990). Therefore, new fungicides and postharvest strategies were sought to reduce the fruit decay in ‘Galia’ muskmelon.

Other treatments including use of Sanosil-25, which is a 48% hydrogen peroxide solution and silver salts (Aharoni et al. 1994), applying Hinokitiol (beta-thujaplicin), a volatile oil extracted from the Japanese Hiba tree (*Thujopsis dolabrata*) at a concentration of 750 ppm in the wax (Aharoni et al. 1993b), other fungicides, such as prochloraz and orthophenylphenol (Aharoni et al. 1992), coating ‘Galia’ fruits with wax containing 2% sodium bicarbonate (Aharoni et al. 1997), and combined treatments, such as hot water (52°C) dipping and γ-irradiation (Barkai-Golan et al. 1994), short hot water (59°C) rinse and rotating brushes (Fallik et al. 2000), and hot water (55°C) treatment and packing in polyethylene bags (Halloran et al. 1999a; 1999b) The optimal treatment to reduce decay and fulfill European import requirements, while maintaining high fruit quality after prolonged storage and marketing simulation in ‘Galia’ type muskmelon is to place the fruits on rotating brushes, and rinse them with ambient water followed by a hot water rinse (HWRB) at 59°C ± 1°C for 15 s (Fallik et al. 2000).

Different strategies have been used in order to delay fruit softening in ‘Galia’ including the use of controlled atmospheres plus adding an ethylene absorbent (Aharoni et al. 1993a), reducing the storage time during transportation by using airfreight systems, instead of seafreight shipping (Bigalke and Huyskens-Keil, 2000), and applying inhibitors of ethylene action, such as 1-methylcyclopropene (Ergun et al. 2005) have been reported.

Aharoni et al. (1993a) carried out a study aimed to determine if ‘Galia’ melons could be stored under controlled atmosphere (CA) (10% CO₂ and 10% O₂) plus an
ethylene absorbent on semi-commercial scale, in order to maintain fruit quality. Fruit were evaluated after 14 days in CA at 6°C and additional 6 days at 20°C. These authors found that ‘Galia’ melons stored in controlled atmosphere plus ethylene absorbent were significantly firmer and had less decay than control treatments.

Bigalke and Huyskens-Keil (2000) studied the influence of different treatments, such as low temperatures (5 and 8°C) and individual fruit plastic packaging, as well as two controlled atmosphere conditions, i.e. 4% and 12% at 80% RH and 8°C for 14 days, on postharvest characteristics of airfreight ‘Galia’ melon. External and internal fruit quality attributes, such as color, firmness, soluble solids content, titratable acidity, and flavor assessment by a consumer panel were evaluated. These authors did not find statistical differences between quality attributes of bagged and non-bagged melon fruits. Also, ‘Galia’ melons stored at 5°C maintained the highest external and internal quality during the storage period, regarding firmness, soluble solids content, titratable acidity and flavor assessment. Controlled atmosphere conditions tested in this study were unfavorable to maintain excellent ‘Galia’ fruit quality characteristics.

Ergun et al. (2005) characterized the physiological responses of ‘Galia’ fruit harvested at green (preripe) and yellow (advanced ripening) stages after treatment with 1-methylcyclopropene (1-MCP) and further storage at 20°C. These authors found that treatment with 1.5 μL·L⁻¹ 1-MCP delayed the climacteric peaks of respiration and ethylene production of green fruit by 11 and 6 days, respectively, and also significantly suppressed respiration and ethylene production maxima. Fruit softening of both developmental stages, green and yellow, was significantly delayed after 1-MCP treatment.
By delaying fruit ripening via reduction in ethylene production ‘Galia’ F₁ hybrid shelf-life might be improved.

2.4.4 Genetic Improvement by Conventional Methods and Biotechnology

‘Galia’ F₁ hybrid has high tolerance to some diseases, such as vine decline and powdery mildew race 1 (Wolf and Miller, 1998, Karchi, 2000) and elevated fruit quality characteristics. ‘Galia’ inbred parental lines have been used in breeding programs to develop improved hybrid with firmer fruit, and also to inbreed other melon accessions lines for multiple virus resistance (Garcia et al. 1998; Lofti et al. 2003). Crossing ‘Ha’Ogen’ x ‘Inodorus’ type has improved shelf-life but favorable fruit quality characteristics, such as flavor, volatiles and sugar levels, were lost (Dr. Karchi, personal communication, 2002).

A reliable and efficient regeneration and transformation system for ‘Galia’ muskmelon has been a desired goal for more than a decade (Gaba et al. 1992). ‘Galia’ muskmelon efficient in vitro regeneration and transformation protocols are lacking (Gaba et al. 1992; 1995; 1996; 1999; Edriss et al. 1996; Kintzios and Taravira, 1997; Galperin et al. 2003a).

Dirks and van Buggenum (1989) described an efficient method for shoot regeneration from leaf and cotyledon explants in ‘Accent’, ‘Galia’, ‘4215’, ‘Preco’, and ‘Viva’ melon cultivars. The best combination of plant growth regulators for shoot induction in cotyledon explants was 1 mg/l BA, without adding IAA. For leaf explants, there were no clear differences on shoot induction among the cultivars tested. A range of 20 to 50 shoots per explant were recorded. These authors also claimed that over 100 shoots were formed in cotyledon explants. The best results on shoot induction were
obtained with ‘Galia’, ‘Accent’, and ‘Preco’. Elongated shoots were rooted on hormone-free MS, and then successfully transferred into the greenhouse.

Leshem (1989), working in the Volcani Center in Israel, reported a plant tissue culture approach in order to study the responsive zone for organogenesis and the time for organ induction in ‘Galia’ melon cotyledons. Calli proliferation as well as root and shoot primordia formation was only confined to the basal side (attachment point to the stem) of the cotyledon or its segments and never appeared on the distal cut edge. The basal segments were always the most regenerative explants than the distal ones. The organ induction period to develop roots required at least one day but no more than three, whereas three days but not more than five were needed to produce shoots. According to Leshem (1989), melon cotyledon responsiveness was apparently achieved by the transverse cut and possibly due to accumulation of a polarly transported endogenous growth factor (maybe IAA). Exogenous plant hormones, such as 0.2 mg L⁻¹ BA and 2 mg L⁻¹ IAA, had a fundamental role for the induction of organ formation. Leshem’s research team, published three more articles (Leshem et al. 1990a; 1990b; 1991), where they studied the role of exogenous cytokinins, some reserve cotyledon polypeptides and polyamines on in vitro melon regeneration. Leshem’s team shifted from using ‘Galia’ melon cultivar to ‘Sweet’n Early’ hybrid in those published articles (Leshem et al. 1990a; 1990b; 1991). Perhaps this decision was taken because fully complete well developed shoots were not obtained in Leshem (1989).

Leshem et al. (1994a; 1994b) used thidiazuron (TDZ), benzyladenine (BA) and paclobutrazol (PC), and the plant hormone IAA in order to examine changes in cotyledonary organogenetic response in ‘Galia’ F₁ hybrid. They studied the effect of
those bioregulators on temporal changes in protein profiles (mostly some reserve
cotyledonary polypeptides with molecular weights of 20-25 kDa -P20-P25-) in cultured
melon cotyledons. These authors found that TDZ exerted a cytokinin-like influence by
inducing de novo shoot proliferation and inhibition of root initiation, while PC was a
potent root-inducer, due to its ability to overcome the inhibitory effect of BA on melon
rooting. However, PC was not able to induce roots at the basal edge of the cotyledon
when TIBA (inhibitor of auxin transport) was incorporated into culture medium.

Exogenous cytokinin (2.25 mg L^-1 BA) enhanced mobilization of reserve proteins (P20-
P25) and also induced shoot regeneration in cultured cotyledons. These polypeptides
disappeared from cotyledonary tissue on day four if the media culture contained BA,
whereas a constant level of those polypeptides was maintained through the culture period
if 1.75 mg L^-1 IAA was present. Regeneration correlated well with the P20-P25
disappearance, and shoots were only induced on medium containing 2.25 mg L^-1 BA. A
delayed organogenetic regeneration and P20-P25 breakdown was observed when IAA
was present in the media. The incorporation to the medium of an inhibitor of
carboxypeptidase (PMSF) inhibited both P20-P25 disappearance and root regeneration,
but not shoot initiation.

Gaba et al. (1996) reported that ancymidol, an anti-gibberellin compound,
improved in vitro bud development in cotyledonary explants of ‘Galia’ melon. This anti-
gibberellin substance had a synergistic effect when BA was incorporated to the media.
The presence of 5 mg/l GA₃ reverted that rate of regeneration induced by BA alone or
BA plus ancymidol.
It has been suggested that cytokinin and white light operate together to enhance polypeptide metabolism and induce shoot regeneration in ‘Galia’ cultured cotyledons (Leshem et al. 1995; Kintzios and Taravira, 1997). Leshem et al. (1995) studied the effect of different light intensities (PPFD 0, 3, 7, 14, 30 and 50) on shoot regeneration incidence on medium containing 2.25 mg L⁻¹ BA and disappearance of P20-P25 polypeptides in melon cotyledons. They found that BA enhanced the polypeptide loss under light conditions (50 µmol m² s⁻¹), but it failed when cotyledons were cultured in total darkness. A reduction in white light fluency to 14 µmol m² s⁻¹ was enough to observe the polypeptide pattern as in darkness culture. The incidence of shoot regeneration was reduced about half when cotyledons were cultivated at 14 µmol m² s⁻¹ white light fluency, and only 5% of cotyledons were responsive to shoot induction under total darkness. These authors suggested that BA loses its shoot induction capacity in the absence of white light, therefore, it is not able to support any growth response, even if BA concentration is increased (2.25 mg L⁻¹).

Kintzios and Taravira (1997) studied several morphogenetic pathways, such as calli induction, somatic embryogenesis and plant regeneration response, of 14 commercial melon cultivars to two levels of photosynthetic photon flux densities (PPFD), i.e. 50 or 250 µmol m² s⁻¹. ‘Galia’ was able to undergo shoot induction only when PPFD was 250 µmol m² s⁻¹.

Micropropagation of ‘Galia’ F₁ melon explants, using either shoot tips or axillary buds, has been reported as well (Spetsidis et al. 1996; Edriss et al. 1996). Elevated levels of BA (2 mg/l) were needed to reach the greatest shoot production, and the presence of 0.5 mg/l IAA did not affect that number.
The first report on ‘Galia’ melon genetic transformation through plant biotechnology was described by Gaba et al. (1992; 1995). They used particle gun bombardment to transform cotyledon explants and detect transient GUS expression. Permanent GUS transformation was observed only after a strong selection procedure (350 mg/l kanamycin). They were able to detect the presence of that transgene by PCR methods, but only 1% of the explants produced transformed shoots. Gaba et al. (1999) claimed that despite abundant shoot induction ‘Galia’ melon cotyledons (Dirks and van Buggenum, 1989), the cultivar was recalcitrant to transformation by \textit{Agrobacterium tumefaciens} (Gaba et al. 1995; 1996).

An innovative regeneration system using the proximal zone of the hypocotyl of ‘Galia’ type melon cultivar, i.e. ‘Revigal’ has been reported (Curuk et al. 2002). This protocol was later applied to three melon cultivars, ‘Revigal’, ‘Topmark’ and ‘Kirkagac’, and to one cucumber cultivar, i.e. ‘Taoz’ (Curuk et al. 2003). Regeneration from hypocotyl explants resulted in almost 100% diploid shoots, therefore, this is the first report of regeneration from \textit{Cucumis} genus producing a fully diploid plant population. Unfortunatly, these authors did not try to regenerate the original ‘Galia’ F$_1$ hybrid through their system nor to transform it by means of \textit{Agrobacterium tumefaciens} or particle gun bombarment.

Galperin et al. (2003a) screened, through \textit{in vitro} regeneration, 30 melon genotypes to measure their regeneration capabilities. Those melon genotypes embraced wild landraces, breeding lines and commercial cultivars. According to their results, 24 out of 30 genotypes did not produce any normal shoots. Only five out of six genotypes displayed low regeneration efficiency, because a few explants developed normal shoots.
Only ‘BU-21’ had exceptional regeneration ability. Five melon genotypes, among them ‘Galia’, were selected from the original 30 genotypes to determine the organogenetic response on three different regeneration media, i.e. MR, HC and IK (Fang and Grumet, 1990; Blackmon and Reynolds, 1982; Moreno et al. 1985a). The regeneration efficiency reported for ‘Galia’ melon was 0% on those three different media.

So far, there is no any report in the literature on stable genetic transformation in ‘Galia’ F₁ hybrid or in any of its parental lines.
CHAPTER 3
EFFECT OF EXPLANT SOURCE ON REGENERATION AND TRANSFORMATION EFFICIENCY IN ‘GALIA’ MELON (CUCUMIS MELO L.) MALE AND FEMALE PARENTAL LINES

3.1 Introduction

The Cucurbitaceae family consists of mostly frost sensitive, principally tendril-bearing vine plants which are found in sub-tropical and tropical regions around the world (Robinson and Decker-Walters, 1999). Melon (Cucumis melo L.) belongs to this family and is an important horticultural produce. World melon production (Cantaloupe & others) in 2004 was over 27 million metric tons (FAO, 2005). Traditional breeding methods in melon have led to a considerable varietal improvement. However, strong sexual incompatibility barriers at the interspecific and intergeneric levels have restricted potential to readily develop new and enhanced melon cultivars (Niemirowicz-Szczt and Kubicki, 1979; Robinson and Decker-Walters, 1999). Using genetic engineering strategies is feasible to overcome most of the genetic barriers among plants, which are unsurpassable by traditional breeding techniques (Vasil, 1990; 1996; 1998; 2003). Likewise, in order to achieve a successful commercial application from biotechnology in melon a competent de novo regeneration system from in vitro cultures is required (Guis et al. 1998).

In the last 25 years, more than 40 in vitro melon regeneration protocols have been described, some using either organogenesis, somatic embryogenesis or both regeneration pathways (Blackmon et al. 1981a; Blackmon et al. 1982; Moreno et al. 1985a; Mackay et al. 1988; Punja et al. 1990; Chee, 1991; Shetty et al. 1992; Hosoi et al. 1994; Singh et al.
Several biological and physical factors influence in vitro regeneration efficiency rate, and all have to be considered in order to develop a reproducible and reliable melon regeneration protocol. All the systems had diverse regeneration rates depending on genotype, culture conditions and explant source. Melon plant regeneration has been reported from roots (Kathal et al. 1994), calli (Moreno et al. 1985b), petioles (Punja et al. 1990), hypocotyls (Moreno et al. 1985b), leaves (Yadav et al. 1996), protoplasts (Moreno et al. 1985a), somatic embryos (Gray et al. 1993), shoot primordia (Adelberg et al. 1999), and cotyledons (Guis et al. 1997a).

