DEVELOPMENT AND UTILIZATION OF GENOMIC TOOLS TO IDENTIFY CANDIDATE GENES FOR MELON (CUCUMIS MELO) FRUIT QUALITY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2009

DEVELOPMENT AND UTILIZATION OF GENOMIC TOOLS TO IDENTIFY CANDIDATE GENES FOR MELON (CUCUMIS MELO) FRUIT QUALITY

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Cornell University 2009

Melon species include a wide variety of cultivars producing fruits differing in many fruit traits. Great efforts have been made trying to understand the genetic mechanisms underlying these traits, but, without genomic sequences, research in this area is limited.

In this experiment, a total of 3269 unigenes have been developed, adding in about 30-fold gene sequence to the current melon ESTs collection. In addition, a melon validated cDNA microarray has also been generated and was used to a transcriptome comparison of fruit maturation in a climacteric (Dulce) and non-climacteric (Rochet) melon, which display diversity in multiple traits of interest including flesh color, aroma volatile production, sugar content and softening process. Our goal was, first, to introduce to the melon research community new genomic tools and resources including ESTs and microarrays, to provide public genomics infrastructure to assist research at the molecular level for species of the Cucurbitaceae family. Second, to shed light on molecular mechanisms that underlie ripening while simultaneously increasing the reservoir of ripening related genes for melon species.

By focusing our analysis on the expression patterns of genes that may participate in biological pathways related to the fruit quality traits, we were able to identify specific differences that were consistent with the variable fruit traits between these two varieties and including fruit softening, aroma, flavor and carotenoids

biosynthesis. Our results suggest that the quick softening phenotype of Dulce during ripening was mainly caused by the concomitant up regulation of isoforms of genes involved in cell wall degradation including *PGs*, *GALs*, *XTHs*, *EXPs* and PME. Multiple regulatory mechanisms may contribute to the orange color (beta-carotene) of Dulce flesh but their gene targets are clear in that transcriptional regulation of *DXR* and *PDS* appears to be highly consistent with the carotenoid accumulation profiles of Dulce versus Rochet. Aroma variation between Dulce and Rochet is likely due to reduced transcription and enzyme activity of AAT.

BIOGRAPHICAL SKETCH

Yang Liu graduated from Medical School, Tongji University at Shanghai in China. Before she came to the United States, she was a lecture in Department of Immunology and Microbiology at Tongji University. She joined the Giovannoni lab as a technician for two years and then became a graduate student in Department of Horticulture at Cornell University.

I would like to dedicate this dissertation to my family, who has supported me throughout this time.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. James J. Giovannoni for providing me with the opportunity to work in his lab and for all his support, advice and encouragement for my research. I am extremely thankful for his guidance, help and patience throughout the course of my thesis work. I would like to extend my sincere thanks to my other committee members, Dr.Susheng Gan and Dr.Geza Hrazdina for their helpful input and suggestions for my research.

I am very grateful to all members of the Katzir laboratory at Israel for generating the cDNA libraries, providing mRNA samples and all the genetic physiological data for my research. Thanks to Dr. Shmulik Wolf and Dr.Arthur Schaffer for generous contributing their gene and EST collections.

I would also like to express my thanks to all the members past and present of the Giovannoni lab: Dr. Zhangjun Fei and his wife Xuemei Tang for making the Melon EST Database and their help in statistical analysis. Dr. Rob Alba for his guidance in my microarray experiment as well as his assistance in trouble-shooting. Dr. Julia Vrebalov for her kindly suggestions in several aspects of my research. Ruth White for her always patient assisting when I need and Ryan McQuinn for his help with qRT-PCR experiment. Thanks to Dr. Miyoung Chung for her friendship and support. She always encourages me and cheers me up when I am depressed.

Special thanks to my husband, Xu Peng and my lovely daughter Jasmine Miao Peng. Thanks for their love, understanding and endless support throughout my study at Cornell. Without this, I would be impossible for me to finish all my projects in time.

Finally, I would like to acknowledge the BARD grant for the financial support of my research.