‘Galia’ muskmelon is an Israeli melon F1 hybrid with exceptional fruit quality characteristics such as 13-15% total soluble solids (TSS), bold flavor and a distinct aroma (Karchi, 2000). Unlike other melon types, such as Cantaloupe Charentais (Ayub et al. 1996; Akasa-Kennedy et al. 2004; Silva et al. 2004), ‘Galia’ muskmelon is not easily cultivated in vitro and full regenerated wild type plants are especially difficult to obtain (Leshem 1989; Leshem et al. 1994a; 1994b; Gaba et al. 1994; Gaba et al. 1996; Edriss et al. 1996; Kintzios and Taravira 1997; Galperin et al. 2003a). Additionally, Gaba et al. (1999) reported ‘Galia’ muskmelon to be recalcitrant to transformation by Agrobacterium tumefaciens. Therefore, a reliable and efficient in vitro protocol for regeneration and transformation in ‘Galia’ muskmelon has been a desired objective for more than a decade (Gaba et al. 1992). Because different melon explants have diverse in vitro regeneration responses, it is important to accurately assess that parameter in ‘Galia’ parental lines. The goal of this research was to measure the source of the explant on regeneration and
transformation efficiency using two reporter genes (GUS and GFP) in ‘Galia’ melon male and female parental lines.

3.2 Materials and Methods

3.2.1 Plant Material

Mature seeds of ‘Galia’ male (Cucumis melo L. var. reticulatus Ser. cv. ‘Krimka’) and female (Cucumis melo L. var. cantaloupensis Naud. cv. ‘Noy yizre’el’) parental lines were surface sterilized and germinated on hormone-free half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) according to Nuñez-Palenius et al. (2005a) protocol. In vitro regeneration of both parental lines was carried out on explants from cotyledons, hypocotyls, and non-expanded true leaves following the procedures and plant-hormone balances reported by Nuñez-Palenius et al. (2005a), Ramirez-Malagon and Ochoa-Alejo (1996), and Guis et al. (2000), respectively. De novo shoot regeneration response was measured as bud forming capacity index [BFC index= (average number of shoots per explant) X (% explants forming shoots) / 100] (Martinez-Pulido et al. 1992). Seedling growth and plant regeneration were conducted in a growth chamber (Lab-Line Instruments, Inc. Melrose Park, IL) under 100 \( \mu \text{mol} \cdot m^{-2} \cdot s^{-1} \) light and a 16 h photoperiod provided by cool-white fluorescent lamps and constant 25 ± 1°C temperature.

3.2.2 Agrobacterium Inoculation and Plant Transformation

Agrobacterium tumefaciens strain ABI containing a binary vector, pMON17204, harboring a selectable glyphosate resistance marker [CP4 syn gene encoding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)], and the uidA gene encoding for \( \beta \)-glucuronidase (GUS) was used (Figure 3.1a). CP4 syn gene was under control of the Figwort Mosaic Virus (FMV) promoter (Richins et al. 1987), whereas uidA gene was under control of the CaMV 35S promoter (35S). Agrobacterium tumefaciens strain LBA-
4404 containing a binary vector, pCAMBIA 2202-sGFPS65T, harboring a selectable kanamycin-resistance marker, NPT-II gene encoding for neomycin phosphotransferase, and a fully functional green fluorescent protein (GFP) gene was used (Figure 3.1b). Both Agrobacterium tumefaciens strains, ABI and LBA4404, were transferred to 5 ml of Luria-Bertani Broth (LB) medium (pH 7.5). The former was supplemented with 25 mg·L⁻¹ chloramphenicol, 50 mg·L⁻¹ kanamycin, and 100 mg·L⁻¹ spectinomycin, whereas the second strain was supplemented with 50 mg·L⁻¹ carbenicillin and 100 mg·L⁻¹ chloramphenicol. Both strains were then incubated on an orbital shaker (200 rpm) for 12 hours at 25°C. Afterward, the cultures (5 ml aliquot) were transferred to 250 ml baffled culture flask containing 50 ml liquid LB medium with the same antibiotics and concentrations previously used, and incubated at 25°C on an orbital shaker (150 rpm) for an additional 14-20 hours until an A₆₀₀= 0.7-1.0 was reached.

Cotyledon and non-expanded leaf explants from both ‘Galia’ melon parental lines were immersed in Agrobacterium suspension for 20-30 min with orbital shaking (50 rpm), and then were blotted three times onto sterile filter paper (Whatman No. 1) to remove the excess bacterial suspension. Cotyledon and true leaf explants were then cultured and de novo shoots were obtained as previously described by Nuñez-Palenius et al. (2005) and Guis et al. (2000), respectively. Hypocotyls were inoculated with a Agrobacterium tumefaciens suspension using a sterile syringe needle (0.72 mm gauge and 32 mm length) following the protocol described by Ramirez-Malagon and Ochoa-Alejo (1996).
3.2.3 Histochemical Staining for GUS Activity and GFP Detection

Histochemical localization for β-D-glucuronidase (GUS) activity in putative T₀ transformant tissues was performed as described by Gama et al. (1996). Plant organ and tissues were incubated in GUS-staining solution [50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA (pH 8.0), 0.06% (v/v) Triton X-100, 0.35 mg·mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (X-Gluc)] at 37°C for 12-36 h. After incubation, the samples were then
transferred to 70% (v/v) ethanol to remove chlorophyll. GUS-positive tissues were photographed with a Zeiss MC63 photomicrographic camera coupled to a DRC stereomicroscope.

Putative GFP-positive plant tissues were examined directly to detect fluorescence using a Zeiss Stemi SU11 fluorescence stereomicroscope having a mercury lamp and a FITC 535 filter.

3.2.4 PCR Assay

Total DNA isolation from wild type and transgenic GUS T1 melon leaflets (0.25-0.5 g) was performed using a modified CTAB protocol (Doyle and Doyle 1987; 1990). The forward and reverse primers for uidA gene (GUS) were 5’-CAACGAACTGAGAGCAG-3’ and 5’-CATCACCACGCTTGGGTG-3’, respectively, amplifying a fragment of 750 bp. The GUS fragments in total DNA were amplified under the following conditions: a pre-incubation period at 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 sec for denaturation, 55 °C for 30 sec for primer annealing, and 72 °C for 45 sec for extension, and a final extension period at 72 °C for 5 min. The amplified PCR products (10 µl) were subjected to electrophoresis on a 0.8% agarose gel and visualized by UV light.

3.2.5 Experimental Design and Statistical Analysis

The experiment to measure the BFC index on three different melon explants was carried out under a factorial design (2 x 3) with six replicates, having two levels for genotype, and three levels for explant type. The experimental design for transformation experiment was a factorial (2 x 3 x 2) with three replicates, having two levels for genotype, three levels for explant type, and two levels for reporter gene used. Original data from transformation experiment was transformed by the arcsine square root in order
to be analyzed for analysis of variance (ANOVA). Means were separated by Tukey’s Studentized Range Test at $P \leq 0.05$ (SAS Institute, Cary, N.C.).

3.3 Results and Discussion

3.3.1 Effect of Explant Origin on Regeneration Efficiencies

Cotyledonary explants from ‘Galia’ muskmelon male and female parental line attained the greatest BFC index compared to hypocotyl and true-leaf explants (Figure 3.2). The BFC value for cotyledons was almost 4 times greater than hypocotyls and true-leaves. When the same explant type was used, no significant differences between male and female BFC response were found, except for cotyledons.

![Figure 3.2 Organogenetic response of ‘Galia’ muskmelon parental lines depending on explant source. Bars with the same letter are not significantly different by Tukey’s Studentized Range Test at $P \leq 0.05$. BFC= bud forming capacity index (Martinez-Pulido et al. 1992).](image)

Cotyledonary explants have been the most common plant organ used to induce de novo regeneration through either organogenesis or somatic embryogenesis in *Cucumis melo* (Blackmon et al. 1981a; 1982; Trulson and Shahin, 1986; Mackay et al. 1988;
Branchard and Chateau, 1988; Dirks and Van Buggenum, 1989; Niedz et al. 1989; Leshem et al. 1989; Chee, 1991; Tabei et al. 1991; Kageyama et al. 1991; Homma et al. 1991; Fassuliotis and Nelson, 1992; Shetty et al. 1992; Debeaujon and Branchard, 1992; Roustan et al. 1992; Oridate et al. 1992; Gray et al. 1993; Adelberg et al. 1994; Ezura and Oosawa, 1994b; Hosoi et al. 1994; Ficcadenti and Rotino, 1995; Yadav et al. 1996; Singh et al. 1996; Kintzios and Taravira, 1997; Guis et al. 1997a; Stipp et al. 2001; Curuk et al. 2002a; Galperin et al. 2003a; 2003b; Akasa-Kennedy et al. 2004). The use of cotyledon as explants for melon in vitro culture has several advantages over other plant tissues, i.e. a) cotyledons have a quick morphogenetic response producing high number of shoots or somatic embryos, b) cotyledons are ready to be used as explants in a short period of time (0-5 days), and c) several cotyledonary explants (8-12) are effortlessly acquired from a single seed (Table 3.1). Moreover, de novo shoots from cotyledon are ready to be ‘hardened’ as early as 9 weeks after the initiation of in vitro culture, whereas hypocotyl and true-leaf systems require 23 and 17 weeks, respectively (Figure 3.3).

Overall, the cotyledon was the best explant to regenerate complete plants for both ‘Galia’ muskmelon male and female parental lines compared to hypocotyl and true-leaf explants.

### 3.3.2 Plant Transformation

In order to assess the effect of source explant on transformation efficiency in ‘Galia’ male and female parental lines, two *Agrobacterium tumefaciens* strains, ABI and LBA4404, harboring GUS and GFP reporter genes, respectively, were tested on cotyledon, hypocotyl and true-leaf explants. GUS-positive tissues were detected by a histological GUS assay (Gama et al. 1996), while GFP-positive tissues were identified by fluorescence microscopy.
Table 3.1 Regeneration efficiency in ‘Galia’ muskmelon parental lines depending on explant source.

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<td>12</td>
<td>6.31</td>
<td>4-5</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>1</td>
<td>1.51</td>
<td>19-20</td>
</tr>
<tr>
<td>True-leaf</td>
<td>10</td>
<td>1.11</td>
<td>11-13</td>
</tr>
</tbody>
</table>

*BFC= bud forming capacity index (average number of shoots per explant) X (% explants forming shoots) / 100. (Martinez-Pulido et al. 1992).

*yPeriod needed to achieve complete developed shoots. Data are the average for male and female ‘Galia’ parental lines results.

Transformed cotyledon and hypocotyl explants from both ‘Galia’ male and female parental lines produced *de novo* shoots and roots, which were positive for GUS and GFP expression (Figure 3.4). Although *de novo* shoots were obtained, only ‘escapes’ (non GUS- or GFP-positive shoots and roots) were observed when true-leaf explants were used (Figures 4-5 and 4-6). It has been reported that different plant explants have diverse susceptibility to be transformed by *Agrobacterium tumefaciens* (Febres et al. 2003). This differential susceptibility has been described in melon (Bordas et al. 1997; Guis et al. 1998) cucumber (Rajagopalan and Perl-Treves, 2005), and other plant species (Tu et al. 2005). Also, the production of ‘escapes’ after Agrobacterium- or biolistic-mediated transformation in *Cucumis melo* is a commonly observable result (Guis et al. 1998). Indeed, Akasaka-Kennedy et al. (2004) have claimed that in order to reduce the problem of high production of ‘escapes’ by melon *in vitro* cultures, alternative regeneration and transformation systems have still to be obtained.
Figure 3.3 Time schedule for de novo shoot regeneration in hypocotyl, cotyledon and true-leaf explants of ‘Galia’ muskmelon parental lines. The numbers between the top and bottom photographs represent the number of weeks to attain from mature seeds the in vitro rooted seedlings. Hypocotyl: (a) seedlings, (b) hypocotyl injury (arrow), (c) decapitation, (d) decapitated seedlings, (e) de novo shoots, (f) in vitro seedling. Cotyledon: (a) cotyledons from 2-day-old seedlings, (b) de novo shoots, (c) in vitro seedling. True-leaf: (a) seedlings, (b) true-leaves, (c) de novo shoots, (d) in vitro seedlings.
A greater number of T₀ GUS-positive seedlings for both ‘Galia’ male and female parental lines was obtained when cotyledons were used as explants compared to hypocotyls (Figure 3.5). Moreover, significant differences were observed between both explants. Conversely, when the Agrobacterium tumefaciens strain LBA4404, containing a binary vector pCAMBIA 2202-sGFPS65T, was used to transform both ‘Galia’ parental lines, only female-hypocotyl explants produced a higher amount of GFP-positive seedlings than cotyledons (Figure 3.6). It was found that 13% of hypocotyl-explants produced transgenic shoots. ‘Galia’ male parental-hypocotyl explants had a transformation efficiency value similar to ‘Galia’ male- and female-cotyledon explants. The results obtained on Cucumis melo transformation efficiency in this work exceed what it has been previously reported. In general, the genetic transformation rate efficiency in most of melon systems is normally lower than other plant organisms (Fang and Grumet, 1990; Dong et al. 1991; Gaba et al. 1992; Gonsalves et al. 1994; Bordas et al. 1997; Akasaka-Kennedy et al. 2004). Several transformation rate efficiency values have been described according to transformation protocol and melon cultivar used, among them, average efficiencies such as 3-7% (Fang and Grumet, 1990), 4-6% (Dong et al. 1991), 1% (Gaba et al. 1992), 0.0%, 0.9%, and 1% (Gonsalves et al. 1994), 0.7-3% (Bordas et al. 1997), 2.4% (Guis et al. 2000), 10% (Nuñez-Palenius et al. 2005a), and 2.3% (Akasaka-Kennedy et al. 2004) has been depicted.

A PCR assay was performed on total DNA isolated from T₁ GUS positive plants in order to detect the presence of uidA gene and corroborate their transgenic status. We were able to detect the presence of a GUS-specific 750 bp fragment which belongs to the GUS
reporter gene, in male and female ‘Galia’ muskmelon transgenic lines but not in non-transgenic lines (Figure 3.7).

In summary, it is possible to obtain transgenic ‘Galia’ male and female parental plants using either cotyledon or hypocotyl explants. Moreover, cotyledonary explants have the advantage to attain those transgenic plants in a shorter period (Figure 3.8).

Figure 3.4 Stable expression of GFP and GUS genes in T₀ shoots and roots of ‘Galia’ muskmelon parental lines. A) GUS non-positive tissue, B) and C) GUS positive tissue. D) GFP non-positive tissue, E) and F) GFP positive tissue.
Figure 3.5 Transformation efficiency on ‘Galia’ muskmelon parental lines. GUS reporter gene was used to transform different explant sources. Bars with the same letter are not significantly different by Tukey’s Studentized Range Test at $P \leq 0.05$.

Figure 3.6 Transformation efficiency on ‘Galia’ muskmelon parental lines. GFP reporter gene was used to transform different explant sources. Bars with the same letter are not significantly different by Tukey’s Studentized Range Test at $P \leq 0.05$. 
Figure 3.7 PCR assay for transgenic GUS ‘Galia’ melon male and female line plants. The amplification product for GUS gene was 750 bp. M: 100 bp Ladder, 1: negative control (DNA from wild type melon plant), 2 and 3: DNA from ‘Galia’ male GUS positive plant, 4 and 5: DNA from ‘Galia’ female GUS positive plant, 6: positive control (DNA from GUS plasmid).
Figure 3.8 Time schedule for cotyledon and hypocotyl transformation systems in ‘Galia’ melon parental lines. A. Explant transformation B. Regeneration of transgenic shoots. C. Elongation of transgenic shoots D. Rooting E. ‘Hardening’ of seedlings.
3.3.3 Summary

The goal of this work was to measure the source of the explant on regeneration and transformation efficiency in ‘Galia’ melon parental lines. Cotyledon, hypocotyl and true-leaves of both female and male ‘Galia’ parental lines were used for transformation. The ABI strain of *Agrobacterium tumefaciens*, containing a construct harboring the GUS gene under the constitutive 35S promoter, and a glyphosate tolerance gene under the constitutive FMV promoter as selectable marker were used. The LBA4404 strain of *Agrobacterium tumefaciens* carrying a plasmid vector harboring the GFP and NPTII genes both under the constitutive 35S promoter was also used. Regeneration of ‘Galia’ melon parental line shoots was achieved from hypocotyls, true-leaves and cotyledons. Hypocotyls were transformed according the protocol described in Ramirez-Malagon & Ochoa-Alejo (1996). Cotyledons were transformed with a protocol previously developed in our lab (Nuñez-Palenius et al. 2005a) and true-leaf explants were transformed using the methodology of Guis et al. (2000). The greatest numbers of shoots were regenerated from cotyledons. These explants also produced the highest number of GUS positive shoots and roots. Using hypocotyls as explants gave the greatest number of GFP positive roots and shoots. We were not able to obtain any transgenic shoots using either the GUS or GFP constructs when true-leaves were used as explants. According to our results, ‘Galia’ melon parental lines are readily transformable with *Agrobacterium tumefaciens* using the hypocotyl or cotyledon-protocol. It is possible to have full regenerated transgenic plants in 3-4 months using the cotyledon-protocol, whereas at least 6-7 months are needed when the hypocotyl-protocol is used.
CHAPTER 4
TRANSFORMATION OF A MUSKMELON ‘GALIA’ HYBRID PARENTAL LINE (CUCUMIS MELO L. VAR. RETICULATUS SER.) WITH AN ANTISENSE ACC OXIDASE GENE

4.1 Introduction

‘Galia’ muskmelon (Cucumis melo L.) is an Israeli melon F₁ hybrid bred by Dr. Zvi Karchi at the Newe Ya’ar research center of the Agricultural Research Organization (A.R.O. - Israel) and released in 1973 (Karchi 2000). This cultivar has green-flesh characteristics of the ‘Ha’Ogen’ melon cultivar, which was used as the female parent, and netted rind from ‘Krymka’, which was used as the male parental line. It has exceptional fruit quality with 13-15% total soluble solids (TSS), bold flavor and a distinct aroma, leading to rapid adoption in both local and export markets. ‘Galia’ type melons have been the mainstay in muskmelon sales in the European market for more than 30 years (Karchi, 2000). One disadvantage of ‘Galia’ is its storage life, which is limited to two to three weeks. Timing of harvest is critical for ‘Galia’ fruit because in order to develop peak flavor and aroma, the melon should be picked at maturity (Karchi, 2000). ‘Galia’ F₁ hybrid tends to be extremely soft at and past peak fruit maturity. Traditional breeding methods have helped breeders develop a strategy to obtain ‘Galia’ muskmelons with a long shelf life; however, this approach can result in a loss of favorable fruit quality characteristics.

Plant biotechnology has the potential to genetically transform plants and transfer novel characteristics. In order for plant transformation to be successful a reliable plant in vitro regeneration system must first be developed (Guis et al. 1998). Using
Agrobacterium and particle-gun bombardment methods, several transgenes, which provide different phenotypic characteristics, have been transferred into *Cucumis melo* explants. These have included selectable marker and reporter genes (Fang and Grumet, 1990; Dong et al. 1991; Gaba et al. 1992; Vallés and Lasa, 1994; Gray et al. 1995; Akasa-Kennedy et al. 2004), virus-resistance genes (Yoshioka et al. 1992; 1993; Fang and Grumet, 1993; Gonsalves et al. 1994; Clough and Hamm, 1995), halotolerance-genes (Bordas et al. 1997), and genes to improve fruit quality (Ayub et al. 1996; Ezura et al. 1997a; Clendennen et al. 1999; Guis et al. 2000; Shellie 2001; Silva et al. 2004). Improved quality of Charentais type muskmelon has been achieved by inserting ACC oxidase genes in antisense orientation in order to reduce fruit ethylene biosynthesis (Ayub et al. 1996; Guis et al. 1997b; Silva et al. 2004).

Increasing the shelf life of ‘Galia’ F<sub>1</sub> muskmelon by transformation of parental lines is a feasible alternative. Unlike other melon types, such as Cantaloupe Charentais (Ayub et al. 1996; Akasa-Kennedy et al. 2004; Silva et al. 2004), ‘Galia’ muskmelon is not easily cultivated *in vitro* and complete regenerated wild type plants are especially difficult to obtain (Leshem 1989; Leshem et al. 1994a; 1994b; Gaba et al. 1994; Gaba et al. 1996; Edriss et al. 1996; Kintzios and Taravira 1997; Galperin et al. 2003a). Moreover, Gaba et al. (1999) reported ‘Galia’ muskmelon to be recalcitrant to transformation by *Agrobacterium tumefaciens*.

The goal of this research was to transform the male parental line (cv. ‘Krymka’) of ‘Galia’ muskmelon with the ACC oxidase (CMACO-1) antisense gene in an attempt to delay fruit ripening.
4.2 Materials and Methods

4.2.1 Plant Material

Coats of 50 male parental line ‘Galia’ (*Cucumis melo* L. var. *reticulatus* Ser.) cv. ‘Krymka’ seeds were removed, and the embryos surface sterilized in 1.2% sodium hypochlorite (20% commercial bleach solution containing two drops of Tween 20™ per 100 ml) for 15 min in a sterile Erlenmeyer flask (50 ml), and washed three times with sterilized distilled water. Embryos were then cultivated for 2 days in regeneration medium (RM), which consisted of Murashige and Skoog salts (MS) (Murashige and Skoog, 1962), supplemented with 30 g L⁻¹ sucrose, 0.1 g L⁻¹ myo-inositol, 0.001 g L⁻¹ thiamine-HCl, 0.05 mg L⁻¹ pyridoxine-HCl, 0.05 mg L⁻¹ nicotinic acid, 2 mg L⁻¹ glycine, 1 mg L⁻¹ benzyladenine (BA), 0.001 mg L⁻¹ α-Naphthaleneacetic acid (NAA), and 0.7 g L⁻¹ phytagar. Seedling growth and plant regeneration were conducted in a growth chamber (Lab-Line Instruments, Inc. Melrose Park, IL) under 100 µmol m⁻² s⁻¹ light and a 16 h photoperiod provided by cool-white fluorescent lamps and constant 25°C temperature.

4.2.2 Plant Regeneration

Cotyledons from 2-day-old seedlings, grown aseptically in RM, were dissected and cut transversely into four equal pieces, then placed with their abaxial side on the surface of RM. Each cotyledon slice constituted an explant. Complete shoots were obtained *de novo* from these cotyledon explants after four weeks of culture. Regeneration efficiency was calculated as BFC index, BFC index= (average number of shoots per explant) X (% explants forming shoots) / 100 (Martinez-Pulido *et al.*, 1992). Shoots were excised from cotyledon explants and incubated on elongation medium (EM), which consisted of MS...
salts, supplemented with 30 g L\(^{-1}\) sucrose, 0.1 g L\(^{-1}\) myo-inositol, 0.001 g L\(^{-1}\) thiamine-HCl, 0.05 mg L\(^{-1}\) pyridoxine-HCl, 0.05 mg L\(^{-1}\) nicotinic acid, 2 mg L\(^{-1}\) glycine, 0.025 mg L\(^{-1}\) BA and 0.8 g L\(^{-1}\) phytagar, for three more weeks. Elongated shoots were then transferred to rooting media consisting of ½ strength MS medium supplemented with 1 mg L\(^{-1}\) indole-3-acetic acid (IAA). After three weeks of culture, shoots developed a complete and normal root system. Rooted shoots were hardened using common practices and then transferred to glasshouse. Plants were grown in an evaporative-cooled fan and pad glasshouse, which maintained temperatures of 28°C day and 20°C night, in plastic pots (11.3 L) filled with soil-less media and following common growing practices recommended by Rodriguez and Cantliffe (2001). A complementary light regime was supplied by Metalarc lamps with a light intensity of 350 – 530 µmol · m\(^{-2}\) · s\(^{-1}\). The light period was set to 18 hours per day.

4.2.3 Agrobacterium Inoculation and Plant Transformation

*Agrobacterium tumefaciens* strain ABI containing a binary vector, pCmACO1-AS, harboring a selectable glyphosate resistance marker [CP4 syn gene encoding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)], and the ACC oxidase (CMACO-1) gene in antisense orientation was used (Figure 4.1). Both genes were under control of the Figwort Mosaic Virus promoter (Richins et al. 1987) (Figure 4.1). Agrobacterium was transferred to 5 ml of Luria-Bertani Broth (LB) medium (pH 7.5) supplemented with 25 mg · L\(^{-1}\) chloramphenicol, 50 mg · L\(^{-1}\) kanamycin, and 100 mg · L\(^{-1}\) spectinomycin, and then incubated on an orbital shaker (200 rpm) for 12 hours at 25°C. Afterward, the culture (5 ml aliquot) was then transferred to 250 ml baffled culture flask containing 50 ml liquid LB medium with the same antibiotics and concentrations previously used, and
incubated at 25°C on an orbital shaker (150 rpm) for an additional 14-20 hours until an 
A$_{600}$ = 0.7-1.0 was reached.

Cotyledonary explants were immersed in Agrobacterium suspension for 20-30 min 
with orbital shaking (50 rpm), and then were blotted three times onto sterile filter paper 
(Whatman No. 1) to remove the excess bacterial suspension. Explants were then cultured 
and de novo shoots were obtained as previously described in Plant Regeneration section, 
except that RM was supplemented with 50 µM Glyphosate and 150 mg L$^{-1}$ Timentin 
(GlaxoSmithKline, Research Triangle Park, NC). Glyphosate selection was carried out 
only on RM.

4.2.4 Flow Cytometry Analysis

Plant nuclei were freshly isolated from the 3rd leaf below the shoot apex of 
acclimatized plants grown in a greenhouse. Briefly, plant material (4 cm$^2$) was chopped 
for three minutes (at 4°C) with a razor blade in a Petri dish containing 5 ml of 
Quesenberry (1995) extraction buffer. The nuclei suspension was filtered through 
Spectra/Mesh® nylon filter (60- m mesh size) to remove cell debris. The nuclei-filtered 
suspension was stained with propidium iodide by adding 1 ml of a propidium iodide 
stock solution (1 mg.ml$^{-1}$) to 2 ml of nuclei suspension. After gently stirring, the nuclei 
mixture was incubated for 5 min and then analyzed by flow cytometry. DNA content of 
the isolated plant nuclei was analyzed with a flow cytometry apparatus (FACScan. BD- 
Biosciences, San Jose, Cal). It was calibrated using the 2C peak from nuclei of young 
leaves of diploid plants derived from seed. A minimum of 10,000 nuclei were measured 
for each sample.
4.2.5 Detection of Transgenes

Total DNA isolation from wild type and putative transgenic melon leaflets (0.25-0.5 g) was performed using a modified CTAB protocol (Doyle and Doyle 1987; 1990). The forward and reverse primers for CP4syn gene were 5’-

CGGTGCAAGCAGCCGTCACGTC-3’ and 5’-CCTTAGTGTCGGAGTTCG-3’, respectively, amplifying a fragment of 1,400 bp (1.4 Kb). The forward and reverse primers for ACC oxidase gene were 5’-GCAATTATCCGCCTGTC-3’ and 5’-

TCTTCAAACACAAACTTGGGG-3’, respectively. These primers will produce a 503 bp fragment from the native (genomic) ACC oxidase gene and a 378 bp product from the transgene. The ACC oxidase and CP4syn fragments in total DNA were amplified under the following conditions: a pre-incubation period at 94 °C for 7 minutes followed by 40 cycles of 94 °C for 1 min for denaturation, 60 °C for 1 min for annealing, and 72 °C for 1 min for extension, and a final extension period at 72 °C for 10 min. The amplified PCR products (25 µl) were subjected to electrophoresis on a 1% agarose gel and visualized by UV light.
4.2.6 Southern Blot Analysis

Total melon DNA was isolated from young leaves of wild type and transgenic T₁, TGM-AS-1 and TGM-AS-2 lines (TGM-AS stands for Transgenic Galia Male AntiSense line), using a modified CTAB protocol from Doyle and Doyle (1987; 1990). Twenty micrograms of this DNA were digested overnight at 37° C with BamHI and separated by electrophoresis in a 1.3% agarose gel. Because BamHI has one cut-site within T-DNA, a fragment bigger than 0.398 Kb is expected for each insertion event. DNA was then denatured and transferred to N⁺ Hybond membrane. The nylon membrane was prehybridized 4 h at 42° C with constant shaking at 60 rpm, in a solution containing 50% (v/v) formamide, 5X Denhardt’s, 1% sodium dodecyl sulfate (SDS), 5X SSPE (1X SSPE is 15 mM NaCl, 10 mM NaH₂PO₄·H₂O and 1 mM EDTA), and 100 µg · mL⁻¹ denatured salmon sperm DNA. Hybridization was carried through overnight at 42° C with constant shaking at 60 rpm, in a solution containing 50% (v/v) formamide, 5X Denhardt’s, 1% sodium dodecyl sulfate (SDS), 5X SSPE, and 100 µg · mL⁻¹ denatured salmon sperm DNA plus 1 X 10⁶ cpm ml⁻¹ denatured ³²P-labeled 1.4 Kb PCR-product from CP4 syn gene. All further washes were performed with constant shaking at 60 rpm. Membranes were first washed in a solution containing 2X SSPE, 0.05% sarkosyl and 0.01% sodium pyrophosphate at 42°C during 20 min. The second wash was performed in a solution containing 2X SSPE, 0.05% sarkosyl and 0.01% sodium pyrophosphate at 65°C for 20 min. The third and last (fourth) washes were carried out in a solution containing 0.1 X SSPE, 0.05% sarkosyl and 0.01% sodium pyrophosphate for 20 min at 65°C. Membranes were exposed to Kodak BIOMAX MR Film at -80°C for 3-5 days.
4.2.7 Segregation Analysis of Transgenes in Primary Transformants

In order to analyze the segregation pattern of transgenes, 62 randomly selected T₁ seedlings of TGM-AS-1 and TGM-AS-2 transgenic lines of ‘Galia’ male parental line were evaluated. DNA was extracted at least three times from each individual seedling according to the modified CTAB protocol (Doyle and Doyle 1987; 1990). A PCR assay with specific primers for CMACO-1 and CP4 syn genes was performed for each DNA sample as described above for transgene detection.