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CHAPTER I

INTRODUCTION

I. Fruit Ripening: An Overview

Fruits are important agriculture commodities. Fleshy fruits have long been an indispensable component in the daily diets of humans. Fruit provide not only attractive color, pleasant flavors and aromas to our diets but also many essential nutrients and dietary constituents that are important for human health, including vitamins, minerals, fiber and antioxidants that can prevent chronic and debilitating diseases. In addition to their roles in our diets, fruit are unique organs specific to the plant kingdom. Fruit represent the last step of floral development and are ultimately responsible for seed dispersal. Fruit thus have long received considerable attention from scientific researchers attempting to understand the molecular mechanism underlying and in order to optimize the systems associated with fruit development, quality and post-harvest manipulation (Giovannoni, 2001; Giovannoni, 2004; Goff and Klee, 2006).

Fruit ripening is a genetically programmed and environmentaly influenced process that is regulated by many endogenous and exogenous factors including transcription factors, hormones, light and temperature. While fruit species are phenotypically variable and the specific process leading to ripening phenomena in different species may represent distinct pathways, during ripening most fleshy fruits undergo physiological and biochemical modifications that fall into similar categories and include pigment biosynthesis, flavor and aroma development, sugar accumulation,

textural alteration, cell wall degradation and a general propensity to become susceptible to opportunistic pathogens (Giovannoni, 2007). The genetically and environmentally regulated coordination of these changes renders fruit attractive and desirable for consumption by both human beings and seed dispersing organisms.

I-1. Classically Defined Ripening Physiology

Based on ripening physiology, fruits can be classified into two major groups, the climacteric and non-climacteric fruits (Biale, 1960). The climacteric fruits, such as tomato, most stone fruits, apple and banana, exhibit a concurrent upsurge in ethylene production and respiration rate at the onset of fruit ripening. In addition, ethylene typically plays an indispensable role in regulating the ripening of fruits in this group. In contrast, neither ethylene induction nor a notable respiration rise is observed in fruits that belong to the non-climacteric classification. They are also apparently capable of ripening in the absence of induced ethylene. Economically important fruits that fall into this group include strawberry, cherry, citrus and grapes.

Ethylene is a gaseous hormone that plays important roles in plant development, stress response, seed germination, root growth, flower senescence, wounding and fruit ripening. Dividing fruits into climacteric and non-climacteric categories is useful for practical purposes including and especially for assigning post-harvest handling strategies and practices. In this regard, the ripening process of climacteric fruit is controlled and manipulated by regulating ethylene or ethylene signal transduction to facilitate post-harvest storage, transportation and thus reduce economic loss (Cardarelli et al., 2002; Fabi et al., 2007; Martinez-Romero et al., 2007).

I-2. Ethylene and Fruit Ripening

Molecular research on fruit ripening has been focused primarily on climacteric fruits and on ethylene synthesis (and more recently ethylene response) using tomato as the primary model system. Much of the work defining the important steps in ethylene biosynthesis and leading to the definition of the Yang cycle was performed in apple (Adams and Yang, 1979) with molecular demonstration of the role of the ethylene biosynthesis genes ACC synthase and ACC oxidase first elucidated in tomato (Blume and Grierson, 1997; Hamilton et al., 1991; Lincoln et al., 1993; Rottmann et al., 1991). Pioneering work on ethylene signaling pathways was first established in the model plant system Arabidopsis (Bleecker et al., 1998; Chang et al., 1993; Guo and Ecker, 2003; Hua and Meyerowitz, 1998) and was demonstrated to be very conserved among plant species including tomato (Adams-Phillips et al., 2004; Klee and Tieman, 2002; Wilkinson et al., 1997; Yen et al., 1995). During the last decade, considerable progress has been made in characterizing the regulatory mechanism of ethylene during fruit ripening which has led to an emerging blueprint of the conserved ethylene regulatory network and its function during ripening in tomato and other climacteric fruit (Adams-Phillips et al., 2004; Wang et al., 2002).