4.3 Results and Discussion

4.3.1 Transformation Efficiency

A total of six experiments of transformation were completed, under a CR design, using the vector pCmACO1-AS. Glyphosate selection was not completely efficient, because several “escapes” were detected by PCR assays. In addition, these PCR assays aided in detecting the presence of CP4 syn and CMACO-1 antisense genes (Figure 4.2). We were able to observe the presence of 1.4, 0.5, and 0.3 kb fragments that belong to the CP4 syn, the native ACC oxidase and the engineered ACC oxidase genes, respectively.

Using the cotyledon-protocol and the CMACO-1 construct, the individual transformation efficiency for each experiment, assessed by PCR, ranged between 7.5% and 12.5% (Table 4.1). Similar transformation efficiency rates (9.5%) were obtained for muskmelon cv. ‘Krymka’ using another independent transformation system, such as the GUS reporter gene (Figure 4.4). This transformation efficiency obtained for ‘Galia’ male parental line is one of the highest reported for any Cucumis melo transformation protocol (Fang and Grumet, 1990; Dong et al. 1991; Gaba et al. 1992; Gonsalves et al. 1994; Bordas et al. 1997; Guis et al. 2000; Akasaka-Kennedy et al. 2004). This high transformation efficiency could be the result of a combination of factors such as
*Agrobacterium tumefaciens* strain, selectable marker, and construct used (Hellens and Mullineaux 2000; Febres et al. 2003), which may have provided the right conditions to accomplish these positive results.

Figure 4.2 PCR assay for putative transgenic ‘Galia’ muskmelon male line plants. The amplification product for *CP4syn*, native ACO-1, and engineered ACO-1 genes were 1.4 Kb, 0.5 Kb, and 0.3 Kb, respectively. M: HyperLadder (Bioline), 1 through 6: putative transgenic plants, 7: DNA from positive plant, 8: DNA from negative plant, 9: PCR reaction mixture.

Transgenic explants had an epinastic response while they were growing *in vitro* on Glyphosate selection (Figure 4.5). However, this abnormality was not observed once transgenic shoots were rooted and transferred to *ex vitro* conditions. Glyphosate is a strong herbicide. It is able to induce ‘stress’ responses even in Glyphosate-tolerant
Table 4.1 Transformation efficiency of ‘Galia’ male parental line with CMACO-1 antisense construct.

<table>
<thead>
<tr>
<th>Number of explants</th>
<th>BFC</th>
<th>PCR+ plants</th>
<th>% of efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0.18</td>
<td>12 ± 3(^x)</td>
<td>10 ± 2.5</td>
</tr>
</tbody>
</table>

Data are the average of six different experiments, each one with 120 explants. 
\(^x\)BFC = bud forming capacity index (average number of shoots per explant) \times (\% explants forming shoots) / 100. (Martinez-Pulido et al. 1992).
\(^y\)PCR+ = Polymerase chain reaction positive for transgenes.
\(^x\)Mean value plus-minus standard error.

plants (Saroha et al. 1998; Raymer and Grey, 2003). Also, it is well known that ethylene is a plant hormone related to ‘stress responses’ (Klee and Clark, 2002). Therefore, it is plausible that epinastic phenotype was induced by Glyphosate. A similar response was observed in positive explants for \(\beta\)-d-glucuronidase gene (GUS) growing on Glyphosate as well (Nunez-Palenius et al. unpublished results).

In summary, transgenic shoots were obtained by using Glyphosate as a selectable agent and were identified by means of PCR assay. This PCR system also allows us to identify those shoots which were non-transgenic.

4.3.2 Ploidy Level of Primary Regenerants

Melon plants regenerated through plant tissue culture methodologies are very susceptible to increases in ploidy level while they are in vitro cultivated (Bouabdallah and Branchard, 1986; Fassuliotis and Nelson, 1992; Ezura et al. 1992a; 1992b; Debeaujon and Branchard, 1992; Kathal et al. 1992; Ezura and Oosawa, 1994a; Ezura et al. 1994; Guis et al. 1998). Therefore, flow cytometry analyses were performed to determine the ploidy level of primary regenerants. Young leaves from all in vitro transgenic regenerated plants and also from wild-type plants obtained from seed were
used. This was done with the aim of discarding any triploid, aneuploid, and tetraploid or mixoploid plants. Based on these analyses, only diploid plants were used in subsequent experiments.

Unexpectedly, some leaves from plants obtained from seeds resulted as tetraploids. This condition has been reported in melon plants field conditions as well (Nugent and Ray, 1992). In Arabidopsis it has been described that ethylene and gibberellins might have an important role in inducing the cellular endoreduplication process, which leads to
Figure 4.4 Transgenic (left) and non-transgenic (right) in vitro ‘Galia’ male line explants. Transgenic explants, growing on 50 μM Glyphosate, had curled leaves and shorter internodes than wild type.

an increase in ploidy level (Kondorosi et al. 2000). Figure 4.6 depicts charts for wild-type diploid and tetraploid plants, as well as transgenic diploid and tetraploid plants. In Figure 4.6a, nuclei sample was obtained from the 3rd leaf below shoot apex (diploid), whereas nuclei in Figure 4.6b were obtained from shoot re-growth from the plant base (4th and 5th node distal to the cotyledonary-leaf). For subsequent experiments using flow cytometry analysis, all leaf samples were harvested from the 3rd leaf below shoot apex. In Figure 4.7a and 4.7b, a transgenic diploid and tetraploid plant is shown. Using flow cytometry analysis it was found that only 20% of the transgenic plants were diploid. This number of diploid melon plants attained through in vitro culture is lower compared with results previously reported (Guis et al. 2000; Curuk et al. 2003). This is because our system is based on using cotyledons as explants, which is a tissue with high propensity to bear
tetraploid cells since the mature seed stage in cucurbits (Colijn-Hooymans et al. 1994). After an entire flow cytometry evaluation (at least seven leaves from different nodes) of all transgenic regenerants, two completely diploid independent transgenic plants were selected, which were named as TGM-AS-1 and TGM-AS-2. Plants that were PCR positive and completely diploid were advanced to the next generation by selfing.

4.3.3 Southern Blot Analysis

The presence of the introduced \textit{CP4 syn} gene in T\textsubscript{1} plants, both TGM-1 and TGM-2 lines, was established by genomic Southern blot analysis (Figure 4.8). Likewise, this assay allowed determining the copy number of the transgene insertion. The \textsuperscript{32}P-labeled 1.4 Kb PCR-product from \textit{CP4 syn} gene used as a probe hybridized with a single \textit{Bam}H\textsubscript{I}-released fragment of genomic DNA isolated from three individual T\textsubscript{1} plants from TGM-1 (Figure 4.8B, lanes five, six and seven) and three individual T\textsubscript{1} plants from TGM-2 lines (Figure 4.8B, lanes one, two and four). These data indicated that a single copy of the T-DNA was incorporated into the ‘Galia’ male parental line genome in TGM-AS-1 and TGM-AS-2 lines. There was no hybridization signal in any non-PCR positive T\textsubscript{1} plants (Figure 4.8B, Lanes 3 and 8).

4.3.4 Transgene Inheritance in the T\textsubscript{1} Progenies of Primary Transformants

On a population of 62 T\textsubscript{1} plants randomly chosen from each transgenic line (TGM-AS-1 and TGM-AS-2), DNA was extracted individually, at least three times, to determine the transgene distribution inheritance pattern. PCR analysis of the progeny of TGM-AS-1 and TGM-AS-2 using CP4 syn- and ACO-antisense specific primers revealed segregation for the presence and absence of both CP4 syn and ACO-antisense fragments. This observed distribution of the ACO-antisense and CP4 syn genes was consistent with a 3:1 ratio (X\textsuperscript{2} value for TGM-AS-1 and TGM-AS-2 was 0.193 and 0.021, with a
probability of 66% and 88%, respectively), which corresponded to the segregation of one single copy insertion of T-DNA. This conclusion is supported by the Southern blot analysis results as well

### 4.4.1 Conclusion

We have successfully transformed a parental line of ‘Galia’ hybrid muskmelon with a gene of interest and obtained two completely diploid transgenic regenerants. Analysis of T1 progenies from TGM-AS-1 and TGM-AS-2 by segregation and Southern blot revealed that both transgenic lines had a single T-DNA insertion.

Preliminary analysis of the transgenic fruits from ACO-1 antisense male line plants has revealed that these fruits produced less ethylene and have a lower ACC oxidase activity in vivo than their wild type counterpart.
Figure 4.5 Flow cytometry analysis of propidium iodide-stained nuclei from wild type leaf tissue of ‘Galia’ male parental line. 10,000 individual nuclei were measured for each sample. (A) wild-type plant having most of nuclei (55%) at diploid level (M1). (B) wild-type plant having most of nuclei (51%) at tetraploid level (M2).
Figure 4.6 Flow cytometry analysis of propidium iodide-stained nuclei from transgenic leaf tissue of ‘Galia’ male parental line. 10,000 individual nuclei were measured for each sample. (A) transgenic plant having most of nuclei (64%) at diploid level (M1). (B) transgenic plant having most of nuclei (58%) at tetraploid level (M2).
Figure 4.7 Southern blot hybridization assay of male muskmelon transgenic plants. (A) Total genomic DNA (20 µg) extracted from leaves of T₁ plants was digested and electrophoresed according to Material and Methods section. M: HyperLadder (Bioline), WT: wild type ‘Galia’ male plant. 1 through 4: T₁ plants from T₀ TGM-AS-2 line. 5 through 8: T₁ plants from T₀ TGM-1 line. (B) Southern blot hybridization assay for BamHI-digested genomic DNA. 1, 2 and 4: PCR-positive T₁ plants of T₀ TGM-AS-2 line. 3: PCR-negative T₁ plant of T₀ TGM-AS-2 line. 5, 6 and 7: PCR-positive T₁ plants of T₀ TGM-AS-1 line, 8: PCR-negative T₁ plant of T₀ TGM-1 line.

4.5.1 Summary

‘Galia’ muskmelon (Cucumis melo L. var. reticulatus Ser.) has been recalcitrant to transformation by Agrobacterium tumefaciens. Transformation of the ‘Galia’ male parental line with an ACC oxidase (CMACO-1) gene in antisense orientation is described herein. Explants were transformed using Agrobacterium tumefaciens strain ABI, which contained a vector pCmACO1-AS plasmid, bearing an antisense gene of CMACO-1 and the CP4 syn gene (glyphosate-tolerance). Both CMACO-1 and CP4 syn genes were assessed by a polymerase chain reaction method. Flow cytometry analysis was performed
to determine plant ploidy level of primary transformants. Two completely diploid independent transgenic plants were obtained. Southern blot and segregation analysis in the T1 generation determined that each independent transgenic line had one single insertion of the transgene. These transgenic muskmelon male parental lines have potential for use in the production of ‘Galia’ F1 hybrids with improved shelf life.
CHAPTER 5
EMBRYO-RESCUE CULTURE IN ‘GALIA’ MALE PARENTAL LINE MELON
(CUCUMIS MELO L. VAR. RETICULATUS SER.)

5.1 Introduction

As part of specific hybridization and breeding programs, crosses between distant elite-plant genotypes sometimes fail to produce a complete embryo inside the seed, due to embryo abortion during the process of embryogenesis. In other cases, even though the embryo is formed, the seed fails to germinate and grow. Several embryo rescue techniques, such as ovule, ovary and embryo culture, have been applied to save valuable dicot and monocot plant materials (Bridgen, 1994; Brown et al. 1997; Kumlehn et al. 1997; 1998; 1999; Momotaz et al. 1998; Sukno et al. 1999; Chrungu et al. 1999; Ramming et al. 2000; Kato et al. 2001; Ishikawa et al. 2001; Ribeiro and Giordano, 2001; Yamada 2001; Faure et al. 2002a; 2002b; 2002c). These embryo rescue techniques have also been useful to enhance hybridization, reduce generation time of elite germplasm, and obtain valuable haploid plants in many crops such as onion (Martinez et al. 1997), wheat (Xynias et al. 2001), maize (Weymann et al. 1993), potato (Eijlander et al. 1994), sunflower (Faure et al. 2002b; 2002c), rice (Alemanno and Guiderdoni, 1994), melon (Lotfi et al. 2003), squash (Metwally et al. 1998a; 1998b), and barley (Hoekstra et al. 1992). Not all attempts to obtain plant material through embryo rescue have been successful; embryos generally failed to undergo complete differentiation (Dryanovska and Ilieva, 1983; Shail and Robinson, 1987; Marcellan and Camadro, 2000; de Oliveira et al. 2003).
In cucurbits, embryo rescue techniques have recovered normal seedlings from anther, ovule, and zygotic-embryo cultures to obtain interspecific hybrids or dihaploid lines (Lazarte and Sasser 1982; Shail and Robinson, 1987; Metwally et al. 1998a; 1998b; Chen et al. 1997; Chen et al. 2002; Gemes-Juhasz et al. 2002; de Oliveira et al. 2003; Sisko et al. 2003; Lotfi et al. 2003; Chen et al. 2003). Similar to other plant species, embryo-rescue culture for cucurbits is affected by genotype (Malepszy et al. 1998; Mackiewicz et al. 1998; Lotfi et al. 2003), formulation of media culture (Malepszy et al. 1998; de Oliveira et al. 2003), plant hormone-type and -dosage included into the media (Beharav and Cohen, 1995a; 1995b; Metwally et al. 1998b), environmental factors such as temperature and light (Metwally et al. 1998a; Gémes-Juhász et al. 2002), components added to the media such as carbohydrate source (Metwally et al. 1998b), and embryo-developmental stage at the time of explant isolation (Ezura et al. 1994a; 1994b; Beharav and Cohen, 1995).

Because of these factors which affect efficiency rate of embryo culture, conditions need to be predetermined for success with specific germplasm. This paper describes an improved procedure for recovery of normal seedlings from wild type and transgenic ‘Galia’ muskmelon male parental line zygotic-embryos. Results are presented on the influence of six new supplements for E-20A basic medium (Sauton and Dumax de Vaulx, 1987), five different fruit harvesting dates and two embryo-inoculation methods for plant materials for which no information on embryo rescue exists.

5.2 Materials and Methods

5.2.1 Plant Material

Transgenic muskmelon plants with an ACC oxidase gene in antisense orientation were obtained using the protocol previously described (Nunez-Palenius et al. 2005a).
Mature seeds of wild type and transgenic (ACC oxidase antisense) ‘Galia’ (*Cucumis melo* L. var. *reticulatus* Ser.) male parental line were germinated on a mixture of 70% Terra Lite Plug Mix (Terra Asgrow, Apopka, FL) and 30% coarse vermiculite in polystyrene flats (cell size 2.25 cm² and 164 cells per flat, Speedling, Bushnell, FL). Seedlings were grown under drip fertigation in plastic pots (11.3 L) filled with soil-less media (coarse-grade perlite) in an evaporative-cooled fan and pad glasshouse, with temperatures maintained at 28°C day and 20°C night. Growing practices were those recommended by Rodriguez and Cantliffe (2001). An integrated pest management program (IPM) was used to control pests (http://www.hos.ufl.edu/protectedag/). A complementary light regime (18 h) was supplied by Metalarc® lamps (Osram Sylvania, Inc.) with a light intensity of 350 – 530 µmol · m⁻² · s⁻¹. Hermaphrodite flowers were isolated from foreign pollen with a twist-tie band in the evening, one day before anthesis, and pollinated by hand on the next morning (7:00-10:00 AM) using at least three male flowers. Stigmas were covered with a gelatin capsule (Capsuline™ size 1, Capsuline, Inc. Pompano Beach, FL) after pollination to avoid excessive pollen dehydration.

### 5.2.2 Embryo Culture

Wild type and transgenic fruits were harvested at 4, 10, 17, 24 and 30 days after pollination (DAP) in the morning (6:00 AM), and taken to the lab for subsequent rescue procedures. The surface of the fruit was thoroughly washed with liquid detergent (5% Liqui-Nox®), rinsed with tap water, and at room temperature. Washed fruits were then surface-sterilized with 70% ethanol for 10 min, followed by a 40-min soak in 1.2% sodium hypochlorite (20% commercial bleach solution containing two drops of Tween
20™ per 100 ml) in a sterile beaker. Fruits were then rinsed three times with sterilized distilled water under axenic conditions in a laminar-flow cabinet.

In fruits harvested 4 and 10 DAP, seeds were aseptically dissected from wild type and transgenic fruits using a stereomicroscope. For 17, 24 and 30 DAP-fruits, seeds were large enough to be visually dissected. Excised seeds were kept in 9% sterile sucrose solution during the process (Ondrej et al. 2002). Embryos were excised from seeds 17, 24 and 30 DAP using a stereomicroscope. Intact seeds and embryos were cultured immediately in 100X15-mm Petri dishes containing either E-20A (Table 5.1) or E-21 media [E-20A medium supplemented with 5% coconut water, 0.02 mg L⁻¹ xylose, 1 mg L⁻¹ glutamine, 0.25 mM putrescine, 0.01 mg L⁻¹ indole-3-butyric acid (IBA), and 0.01 mg L⁻¹ 6-benzylaminopurine (BA)]. Two inoculation systems were used to culture the embryos. In one system, immature embryos were excised only from seeds 17, 24 and 30 DAP and directly placed on either E-20A or E-21 media. In the other system (intact seed), immature embryos from seeds 4, 10, 17, 24 and 30 DAP were not removed from the seeds but left to develop, by directly placing the whole seed in the Petri dish with the hilum facing the medium.

Cultures were incubated for 35 days in a growth chamber (Lab-Line Instruments, Inc. Melrose Park, IL) in dark and constant 25±1°C temperature. After the incubation period, plant material was transferred to maturation medium (½ strength E-21 medium supplemented with 0.7% phytagar) and cultured between 2 to 5 more weeks under 100 µmol m⁻² s⁻¹ light and a 16 h photoperiod provided by cool-white fluorescent lamps and constant 25±1°C temperature. Well-developed seedlings (cotyledonary stage) were
Table 5.1 Components of E-20A nutrient medium.

<table>
<thead>
<tr>
<th>Macroelements (mg L⁻¹)</th>
<th>Microelements (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃ 1,075.0</td>
<td>MnSO₄ 11.065</td>
</tr>
<tr>
<td>NH₄NO₃ 619.0</td>
<td>ZnSO₄·7H₂O 1.812</td>
</tr>
<tr>
<td>MgSO₄·7H₂O 206.0</td>
<td>H₃BO₃ 1.575</td>
</tr>
<tr>
<td>CaCl₂·2H₂O 156.5</td>
<td>KI 0.345</td>
</tr>
<tr>
<td>KH₂PO₄ 71.0</td>
<td>Na₂Mo₄·2H₂O 0.094</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O 25.0</td>
<td>CuSO₄·5H₂O 0.008</td>
</tr>
<tr>
<td>NaH₂PO₄·4H₂O 19.0</td>
<td>CoCl₂·6H₂O 0.008</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 17.0</td>
<td>Na₂EDTA 37.3</td>
</tr>
<tr>
<td>KCl 3.5</td>
<td>FeSO₄·7H₂O 27.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organics (mg L⁻¹)</th>
<th>Plant growth regulators (mg L⁻¹) and other supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-Inositol 50.300</td>
<td>Indole-3-acetic acid 0.01</td>
</tr>
<tr>
<td>Pyridoxine-HCl 5.500</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid 0.700</td>
<td>Sucrose (g L) 20</td>
</tr>
<tr>
<td>Thiamine 0.600</td>
<td>Agar (g L) 10</td>
</tr>
<tr>
<td>Ca-d-Pantothenate 0.500</td>
<td>pH 5.9</td>
</tr>
<tr>
<td>d-Biotine 0.005</td>
<td></td>
</tr>
<tr>
<td>Glycine 0.100</td>
<td></td>
</tr>
</tbody>
</table>

(Sauton and Dumas de Vaulx 1987).
transferred to soil (mixture of 70% Terra Lite Plug Mix and 30% coarse vermiculite) in polystyrene flats of cell size 2.25 cm² and 164 cells per flat (Speedling, Bushnell, FL), and grown in a walking-growth chamber (250 µmol m⁻² s⁻¹ light and a 16 h photoperiod provided by cool-white fluorescent lamps and constant 25±1°C temperature) until a 2-3-true-leaf stage was reached. Seedlings were then evaluated for percent regeneration efficiency [(No. of 2-3-true-leaf stage seedlings X 100)/No. of cultured seeds].

5.2.3 Experimental Design and Statistical Analysis

The experimental design was an incomplete factorial (2 x 2 x 2 x 5 or 3) with three replicates, having two levels for genotype, two levels for media, two levels for inoculation system, and five or three levels for harvesting dates. Original data was transformed by the arcsine square root in order to be analyzed for analysis of variance (ANOVA). Means were separated by Tukey’s Studentized Range Test at $P \leq 0.05$ (SAS Institute, Cary, N.C.).

5.3 Results and Discussion

5.3.1 Embryo Development

Seeds were isolated from wild type and transgenic ‘Galia’ male parental line fruits, which were harvested at 4, 10, 17, 24, and 30 DAP. Due to small embryo size at 4 and 10 DAP, it was not possible to dissect the embryos from seeds harvested on that stage, only intact seeds were cultured for those dates (Figure 5.1 and 5.2). Embryos, inside of intact seed at 4 or 10 DAP stage, underwent normal development after being cultured with the hilum facing either E-20A or E-21 media (Figures 5.1 and 5.2). It has been reported that culture of intact ovules, avoids embryo damage during dissection (Monnier, 1976, Thomas, 1976, Momotaz et al., 1998). After one week of culture, embryos from intact seeds 4 DAP became green and elongated forming the torpedo stage of development.
After three more weeks of culture, an immature seedling with apical and radical meristems was observed. These seedlings formed normal plants (2-3-true-leaf stage) after the following steps: one more subculture on either E-20A or E-21 media for two weeks then culture on maturation medium for three weeks and finally transferred to soil. More than 12 weeks were needed to obtain complete wild type and transgenic ‘Galia’ male parental line plants using seeds from 4 DAP fruits.

A similar development pattern was observed when intact seeds from fruits 10 DAP were cultured on E-20A and E-21 media. However, the period to obtain full seedlings was shorter than it was for 4 DAP-intact seeds. After one week of culture on either E-20A or E-21 media, embryos became green and developed primordial cotyledons. Two weeks later, the immature seedling had developed a normal root, bearing root hairs, and had an apical meristem. At this stage, embryos could be transferred to maturation media for further development. Complete muskmelon ‘Galia’ male parental plants were obtained in 9 weeks after seeds were dissected from 10 DAP fruits.

Embryos from fruits 17, 24 and 30 DAP were at different developmental stages and had diverse sizes. Two inoculation systems were applied for these embryos: excising the embryo from the seed and placing it directly on culture media or leaving the immature embryo inside the seed (intact seed) and culturing those seeds with the hilum facing the media. Using both inoculation systems, complete and normal wild type and transgenic ‘Galia’ male parental line plants were obtained after 4-5 weeks of in vitro culture.
5.3.2 Regeneration Efficiency

The regeneration efficiency of wild type and transgenic ‘Galia’ male muskmelon embryos was affected by media, DAP, and inoculation system used (Table 5.2). Greater regeneration efficiency levels were obtained when embryos or intact seeds from both genotypes were cultured on E-21 medium than on E-20A medium (Figure 5.6 and 5.7). These differences were significant (Table 5.3) and observed independently of DAP or inoculation system used; i.e. wild type-30 DAP-intact seed on E-20A had a regeneration efficiency of 50.1%, whereas the regeneration efficiency for wild-type-30 DAP-intact seed on E-21 was more than 90% (Table 5.2).
It has been reported that more developed embryos are easier to rescue than embryos from earlier developmental phases (Liu et al. 1993; Ondrej and Navratilova 2000). Developed embryos may require fewer nutrients and hormones, and a lower osmotic potential to fully develop (Ondrej and Navratilova 2000; Ondrej et al. 2002). A similar
pattern, regarding that older embryos are easier to rescue than younger ones, was observed when embryos and intact seeds were obtained from fruits harvested on different dates (Figure 5.6 and 5.7).

Figure 5.3 ‘Galia’ male parental line fruit at 17 DAP stage (a). *In vitro* embryo development from 17 DAP stage, (b) yellow circle encloses the embryo, (c) inoculated seeds (without dissecting the embryo) with the hilum facing the culture medium, (d) testa removal from seeds after 15 days of *in vitro* culture, (e) seedlings after one week of testa removal.
Indeed, the efficiency of this technique was greater when the time after pollination (4, 10, 17, 24 and 30 DAP) to rescue the embryos was increased (Table 5.4).

Regeneration efficiency between inoculation systems (intact seed and embryo removed) was different (Figure 5.6 and 5.7). Firstly, unlike embryo removed system, using the intact seed method is possible to rescue embryos from earlier stages such as 4 and 10 DAP. Secondly, the greatest amount of plants (90%) was regenerated through the intact seed method (Figure 5.6B). Thirdly, significant differences were observed between both inoculation systems (Table 5.5). Thus, a greater quantity of plants can be obtained in a shorter period of time if the embryo is left inside the seed coat.

It has been reported that plant genotype can have a profound effect on the efficiency of plant regeneration systems, and it is an important factor in establishing cell selection and genetic transformation protocols (Machii et al. 1998; Yamada, 2001; Faure et al. 2002c; El-Itriby et al. 2003). Therefore, plant tissue culture methods which have the
capability to support sustainable growth disregarding plant genotype are considered important tools. Similar regeneration efficiency levels on either media were attained when wild type and transgenic ‘Galia’ male parental line embryos were cultured. Thus, the presence of the transgene did not have any effect on regeneration efficiency values. It might be interesting to test more GMO-melon genotypes on this improved E-21 medium.

Figure 5.5 Normal ‘Galia’ male parental line seedlings obtained from embryo rescue, having well-developed cotyledonary (red arrows) and true leaves (blue arrows). These plants were attained from 4, 10, 17, 24 and 30 DAP stage embryos.
Table 5.2 Regeneration efficiency of ‘Galia’ male parental line immature embryos that underwent development to form normal plants.

<table>
<thead>
<tr>
<th>Media</th>
<th>DAP</th>
<th>Inoculation</th>
<th>Wild type (%)</th>
<th>Transgenic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>system (IS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-20A</td>
<td>4 A</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 A</td>
<td>36.9</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 A</td>
<td>53.9</td>
<td>45.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>29.6</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 A</td>
<td>53.1</td>
<td>54.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>34.5</td>
<td>44.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 A</td>
<td>50.1</td>
<td>51.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>49.7</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td>E-21</td>
<td>4 A</td>
<td>8.8</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 A</td>
<td>48.8</td>
<td>53.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 A</td>
<td>80.6</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>54.1</td>
<td>53.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 A</td>
<td>90.8</td>
<td>90.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>62.1</td>
<td>61.4</td>
<td></td>
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<tr>
<td></td>
<td>30 A</td>
<td>92.3</td>
<td>94.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>66.4</td>
<td>69.3</td>
<td></td>
</tr>
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</table>
**Table 5.2 Continued.**

<table>
<thead>
<tr>
<th>Factorial treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
</tr>
<tr>
<td>* DAP</td>
</tr>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>NS IS</td>
</tr>
<tr>
<td>Media x Genotype</td>
</tr>
<tr>
<td>* Media x DAP</td>
</tr>
<tr>
<td>Media x IS</td>
</tr>
<tr>
<td>* Genotype x DAP</td>
</tr>
<tr>
<td>Genotype x IS</td>
</tr>
<tr>
<td>NS IS DAP</td>
</tr>
<tr>
<td>Media x Genotype x DAP</td>
</tr>
<tr>
<td>Media x Genotype x IS</td>
</tr>
<tr>
<td>Genotype x DAP x IS</td>
</tr>
<tr>
<td>Media x Genotype x DAP x IS</td>
</tr>
</tbody>
</table>

* DAP) days after pollination.
* A) intact seed, B) embryo removed from seed.
* ND) non-determined.
* NS, *Non-significant or significant, respectively, by Tukey’s Studentized Range Test at \( P \leq 0.05 \).