I-3. Molecular Regulation of Ethylene and Ripening Responses

Considerable research points to the fact that ethylene controls the ripening process of climacteric fruits in large part by regulating the expression of genes associated with fruit developmental changes that contribute to ripening phenomena including genes involved in cell wall degradation, carotenoid biosynthesis, aroma

volatile metabolism and ethylene biosynthesis itself (Barry and Giovannoni, 2007; Giovannoni, 2004). While ethylene is important for this process in climacteric fruits, further analysis of several tomato pleiotropic ripening mutations including the non-ripening single locus *ripening-inhibitor (rin), non-ripening (nor) and Colorless non-ripening (Cnr)* mutants has provided new insights toward understanding the mechanisms of overall ripening control beyond ethylene. In all of these three non-allelic mutants, all measured ripening phenomena were inhibited or completely arrested including softening, color change, flavor development and climacteric ethylene evolution. In addition and of special interest, while ethylene production was inhibited, exogenouse ethylene failed to restore the ripening process of any these mutants even though the ethylene signaling pathway was verified to be functional (Giovannoni, 2004; Giovannoni, 2007; Thompson et al., 1999; Vrebalov et al., 2002). These results support the existence of an ethylene-independent regulatory network for the regulation of ripening. Such a pathway might be further anticipated to play a role in ripening of non-climacteric fruits.

The *RIN*, *NOR* and *CNR* genes have been cloned (Manning et al., 2006; Vrebalov et al., 2002) (Giovannoni JJ. unpublished data). *RIN* encodes a ripening-specific MADS-box transcription factor which was localized to the nucleus and only accumulates in ripe tomato fruit. RIN protein formed a stable homedimer after translation and further binds to CArG box DNA sequences typically recognized by MADS-box transcription factors (Ito et al., 2008). While a prior study showed that the expression of *LeACS4* was totally *RIN*-dependent (Barry et al., 2000), no typical CArG motif was found in the *LeACS4* gene promoter suggesting that multiple recognition sites may be targeted by the RIN protein or that *LeACS4* is not a direct responder to *RIN*. While other ripening genes (*LeACS2, LeACO1* and the *Nr* ethylene

receptor) that contained the CArG sequence were identified, only *LeACS2* bound RIN with a high affinity in a CHiP assay, suggesting that RIN may initiate climacteric ethylene biosynthesis by directly inducing transcription of *LeACS2* and indirectly regulating the expression of *LeACS4* and additional ripening genes.

The *Cnr* locus encodes a SBP-box (SQUAMOSA promoter binding protein-like) transcription factor whose homologs have been characterized by their ability to bind a sequence motif in the promoter of *SQUAMOSA* (*SQUA*) family genes. SQUA is a floral meristem identity regulatory transcription factor that belongs to the MADS-box gene family (Huijser et al., 1992). The *Cnr* phenotype was shown to result from an epigenetic mutation resulting in high level methylation of the *CNR* gene promoter leading to the silencing of said gene in the *Cnr* mutant (Manning et al., 2006). Recent analysis indicates that the SBP-box protein binds to DNAs containing the core consensus motif GTAC (Birkenbihl et al., 2005; Cardon et al., 1999; Liang et al., 2008).

The NOR protein has also been cloned by other members of our lab and was also shown to be a transcription factor that in this case belongs to the plant-specific NAC family of regulatory proteins (Vrebalov J and Giovannoni JJ. unpublished data). NAC proteins represent one of the largest families of transcription factors in the Arabidopsis genome and have been associated with numerous biological phenomena including meristem and cotyledon development, disease resistance, and senescence, though until now they have not been associated specifically with fruit ripening. Nevertheless, senescence is considered to be a component of or the final phase of fruit ripening and in this regard it is noteworthy that a number of important senescence genes have been recently described. One of the first genes to be isolated from this

family was the tomato SENU5 gene which was identified from a differential screen of senescent versus healthy leaves (John et al., 1997). The role of NAC genes in leaf senescence was definitively shown in Arabidopsis through mutation of the AtNAP gene (Guo and Gan, 2006). More recently an important regulator of wheat senescence attributable to an important domestication trait of this crop was described (reference needed).