The described improved medium (E-21) and protocol (direct inoculation with the hilum facing the medium) proved to be efficient in regenerating healthy seedlings from wild type and transgenic immature ‘Galia’ male parental line embryos. One novelty of E-21 medium is the amendment with putrescine (polyamine) to rescue embryos from cucurbit seeds (melon). Putrescine has been used in other plant species, such as grape, where adding 2 mM putrescine to media culture was able to significantly increase the percentage of rescued embryos and normal plants (Ponce et al 2002a; 2002b). It is known that polyamines are active regulators of plant growth and have the ability to interact synergistic with plant hormones (Srivastava 2002; Bais and Ravishankar 2002; 2003).
Figure 5.6 Percentage of ‘Galía’ male parental line immature embryos that developed to form normal plants. Complete seeds were directly inoculated with the hilum facing either E-20A (A) or E-21 (B) medium. Lines on bars are SE.
Figure 5.7 Percentage of ‘Galia’ male parental line immature embryos that developed to form normal plants. Immature embryos were dissected and inoculated on either E-20A (A) or E-21 (B) medium. Lines on bars are SE.
Table 5.3 Regeneration efficiency of ‘Galia’ male parental embryos cultured on two different media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Regeneration efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-20A</td>
<td>37.8 b</td>
</tr>
<tr>
<td>E-21</td>
<td>65.3 a&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>z</sup>Mean separation by Tukey’s Studentized Range Test at $P \leq 0.05$.

Table 5.4 Regeneration efficiency of ‘Galia’ male parental embryos depending on harvesting dates.

<table>
<thead>
<tr>
<th>DAP harvesting dates</th>
<th>Regeneration efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5.5d&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>45.2c</td>
</tr>
<tr>
<td>17</td>
<td>55.4b</td>
</tr>
<tr>
<td>24</td>
<td>63.0a</td>
</tr>
<tr>
<td>30</td>
<td>67.3a</td>
</tr>
</tbody>
</table>

<sup>z</sup>Mean separation by Tukey’s Studentized Range Test at $P \leq 0.05$.

Table 5.5 Regeneration efficiency of ‘Galia’ male parental embryos depending on inoculation system (IS).

<table>
<thead>
<tr>
<th>Inoculation system</th>
<th>Regeneration efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact seed</td>
<td>70.3 a&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryo removed</td>
<td>50.4 b</td>
</tr>
</tbody>
</table>

<sup>z</sup>Mean separation by Tukey’s Studentized Range Test at $P \leq 0.05$. 
In cucurbits, several regeneration rates for different zygotic embryo-rescue methods have been reported. Values such as 9% (Fassuliotis and Nelson 1988), 37.3% (Chen et al. 1997; 2002; 2003), 9.1-74% (Metwally et al. 1998a; 1998b), 59.4% (Malepszy et al. 1998), 4.5-39.0% (Mackiewicz et al. 1998), 7.1% (Gémes-Juhász et al. 2002), 27.2% (Ondrej et al. 2002), 28% (Sisko et al. 2003), and 66.7% (de Oliveira et al. 2003) have been described. In *Cucumis melo*, regeneration rates from 0.09% to 76.8% have been obtained (Ezura et al. 1994a; Beharav and Cohen 1995a; 1995b; Lotfi et al. 2003). The regeneration rates (90%) obtained for melon in the present study exceeds significantly those previously reported.

Unlike other embryo rescue methods for cucurbits, some of which require double-layer culture with two or more different media, and several subcultures in order to regenerate healthy and complete plants (Ondrej et al. 2002), this competent embryo-rescue technique for melon, using only one medium culture type, can be applied to save valuable GMO hybrid-melon embryos.

5.4 Summary

In order to obtain a reliable embryo-rescue technique for wild type and transgenic ‘Galia’ muskmelon male parental line, an improved (five new supplements) nutrient medium (named E-21) from the E-20A basic medium (Sauton and Dumax de Vaulx, 1987), an inoculation system (removing the embryo from the seed or intact seed), and the use of different fruit harvesting dates were evaluated. Transgenic muskmelon with the ACC oxidase gene in antisense orientation was obtained using the protocol previously described (Nuñez-Palenius et al. 2005a). Wild type and transgenic muskmelon plants were grown using commercial growing practices that included pruning and training to one vertical stem and the use of soil-less media and drip fertigation. Fruits of wild type
and transgenic (ACC oxidase gene in antisense orientation) ‘Galia’ male parental line were harvested at 4, 10, 17, 24 and 30 days after pollination (DAP). Subsequently, seeds were removed from fruit under sterile conditions. These seeds were used either to dissect the embryos or placed directly with the hilum facing E-20A or E-21 medium. Seedlings from all treatments were transferred to E-21 elongation medium, incubated five weeks, and transferred to soil to evaluate growth. The efficiency of this technique was greater when the time after pollination (4, 10, 17, 24 and 30 DAP) to rescue the embryos was increased. Therefore, 30 DAP was the best time to rescue the embryos. The number of rescued embryos using E-21 medium was greater than E-20A basic medium. We did not find any significant differences in survival efficiency rate between wild type and transgenic embryos. We have obtained a competent embryo-rescue technique for wild type and transgenic ‘Galia’ male parental line, which can be applied to rescue valuable GMO hybrid-melon embryos.
CHAPTER 6
FRUIT RIPENING CHARACTERISTICS IN A TRANSGENIC ‘GALIA’ MALE PARENTAL LINE MUSKMELON (CUCUMIS MELO L. VAR. RETICULATUS SER.)

6.1 Introduction

Melon fruit ripening is a genetically determined event that involves a series of changes in color, texture, firmness, aroma, and flavor, making fruit scent and flavor appealing to consumers (Lelievre et al. 1997; Jiang and Fu, 2000). Ethylene is known to regulate the ripening of climacteric fruits, such as melon (Giovannoni, 2001). This plant hormone is produced substantially and accumulates during the climacteric stage, which coincides with the ripening process in melon fruits (Seymour and McGlasson, 1993; Giovannoni, 2001).

‘Galia’ F₁ hybrid muskmelon (Cucumis melo L.) is a climacteric fruit, having a short storage life, which is limited to two weeks or less, even when it is maintained in low-temperature (8°C) storage (Karchi, 2000).

Since ethylene induces ripening of climacteric fruits, it is a potential target for control of ripening (Ayub et al. 1996; Guis et al. 1997b; Clendennen et al. 1999; Silva et al. 2004). Two regulatory enzymes in the ethylene biosynthesis pathway are ACC synthase (ACS) (EC 4.4.1.14) and ACC oxidase (ACO) (EC 1.4.3.-) (Seymour and McGlasson, 1993). RNA antisense technology has permitted regulating the expression of specific genes involved in tomato fruit ripening (Gray et al. 1992; Fray et al. 1993; Chen et al. 1996b). Targeting ACO via antisense technology has been successfully applied to cantaloupe Charentais (cv. ‘Vedrantes’) melon fruits in order to reduce ethylene production, and as a consequence improve fruit quality by delaying ripening and softening processes (Ayub et al. 1996; Guis et
al. 1997b; Silva et al. 2004). The reduction of ethylene production in these transgenic melon fruits was 97-99%, compared to their wild type counterpart. However, this strong ethylene-production inhibition did not delay, but rather arrested completely the ripening process in those transgenic fruits. Therefore, a continuous ethylene treatment was needed in order to restore fruit ripening, as observed by rind yellowing, fruit softening and activation of the peduncle abscission zone (Ayub et al. 1996; Guis et al. 1997b; Silva et al. 2004). A similar RNA antisense technology targeting ACO (CMACO-1) might be applied to ‘Galia’ male parental line for use of ‘Galia’ F₁ hybrids with improved shelf life.

Two completely diploid independent ACC oxidase antisense transgenic plants of ‘Galia’ inbred male parental line (TGM-AS-1 and TGM-AS-2) were previously obtained (Nunez-Palenius et al. 2005a). Experiments were conducted to compare fruit quality characteristics between transgenic ACC oxidase antisense (TGM-AS), azygous-AS, transgenic GUS (T-GUS) and wild type (WT) fruits from plants grown in greenhouse conditions. Physiological and biochemical fruit parameters, such as weight, length, width, rind color, soluble solids, titratable acidity, pH, flesh thickness, firmness, ripening index, seed cavity size, seed number, ACC oxidase activity in vivo and ethylene production were measured during fruit development, specifically, at zero-, half- and full-slip developmental stages.

6.2 Materials and Methods

6.2.1 Plant Material

‘Galia’ male parental line transgenic plants (Cucumis melo var. reticulatus cv. ‘Krymka’), harboring either an ACC oxidase antisense (CMACO-1) or uidA (GUS) gene, were obtained by a cotyledon-protocol described in Nunez-Palenius et al. (2005). Two independent diploid transgenic lines (TGM-AS-1 and TGM-AS-2) were attained with the
CMACO-1 construct, whereas a single independent diploid transgenic line (T-GUS) was obtained using the GUS gene. *In vitro* non-transgenic-AS ‘escape’ seedlings were obtained after *Agrobacterium tumefaciens*-mediated transformation. These plants were called azygous-AS to emphasize the absence of the ACC oxidase antisense transgene.

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

Plants were cultivated inside of an evaporative-cooled fan and pad glasshouse in Gainesville, FL, 28°C day and 20°C night. Plants were grown in plastic pots (11.3 L) filled with soil-less media (course-grade perlite) following common growing practices recommended by Rodriguez and Cantliffe (2001). Complementary light was supplied by Metalarc™ lamps with a light intensity of 350-530 µmol m⁻² s⁻¹ for 18 hours per day. Training, pruning, and fertigation of plants, as well as application of fungicides, were performed according to those recommended by Rodriguez and Cantliffe (2001). An integrated pest management program (IPM) was used to keep pests under control (http://www.hos.ufl.edu/protectedag/).

Hermaphrodite flowers were self-pollinated by hand and tagged for date of pollination. Only three fruits were kept on each plant. A sample of 10 fruit from wild type, azygous-AS and T-GUS were harvested at 37, 42 and 50 days after pollination (DAP), which corresponded to zero-, half- and full-slip developmental stages, respectively. TGM-AS-1 and
TGM-AS-2 fruits were harvested at 42, 50 and 56 DAP, which corresponded to zero-, half- and full-slip developmental stages, respectively. Fruits harvested at full-slip stage were kept on the vine by pantyhose (Figure 6.1).

6.2.2 Determination of Fruit Size

Harvested fruits were weighed, and measured for length and width. In order to avoid microbial-ethylene production, fruits were gently brushed, washed in tap water (~25°C), dipped into 200 µL L⁻¹ chlorinated water for 60 sec and then air-dried for 12 h at room temperature to pursue subsequent analysis.

6.2.3 Determination of Physical and Biochemical Characteristics

Rind color was measured with a Minolta Chroma Meter (Model CR-200 Minolta Camera Co. Ltd Japan) using the Hue angle (°) parameter (Hurr and Huber, 2005). Due to ‘Galia’ muskmelon fruit does not develop a rind-yellowing uniform pattern, and in order to have a more precise rind color assessment, four equidistant and independent point measurements in the equatorial region of each melon fruit were taken.

Fruit firmness was measured as described by Jeong et al. (2002). Whole unpeeled fruits were tested using an Instron Universal Testing Instrument (Model 4411-C8009, Canton, MA) fitted with a flat-plate probe (31 mm diameter) and 50-kg load cell. After establishing zero force contact between the probe and the equatorial zone of the fruit, the probe was driven with a crosshead speed of 50 mm·min⁻¹. The force was recorded at 2 mm deformation and was performed at two equidistant points on the equatorial zone of each fruit.
Each fruit was cut lengthwise from the stem-scarf towards the blossom end and the flesh size was measured in six different points (A1, A2, B1, B2, C1 and C2) in both halves as showed in Figure 6.2. Length, width and flesh measurements were used to calculate the seed cavity area size by means of ellipse equation (Figure 6.2). The area of the quarter that corresponds to the measurements A1, B1 and C1 is given by:

\[
\frac{1}{4} \text{Seed Cavity Size} = \frac{X_1 Y_2 + X_2 Y_1}{2}
\]

Where \((X_1, Y_1)\) correspond to the coordinates of the point P in Figure 6.2, and \(X_2 = a - A1\) and \(Y_2 = b - C1\) (Figure 6.2).

Seeds were collected, washed and dried according to standard seed-harvesting procedures (Desai et al. 1997). Empty and full seeds were separated and counted for each fruit.
Total soluble solids (TSS), titratable acidity (TA), and pH were determined, in triplicate, from juice obtained after the mesocarpic tissue was macerated, centrifuged (28,000xg), and filtered through Whatman paper No.1. TSS, TA, and pH were quantificated using a digital refractometer (Abbe Mark-10480, Buffalo, N.Y.), a Fisher-395 dispenser connected to an electrometer (Fisher 380), and a digital pH meter (model 340, Corning, N.Y.), respectively. TA was expressed as percent malic acid equivalents. The fruit ripening index was calculated as the quotient between TSS and TA.

*Ethylene evolution and ACC oxidase activity in vivo*

Twelve hours after harvest, fruits were placed in airtight plastic containers (1 fruit per container) (3.7 L) and sealed for 2 h at 25°C. Ethylene production for each fruit was determined by measuring the ethylene concentration in the headspace of the containers. Sampling for ethylene was taken through a serum stopper where 1 mL of headspace was drawn and injected into a gas chromatograph (Hewlett Packard 5890 Series II, Avondale, Pa.) as described in Ciardi et al. (2000). The results were expressed as µL ethylene produced per kg of tissue per hour (µL·kg⁻¹·h⁻¹).

ACC oxidase activity was measured *in vivo* in melon mesocarpic tissue based on the conversion of exogenous ACC to ethylene (Smith et al. 1994; Amor et al. 1998). Samples of mesocarpic tissue were taken in triplicated with a No.5 cork-borer from the equatorial region of the fruit. After removing the peel, 1 g of tissue, was incubated for three hours at 25°C in 3 ml reaction buffer, which contained 50 mM Tris HCl (pH 7), 100 mM sucrose, 250 µM ACC, and 100 µM cycloheximide (Amor et al. 1998). Boiled (95°C for 10 min) mesocarpic tissue and reaction buffer alone were used as negative controls. After the incubation period, a 1-mL
gas sample was taken from the head space and analyzed by gas chromatography (Ciardi et al. 2000). Negative controls always had non-detectable ACC oxidase activity.

6.2.4 Experimental Design and Statistical Analysis

A Completely Randomized Design (CRD) was used to set up the experiment. Original data was evaluated for analysis of variance (ANOVA). Means were separated by Duncan’s Multiple Range Test at $P \leq 0.05$ (SAS Institute, Cary, N.C.).