The specific regulatory mechanisms of *RIN*, *NOR* and *CNR* remain to be fully characterized but the common fact that they are all transcription factors substantiates the important role of transcriptional regulation of fruit ripening. It has been suggested that the regulatory system represented by *RIN*, *NOR* and *CNR* serves as a primary control over the overall ripening processes in climacteric fruits (Adams-Phillips et al., 2004; Giovannoni, 2001; Giovannoni, 2004). On one hand, they are necessary for the induction of the ethylene-dependent pathway for fruit ripening through their necessity for the induction of rate limiting ethylene biosynthesis genes and on the other hand they also regulate an ethylene-independent pathway that is a clear and necessary component of ripening control. Attempts to define regulatory hierarchy based on expression of these genes in the three respective mutants suggest that *RIN* is likely to operate upstream of the *CNR* gene (Cara B. *et al*. 2008) and *NOR* may regulate *RIN* (J. Vrebalov and J. Giovannoni, personal communication). It is interesting also to note that a RIN gene which was isolated in strawberry based on homology to tomato *LeMADS-RIN* is expressed during strawberry ripening and thus may represent a conserved ripening function among climacteric and non-climacteric species (Vrebalov et al., 2002). This gene has been repressed in strawberry fruit using RNAi technology in a collaboration between our lab and Warwick University (UK) and a delayed ripening phenotype was observed (G. Seymour, personal

communication). A similar experiment has also been performed in the climacteric *Cucumis melo* (cantaloupe) and a similar result in terms of ripening inhibition was observed (M. Binzel, personal communication). These results together indicated that regulation of ethylene-dependent ripening pathways are conserved among climacteric fruit species and furthermore, that common regulation of ethylene-independent regulatory mechanisms are likely conserved between climacteric and non-climacteric species (Giovannoni, 2004; Vrebalov et al., 2002).

I-4. New Tools for Investigating Ripening Control

In recent years, research on fruit ripening has focused more on mechanisms up stream of ethylene, attempting to understand the basis of initiation of ethylene biosynthesis and overall regulation of both ethylene-dependent and –independent ripening control. Years of molecular and genetic investigation have resulted in abundant resources for molecular analysis of ripening in many species and especially in the model fruit species, tomato. In addition to thousands molecular markers for genetic mapping, a high-density genetic map and high-molecular weight insert genomic libraries, the recent addition of an international genome sequencing project (www.sgn.cornell.edu) provides even more potential for this system. To date, more than150,000 tomato ESTs, derived from 27 cDNA libraries covering more than eight tissue types have been developed and are publicly available (Fei et al., 2006). Over 40,000 of those ESTs were derived from fruits representing a range of different developmental stages. These ESTs collections have been used to identify candidate ripening regulatory genes (Fei et al., 2004) as well as numerous additional genes that may be related to specific ripening associated pathways. For example, to date, 13 ESTs were identified as MADS-box genes that are expressed in ripening fruit. Four of

them are expressed at high levels in ripening fruit (Giovannoni, 2007). RNAi technology is currently being applied to study the biological function of these candidates in transgenic plants in our laboratory. In addition, using the consensus DNA-binding sequence for the SBP-box protein, hundreds of candidate target genes have been identified for this regulator (Liang et al., 2008). Additional expression studies and other experiments, such as chromatin immunoprecipitation (ChIP), will be required to verify the targets of CNR, RIN and NOR. Interestingly, microRNA (micRNA) complementary sequences have been found in some SBP genes suggesting a role for micRNAs in regulating the expression of SBP genes and possibly ripening (Xie et al., 2006). Arabidopsis *AtSPL3*, an orthologue of *LeCNR,* contains the miRNA 156 binding site in its 3'UTR and this micRNA negatively regulates the expression of *AtSPL3* (Gandikota et al., 2007; Wu and Poethig, 2006). The regulatory role of microRNA156 on *LeCNR* is currently under study and may point to the involvement of miRNAs in controlling the expression of key regulators of fruit ripening.

Biological pathways and related metabolic analysis impacting fruit quality and nutritional content have also been a recent focus of fruit molecular biologists interested in improving fruit quality traits such as carotenoid composition and flavor attributes. 76 wild species introgression lines (ILs) of tomato have been developed by Liu and Zamir (1999) resulting in 2795 QTLs associated with fruit quality traits including shape, weight, yield and color (Lippman et al., 2007). Candidate genes resulting from the EST collections and the genome sequencing project can now be associated with these traits to assess potential as candidate genes. In addition, transcriptome comparisons between fruit of the IL lines altered in carotenoid compounds or other quality traits has been used to identify genes whose expression may be altered in association with said QTLs and may point to additional candidate

genes. Finally, both cDNA and oligo microarrays are available for tomato based on existing tomato sequence resources. The tomato cDNA array contains > 13,000 fragments representing 8700 unigenes and has been used to characterize transcriptome activity during fruit ripening (Alba et al., 2005). A database contains integrated metabolite and gene expression date has also been generated (http://ted.bti.cornell.edu). These resources will facilitate investigations into fruit development and ripening control in tomato that will generate hypotheses to be tested for conservation or deviation in other important fruit crop species such as melon.