![Figure 6.2 Flesh size and seed cavity size determination in ‘Galía’ male parental line fruit. Six measurements were made in both halves as indicated by A1, A2, B1, B2, C1 and C2 for flesh size.](image)
6.3 Results

6.3.1 ACC Oxidase Activity In Vivo

When ‘Galia’ male parental mesocarpic tissue was incubated in the presence of reaction buffer mixture, ACC oxidase activity was detected via quantification of ethylene evolution. At zero-slip developmental stage, the enzyme activity in vivo was below 1 nL g⁻¹ h⁻¹ for all genotypes (Figure 6.3). An increase in ACC oxidase activity was observed in WT, azygous-AS and T-GUS mesocarpic tissue at half-slip stage. Conversely, TGM-AS-1 and TGM-AS-2 fruit did not have that increase in ACC oxidase activity at the same developmental stage. Indeed, expression of the ACC oxidase antisense gene in transgenic fruits caused a reduction in ACC oxidase activity, which was four times less compared to WT, azygous-AS and T-GUS fruit at half-slip stage (Figure 6.3). Therefore, significant differences in ACC oxidase activity in vivo were found between ACC oxidase antisense fruit and WT, azygous-AS and T-GUS at half-slip developmental stage (data not shown). At full-slip stage, the lowest level of ACC oxidase activity was detected in TGM-AS-2 mesocarpic tissue, which was significantly different to other genotypes (Figure 6.3).

6.3.2 Ethylene Production

Average ethylene production in WT, TGM-AS-1, TGM-AS-2, azygous-AS and T-GUS fruit harvested at zero-slip stage was 7.6, 7.5, 2, 2, and 1.4 µL kg⁻¹ h⁻¹, respectively (Figure 6.4). On the next developmental stage, half-slip, a substantial increase in ethylene production was observed for WT, azygous-AS and T-GUS fruit, but not for TGM-AS-1 and TGM-AS-2 genotypes (Figure 6.4). Indeed, the ethylene production level for the ACC oxidase antisense lines was below 7 µL kg⁻¹ h⁻¹ at half-slip stage. Therefore, significant differences were observed between ACC oxidase antisense fruit and other treatments at half-slip stage (data not shown).
Figure 6.3 ACC oxidase activity *in vivo* in WT (●), TGM-AS-1 (○), TGM-AS-2 (▼), azygous-AS (∇) and T-GUS (■) fruits. Vertical bars represent standard error of the means (n=10 fruits). Each fruit was assayed by triplicated.

TGM-AS-2 fruit always had a lower ethylene level than TGM-AS-1. A significant (*P*<0.001) regression coefficient (*R*²=0.91) was obtained between ACC oxidase activity and ethylene production data, supporting that both parameters are absolutely related (Figure 6.5).
6.3.3 Firmness, Rind Color and TSS

In order to analyze and compare more easily fruit firmness, rind color and total soluble solids parameters to ethylene production, the firmness data, as well as rind color and total soluble solids data, from WT, azygous-AS and T-GUS genotypes were considered as a single average value at zero-, half- and full-slip stages, and named it as ‘WT’.
Likewise, TGM-AS-1 and TGM-AS-2 data for firmness, as well as rind color and total soluble solids data, were regarded as one single average value at zero-, half- and full-slip stages, and named as ‘TGM-AS’.

Ethylene production data from WT, azygous-AS and T-GUS were averaged to one single value and considered as WT. TGM-AS-1 and TGM-AS-2 ethylene production data were regarded as TGM-AS.

Fruit softening was observed in WT and TGM-AS during the ripening process (Figure 6.6). The maximum decrease in firmness was detected in WT fruit, which had an initial average fruit firmness value of 52 N at zero-slip stage and the final value was below 18 N at full-slip stage. The reduction of ethylene production in TGM-AS fruits induced a delayed
fruit softening process (Figure 6.6). The reduction of firmness in TGM-AS from half-slip stage to full-slip stage was only 28% from WT displayed. At full-slip stage, the TGM-AS firmness value was above 29 N. Therefore, significant differences were observed between WT and TGM-AS fruits at full-slip developmental stage (Figure 6.6).

According to the color-chart (Konica-Minolta, Inc), green color has a higher Hue angle (°) value than yellow. Similarly as most of muskmelon fruits, ‘Galia’ male parental fruit turns from green to yellow rind color during the ripening process. In TGM-AS fruits, the Hue angle (°) value was higher than WT at half- and full-slip developmental stage (Figure 6.7).

Significant differences between WT and TGM-AS treatments were observed at those two stages (data not shown). Despite some ACC oxidase antisense transgenic fruits were last harvested until full-slip stage, they did not reach a full golden-yellow color as WT (Figure 6.8). TSS content of WT and TGM-AS fruits increased along with ripening (Figure 6.9). Therefore, higher TSS contents were found on fruits harvested at full-slip stage. No significant differences on TSS accumulation were detected between WT and TGM-AS fruits at any developmental stage. Although TGM-AS fruits remained a longer time attached to the vine than WT fruits, they did not accumulate more TSS.

6.3.4 Mesocarpic Titratable Acidity, pH and Ripening Index

In contrast to TSS content, which had an increase along ripening, mesocarp TA level had an important decrease, which took place simultaneously with the fruit ripening process (Table 6.1). On zero-slip stage, the lower and greater TA values were observed for WT and T-GUS fruit, respectively, although they were not significantly different. TGM-AS-2 TA value was higher than WT, azygous-AS, T-GUS and TGM-AS-1 at full-slip stage, and it was significantly different (Table 6.1). The regression coefficient ($R^2 = 0.62, y = -0.037x + 0.45$) between TSS and TA had a significant value ($P<0.001$).
Similarly as TSS accumulation pattern, mesocarpic pH values of WT, azygous-AS, T-GUS, TGM-AS-1, and TGM-AS-2 increased concomitantly with fruit ripening (Table 6-1). No significant differences in mesocarpic pH values were found among all treatments at zero-, half- and full-slip stages (Table 6.1). TA and pH were inversely related since they had a significant ($P< 0.001$) regression coefficient and negative slope ($R^2= 0.80$, $y= -7.73x + 7.42$).

Fruit ripening index increased in all treatments from zero-slip to full-slip developmental stages (Table 6.1). In general, TGM-AS-2 fruits had a lower ripening index than WT, azygous-AS, TGM-AS-1, and T-GUS counterpart. Significant differences were
only observed at full-slip developmental stage between TGM-AS-2 fruits and other treatments (Table 6.1).

6.3.5 Determination of Fruit Size and Seed Number

Maximum average fruit weight, length and width of WT fruits occurred at full-slip stage (Table 6.2). These measurements were the same as those from azygous-AS, T-GUS, TGM-AS-1, and TGM-AS-2 counterparts at full-slip developmental stage (Table 6.2). Minimum average fruit weight, length and width were observed at zero-slip stage, the minimum weight parameter was found in WT fruits, whereas minimum length and width were determined in TGM-AS-1 fruits. Similarly as maximum values, there were no significant differences among minimum parameters from different plant genotypes.
Figure 6.8 Rind color in WT (A) and TGM-AS (B) ‘Galia’ male muskmelon fruit.

Figure 6.9 Ethylene production from WT (●) and TGM-AS (○) fruits compared to TSS content of WT (■) and TGM-AS (□) intact fruits. Vertical bars represent standard error of the means (n=30 fruits for WT and n= 20 for TGM-AS).
Table 6.1 Postharvest fruit characteristics of ‘Galia’ male parental line.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Titratable acidity (%)</th>
<th>pH</th>
<th>Ripening index$^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-Slip$^y$</td>
<td>Half-Slip</td>
<td>Full-Slip</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.15</td>
<td>0.106</td>
<td>0.076$^b$</td>
</tr>
<tr>
<td>Azygous-AS</td>
<td>0.19</td>
<td>0.108</td>
<td>0.095$^b$</td>
</tr>
<tr>
<td>T-GUS</td>
<td>0.21</td>
<td>0.099</td>
<td>0.082$^b$</td>
</tr>
<tr>
<td>TGM-AS-1</td>
<td>0.16</td>
<td>0.108</td>
<td>0.095$^b$</td>
</tr>
<tr>
<td>TGM-AS-2</td>
<td>0.17</td>
<td>0.102</td>
<td>0.103$^a$</td>
</tr>
<tr>
<td>Significance$^x$</td>
<td>ns$^y$</td>
<td>ns</td>
<td>*</td>
</tr>
</tbody>
</table>

$^z$Fruit ripening index was calculated as the quotient between total soluble solids (TSS) and titratable acidity (TA).

$^y$Developmental stage.

$^x$Duncan’s Multiple Range Test at $P \leq 0.05$.

$^v$ns= not-significant, * significant.
(Table 6.2). Significant ($P<0.001$) regression coefficients for length-width, weight-width, and weight-length were 0.87 ($y=0.776x+2.6$), 0.56 ($y=0.0034x+8.8$) and 0.60 ($y=0.0042x+8.1817$), respectively.

The minimum value for flesh size on all treatments was recorded in TGM-AS-1 at zero-slip stage, but, it was not significantly different from WT, azygous-AS, T-GUS, and TGM-AS-2 data (Table 6.3). Azygous-AS fruits had the maximum value for flesh size at full-slip stage, although it was not significant different to other treatments (Table 6.3).

The maximum value for seed cavity size was observed in TGM-AS-2 at full-slip stage, whereas minimum seed cavity size was detected in TGM-AS-1 at zero-slip stage. Likewise, as previously evaluated fruit parameters (weight, length, width, and flesh size) there were no significant differences among treatments.

The production of viable and high-vigor seeds within plant breeding programs has a paramount importance. Therefore, full and empty seeds were evaluated on each fruit for all genotypes. The minimum percentage of full seeds (70%) from all treatments was determined in azygous-AS fruits at half-slip stage (Table 6.3). Consequently, the greatest percentage of empty seeds was recorded on those fruits as well. TGM-AS-2 had the highest full seed percentage at half-slip stage. Nevertheless, no significant differences for full seed percentage were observed among treatments on zero- half- and full-slip developmental stages (Table 6.3).

### 6.4 Discussion

Fruit ripening is a genetically determined event, and considered as a highly complex development process (Seymour and McGlasson, 1993). Fruit in general can be classified as either climacteric or non-climacteric on the basis of their respiration pattern
Table 6.2 Postharvest fruit characteristics of ‘Galía’ male parental line.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (Kg)</th>
<th>Width (cm)</th>
<th>Length (cm)</th>
<th>Width (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-Slip</td>
<td>Half-Slip</td>
<td>Full-Slip</td>
<td>0-Slip</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.94</td>
<td>1.12</td>
<td>1.55</td>
<td>12.6</td>
</tr>
<tr>
<td>Azygous-AS</td>
<td>1.05</td>
<td>0.92</td>
<td>1.48</td>
<td>13.1</td>
</tr>
<tr>
<td>T-GUS</td>
<td>1.10</td>
<td>1.21</td>
<td>1.37</td>
<td>12.8</td>
</tr>
<tr>
<td>TGM-AS-1</td>
<td>1.05</td>
<td>1.24</td>
<td>1.39</td>
<td>11.5</td>
</tr>
<tr>
<td>TGM-AS-2</td>
<td>1.21</td>
<td>1.42</td>
<td>1.51</td>
<td>13.9</td>
</tr>
<tr>
<td>Significance</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(^z^\text{Developmental stage.}

\(^y^\text{Duncan’s Multiple Range Test at } P \leq 0.05.

\(^v^\text{not-significant.}
Table 6.3 Postharvest fruit characteristics of ‘Galia’ male parental line.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Flesh size (cm)</th>
<th></th>
<th>Seed cavity size (cm²)</th>
<th></th>
<th>Full seeds (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.0</td>
<td>3.2</td>
<td>3.0</td>
<td>29.2</td>
<td>34.6</td>
<td>46.2</td>
<td>85</td>
<td>85</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Azygous-AS</td>
<td>2.9</td>
<td>2.9</td>
<td>3.6</td>
<td>36.8</td>
<td>25.3</td>
<td>39.1</td>
<td>86</td>
<td>70</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>T-GUS</td>
<td>3.2</td>
<td>2.9</td>
<td>2.9</td>
<td>30.8</td>
<td>28.6</td>
<td>38.1</td>
<td>76</td>
<td>89</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>TGM-AS-1</td>
<td>2.7</td>
<td>3.1</td>
<td>3.0</td>
<td>27.0</td>
<td>34.5</td>
<td>38.1</td>
<td>76</td>
<td>78</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>TGM-AS-2</td>
<td>3.4</td>
<td>3.0</td>
<td>3.0</td>
<td>37.9</td>
<td>31.3</td>
<td>49.0</td>
<td>83</td>
<td>90</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

²Developmental stage.
³Duncan’s Multiple Range Test at $P \leq 0.05$.
⁴not-significant.
and autocatalytic ethylene production peak during ripening (Tucker, 1993). Climacteric fruits, such as most melon varieties, have a respiratory burst and a pronounced autocatalytic ethylene production while the ripening process is proceeding.

The use of ACC oxidase antisense transgenic Charentais melon fruit has allowed discriminating between ethylene-dependent and –independent processes during fruit ripening (Guis et al. 1997b; Guis et al. 1999; Flores et al. 2001a; Flores et al. 2001b; Silva et al. 2004). Indeed, it has been suggested that yellowing of the rind, fruit softening, volatile production, climacteric respiration, and formation of abscission layer in the peduncle are ethylene-dependent events, while sugar accumulation, loss of acidity and pigmentation of the flesh are ethylene-independent processes (Guis et al. 1997b; Guis et al. 1999; Flores et al. 2001a; Flores et al. 2001b). Recently, Silva et al. (2004) suggested that organic acid metabolism in Cantaloupe Charentais melon fruit might not be an ethylene-independent process. However, it can not be excluded that some of those processes involved in melon ripening might encompass ethylene-dependent and –independent mechanisms (Guis et al. 1999), which may be active at the same time. More research, at molecular and physiological level, is needed to address the specific role of ethylene on melon ripening.

The edible part of most melon fruits is the mesocarpic tissue, also commonly known as flesh (Seymour and McGlasson, 1993). Most of the consumers prefer a melon fruit with small seed cavity, and firm, thick and colorful flesh (Yamaguchi, 1977; Artes et al. 1993; Goldman, 2002). Due to the insertion of certain tranregenes, some secondary unwanted and/or deleterious effects on recipient plants have occurred (Birch, 1997; Yoshida and Shinmyo, 2000; Gomez-Lim and Litz, 2004; Halford, 2004). Therefore, in
order to address that possibility, several postharvest quality characteristics on transgenic ACC oxidase antisense ‘Galia’ male parental fruit were evaluated in this study.

The highest ACC oxidase \textit{in vivo} activity was observed in WT, azygous-AS and T-GUS fruits at half-slip stage. Conversely, TGM-AS-1 and TGM-AS-2 did not have an enzyme activity peak at half-slip stage. Smith et al. (1994) reported that the presence of ascorbate in the reaction buffer may have deleterious effect on ACC oxidase activity in ‘Galia’ F\textsubscript{1} hybrid fruit. These authors described that the high initial rate activity declined rapidly after 1 h of incubation if ascorbate was present in the reaction buffer; half-life of 19 min with ascorbate. The ACC oxidase reaction buffer assay utilized in the present study was void of ascorbate in order to circumvent this. Low levels of ethylene production by ACC oxidase antisense melon fruit can be ascribed to a considerable reduction in ACC oxidase activity (Ayub et al. 1996; Guis et al. 1997b; Silva et al. 2004). Certainly, ACC oxidase activity in TGM-AS-1 and TGM-AS-2 fruit was reduced by 4.0- and 186.7-fold, respectively, compared to WT, azygous-AS and T-GUS fruit.

External ethylene production in ‘Galia’ male muskmelon fruit had a similar pattern as mesocarpic ACC oxidase activity during ripening. WT, azygous-AS and T-GUS fruit displayed a rapid increase in ethylene production at half-slip stage. That ethylene peak was not present in any of the TGM-AS lines. Interestingly, ethylene production in TGM-AS-1 fruit was always higher than TGM-AS-2 at any date of harvest. The average ethylene production at zero-, half- and full-slip stages by TGM-AS-1 and TGM-AS-2 was 7.71 µl kg\textsuperscript{-1} h\textsuperscript{-1} and 0.86 µl kg\textsuperscript{-1} h\textsuperscript{-1}, respectively. These differences in ethylene production are not related to the number of T-DNA copies inserted into the melon genome, because both transgenic lines had a single copy of the ACC oxidase antisense
transgene (Nunez-Palenius et al. 2005a). Rather, T-DNA insertion position effects on transgene expression might have played some role in that different ethylene production. T-DNA position effects have been reported in other transgenic plants (Deblock 1993; Zhu et al. 1999; Cotsaftis et al. 2002).

It has been reported that transgenic ACC oxidase antisense Charentais melon fruit attached to the vine, exhibited very low internal ethylene levels: <0.5 µL⁻¹ (Guis et al. 1997b) and ≤0.09 µL⁻¹ (Silva et al. 2004). However, after harvesting at 38 DAP, the internal ethylene content increased up to 4 µL⁻¹ (Guis et al. 1997b). These authors described that detachment of the fruit from the vine induces an increase on fruit ethylene production (Guis et al. 1997; Bower et al. 2002). The value reported by Guis et al. (1997b) on internal ethylene content in detached fruit is similar to the external ethylene production level by TGM-AS-1 fruit separated from the vine. Even though TGM-AS-2 fruits were removed from the plant, ethylene production was 99.9% less than WT, azygous-AS and T-GUS fruit at half-slip stage. In summary, the insertion of CMACO-1 gene in antisense orientation lead to reduced ethylene production in the ‘Galia’ male parental fruit. The average extent of reduction compared to WT, azygous-AS, and a T-GUS fruit was 76.9% and 99.1% in TGM-AS1 and TGM-AS-2, respectively.

Fruit softening observed during ripening is associated with textural changes that are thought to result from modification and disassembly of the primary cell wall (Fischer and Bennett, 1991). Fruit softening and the underlying cell wall structural changes are complex. Softening or loss of firmness of the edible mesocarp of melon fruit may start in the middle (around 30–45 days after anthesis, depending on cultivar) of the development
cycle, along with other typical changes connected with the ripening process (Lester and Dunlap, 1985).

Among the different plant hormones which are involved in melon fruit development, ethylene might have the main role during melon fruit softening, because this gaseous plant hormone regulates the expression of several proteins involved in the softening process (Rose et al., 1997, 1998). According to Guis et al. (1997), Guis et al. (1999), Flores et al. (2001a), Flores et al. (2001b), and Silva et al. (2004), fruit softening in melon is an ethylene-dependent event. Their ACC oxidase antisense transgenic Cantaloupe Charentais fruits had a delayed fruit softening process, compared to wild type counterpart. A similar response was observed in ACC oxidase antisense ‘Galia’ male parental fruits (Figure 6.6). These fruits retained a higher firmness than WT, azygous-AS and T-GUS fruits, mainly at full-slip developmental stage, when significant differences were detected.

Yellowing of the rind in ‘Galia F₁ hybrid fruit is induced by increased chlorophyll degradation during the ripening process, as well as a partial decrease in total carotenoids (Flügel and Gross 1982). Likewise, chlorophyll is degraded by the action of chlorophyllase (EC 3.1.1.14), whose de novo synthesis is ethylene regulated (Jacob-Wilk et al. 1999). Rind color in WT, azygous-AS, and T-GUS fruits, turned from green to golden-yellow during ripening, whilst, ACC oxidase antisense fruit did not reach a full golden-yellow color (Figure 6.8). It seems that lower ethylene production in CMACO-1 proves this assumption correct. The antisense transgenic ‘Galia’ male fruits did not allow complete chlorophyll degradation, leaving partial green-patches on the rind. Analogous results were reported in ACC oxidase antisense transgenic Cantaloupe Charentais melon
fruits (Guis et al. 1997b; Martinez-Madrid et al. 1999; Flores et al. 2001a; Flores et al. 2001b; Martinez-Madrid et al. 2002; Silva et al. 2004).

Sweetness is one of the most important edible quality attribute of ripe melon fruits (Yamaguchi, et al., 1977; Lester and Shellie, 1992; Artes et al. 1993). The trait for sugar accumulation in melon is controlled by a single recessive gene, called suc (Burger et al. 2002). Therefore, sucrose accumulation is controlled through several plant hormones such as auxins and ABA, enzymes such as sucrose phosphate synthase (EC 2.4.1.14) and acid invertase (EC 3.2.1.26), and compartmentation processes, as well as is directly related to fruit ripening (McCollum et al. 1988; Hubbard et al. 1989; Ofosu-Anim and Yamaki, 1994; Lee et al. 1997; Ofosu-Anim et al. 1998; Gao et al. 1999; Gao and Schaffer, 1999; Feusi et al. 1999; Carmi et al. 2003; Volk et al. 2003). The TSS content of WT, azygous-AS, T-GUS, TGM-AS-1 and TGM-AS-2 mesocarp increased in average from 7.69% to 9.89% at zero- and full-slip stage, respectively. Thus, TSS accumulation was a simultaneous event to fruit ripening and an ethylene-independent phenomenon. Similar results have been observed in most of sweet-type melons (McCollum et al. 1988; Seymour and McGlasson, 1993; Guis et al. 1997b; Burger et al. 2002; Burger et al. 2003; Silva et al. 2004; Liu et al. 2004; Ergun et al. 2005)

Although TGM-AS-1 and TGM-AS-2 remained for a longer time attached to the vine than WT, azygous-AS and T-GUS fruits, they did not accumulate more sugars. These results on TSS content for ACC oxidase antisense transgenic fruit are similar to results reported by Guis et al. (1997b), but dissimilar from others (Flores et al. 2001b; Martinez-Madrid et al. 2002; Silva et al. 2004). Guis et al. (1997b) claimed that transgenic (ACC oxidase antisense) Cantaloupe Charentais melon fruit remained attached
to the vine for 10 more days than its wild type counterpart, allowing the accumulation of more sugars, and increasing the soluble solids content from ∼11.3 (wild type) to ∼12 °Brix (antisense). However, differences between wild type and transgenic Cantaloupe Charentais melon TSS values were not significant, since their standard errors overlap (see Figure 3A in Guis et al. 1997b). Flores et al. (2001b) and Martinez-Madrid et al. (2002) studied biochemical and physiological fruit characteristics of some transgenic Cantaloupe melon plants obtained from the Guis’ (1997b) T₀ original line ‘B17’. These authors found that ACC oxidase antisense fruits accumulated significantly more sugars than wild type fruits at 40 DAP, but not at 15, 24, 35 and 37 DAP. Silva et al. (2004) developed transgenic (ACC oxidase antisense from apple) Cantaloupe Charentais melon which were not ripe on 38 DAA, whereas WT fruit were ripe at ∼30-32 DAA. The authors concluded that as transgenic fruit remain attached ∼5-8 more days, they were able to accumulate more TSS. Unlike Guis’ study, transgenic fruit TSS values were significantly different from wild type fruit counterpart.

The differences on TSS accumulation between transgenic ACC oxidase antisense ‘Galia’ male parental muskmelon and transgenic ACC oxidase antisense Cantaloupe Charentais cv. ‘Vedrantais’ fruits (Flores et al. 2001b; Martinez-Madrid et al. 2002; Silva et al. 2004) can be ascribed to the fact that our transgenic melons belonged to variety reticulatus, whereas transgenic melon fruits obtained by those authors correspond to variety cantaloupensis. As it has been reported, genetic background has a profound effect on melon fruit sugar accumulation (Seymour and McGlasson, 1993; Stepansky et al. 1999; Burger et al. 2002; Burger et al. 2003; Liu et al. 2004). Moreover, different plant growing environmental conditions might have played a role on fruit-TSS buildup.
Sugar accumulation in melon fruit is affected quantitatively by environmental and physiological factors, such as salinity, nutrient availability, shading, cellular size in the fruit, and available foliar area (Hubbard et al. 1990; del Amor et al. 1999; Nishizawa et al. 2000; 2002; 2004; Kano, 2002; 2004). ACC oxidase antisense gene insertion did not reduce or increase the fruit TSS content in any of CMACO-1 transgenic lines at fruit maturity.

Unlike other ripe fruits, such as strawberry, pineapple and apricot, where high organic acids content have an imperative role in developing acceptable taste for consumers, sweet melons have a low organic acid content. Nevertheless, sensory quality of melon fruit is also determined by organic acid levels (Sweeney et al. 1970; Yamaguchi et al. 1977). In ‘Galia’ male parental line, TA and pH appear to be inversely related. While TS in all treatments had a sharp decrease since the first date of harvest, pH values started to increase steadily. Although, differences in TA and pH among all treatments were observed, they were not significant. Similar results were obtained by Guis et al. (1997b) and Martinez-Madrid et al. (2002). These authors reported that TA had a sharp decrease in WT Cantaloupe Charentais melon fruit during ripening, and a similar trend was observed in transgenic ACC oxidase antisense fruit.

Fruit ripening index calculation is commonly used as a factor to measure fruit sensory quality, and is obtained as the quotient between the TSS content and the TA level (Leshem et al. 1986). A similar ripening index was observed in WT, azygous-AS, T-GUS, TGM-AS-1 and TGM-AS-2 fruits on all fruit development stages, except at full-slip stage where significant differences were found between TGM-AS-2 and other treatments. It has been suggested by Silva et al. (2004) that organic acid metabolism in
melon fruit is an ethylene-dependent process. Likewise, Flores et al. (2001b) and Martinez-Madrid et al. (2002) reported that transgenic (ACC oxidase antisense) had a greater ripening index than wild type Cantaloupe Charentais melon fruit. Considering that TGM-AS-2 genotype had the lowest ethylene production level among treatments, it is possible that this low-ethylene level in TGM-AS-2 has contributed to attain a high TA value at full-slip stage, consequently, the lowest fruit ripening index level at full-slip stage was observed for TGM-AS-2.

Although differences in weight, length, width, flesh size and cavity seed size were found among WT, TGM-AS-1, TGM-AS-2, azygous-AS and T-GUS fruits, they were not significantly different. Moreover, comparable variation in fruit weight and size has been reported in other muskmelon cultivars (Kultur et al. 2001; Nerson et al. 2002; Liu et al. 2004). Overall, the CMACO-1 gene single insertion in antisense orientation did not have either a positive or negative effect on fruit size of ‘Galia’ male parental line. Because there were not significant differences in total number of seeds, as well as number of full seeds among treatments, it is clear that CMACO-1 gene single insertion did not have any effect on seed development in ‘Galia’ male parental line fruit.

In summary, the results of these experiments describe that the insertion of ACC oxidase gene in antisense orientation in ‘Galia’ male parental inbred reduced the ethylene synthesis in TGM-AS T₀ fruits. That inhibition was more evident in TGM-AS-2 than TGM-AS-1 line. Reduced ethylene production may be a direct consequence of a low ACC oxidase activity detected in those fruits. As a result of low ethylene production by TGM-AS fruits, several parameters such as, yellowing of the rind, ripening index, and fruit softening were delayed. Other traits, such as fruit size, seed development, and
mesocarpic total soluble solids, titratable acidity and pH were not affected by the transgene presence. TGM-AS-1 and TGM-AS-2 T1 seeds, obtained from these T0 evaluated fruits, will be used to obtain and select future improved lines with extended shelf-life.

6.5 Summary

‘Galia’ is a high-quality muskmelon cultivar that is grown in greenhouses or tunnels to maximize fruit quality and yield. Maximum fruit quality and flavor are achieved when ‘Galia’ fruits are harvested at ¾ to full-slip stage. One disadvantage of ‘Galia’ is its storage life, which is limited to two to three weeks due to rapid fruit softening. *In vitro* regeneration and transformation of ‘Galia’ melon parental lines with antisense technology, targeting enzymes involved in ethylene biosynthesis pathway, is a feasible strategy that can be used to increase its fruit shelf-life. The male parental line of ‘Galia’ muskmelon has been transformed with two different constructs according to the protocol described by Nunez-Palenius et al. (2005a): one plasmid was bearing the GUS gene and another had an ACC oxidase gene (CMACO-1) in antisense orientation (Nunez-Palenius et al. 2005a). Transgenic ACC oxidase antisense (TGM-AS), azygous-AS (PCR negative), transgenic GUS (T-GUS) and wild type (WT) fruits, from plants grown in the greenhouse, were harvested at zero-, half-, and full-slip developmental stage. Wild type, azygous-AS and transgenic (both TGM-AS and T-GUS) weight, length, width, soluble solids, pH, flesh thickness, seed cavity size, and full seed percentage parameters were not significantly different on all fruit developmental stages. Fruit firmness from full-slip TGM-AS was almost twice than wild type, azygous-AS and T-GUS. Ethylene production and ACC oxidase from half-slip wild type, azygous-AS and T-GUS fruits were greater.
than from TGM-AS fruits. TGM-AS ‘Galia’ male parental melon fruits had a delayed fruit ripening process compared to wild type fruits.
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BIOGRAPHICAL SKETCH

Hector Gordon Nuñez-Palenius was born on January 19, 1962, in Guadalajara, Jalisco state, México. He got his Master of Science degree in December, 1997 in plant biology from the Genetic Engineering Department at the Unit of Biotechnology and Plant Genetic Engineering in the Center of Research and Advanced Studies (CINVESTAV-IPN. Unidad Irapuato). Afterwards, Hector G. Nuñez-Palenius started a Ph.D. program at the University of Florida, Horticultural Sciences Department, Seed Physiology Lab. His research focused on transformation of ‘Galia’ muskmelon to improve fruit quality, specifically on in vitro regeneration and Agrobacterium-mediated transformation of ‘Galia’ parental lines. His future plans are to continue working in the area of biotechnology of cucurbits, with special emphasis on genetic improvement.