II. Melon fruit ripening

Melon belongs to the Cucurbitaceae family. Other important crops in this family include watermelon, cucumber, pumpkin and squash. Melon (*Cucumis melo*) is one of the most intensively studied species in terms of fruit ripening and is becoming an increasingly important economic fruit crop. During 2003-2005, the average global production of melon reached 60.4 billion pounds, ranking at the 16th position and sharing 1.3 % of the world production of fruits and vegetables (FAOStat database, 05/2006). The United States is one of the leading producers in the output of melons (cantaloupe and miscellaneous melons) world wide, only behind China and Turkey, which yields an average farm value about 495 million dollars per year (Vegetables and Melons Outlook/VGS-279/June 20,2003). In addition, melon is also one of American's favorite fruits for dessert and salad uses because of its unique flavor. The average per capita consumption of melon in the U.S. has been increasing consecutively each decade since the 1960s. During the past few years (2000-2006), the estimated average U.S. per capital consumption exceeded 12 pounds per year, an 8 %

rise from 1990-1999 (Vegetables and Melons outlook/VGS-320/April 19,2007. Economic Research Service, USDA).

Compared with other major crops in the cucurbit, family *C.melo* is one of the species producing fruits with the greatest amount of genetic and morphological diversity. Melon includes a wide variety of cultivars producing fruit deferring in many traits including fruit shape (round, flat, elongated), size (large: 15kg to small:50g), flesh color (orange, light green, white), sweetness (higher sugar content or low), aroma volatiles and fruit texture (Nunez-Palenius et al., 2008). In addition, melon fruit also have significant variation in ripening behavior. Melon fruits can be categorized as either climacteric or non-climacteric types based on their ripening related respiration rate and ethylene evolution profiles. Usually fruit in *C.melo var.cantalupensis* Naud and *C.melo var.reticulatus* Naud are considered as climacteric types, and include Cantaloup, Vedrantaise (VED), Noy Yizre'el (NY) and Dulce (DUL) cultivars that are focused on in this study. Fruits from *C.melo var.inodorus* Naud are generally classified as non-climacteric types and include for example the Tam Dew (TD) and Rochet (ROC) cultivars. In addition to ripening physiology, climacteric and non-climacteric melons also differ in additional phenotypes. Most of the climacteric melons have orange flesh, high aroma and quick softening upon ripening, the latter leading to difficulties in post-harvest handling. While non-climacteric melons usually display pale-green flesh, low level of aroma and slow softening resulting in typically longer shelf life than climacteric varieties. It is interesting to note that climacteric and non-climacteric varieties exist in the same species implying that in at least the case of melon, these differences are more likely the result of genetic differences in ethylene synthesis or response and probably do not reflect major differences in primary ripening programs (Giovannoni, 2007).

Extensive molecular and genetic studies have been carried out in order to better understand the regulatory mechanisms underlying these traits with the aim to improve melon fruit quality and to extend storage time. Traditional breeding has produced numerous cultivars with enhanced fruit traits in melon, especially disease resistance and environmental tolerance. Traditional breeding, while highly effective, is generally slow and limited by many factors including sexual compatibility and saturation of genetic potential (Niemirowiczszczytt and Kubicki, 1979; Nunez-Palenius et al., 2008; Pitrat et al., 1999). Biotechnological methods have been applied to generate transgenic melon in order to produce more desirable agronomic traits, particularly to reduce softening and extend shelf life. Several lines of transgenic melon with suppression of the expression of an ACC oxidase ethylene synthesis gene using antisense strategies have been generated for climacteric melon varieties (Ayub et al., 1996; Bauchot et al., 1998; Flores et al., 2002; Nunez-Palenius et al., 2007; Nunez-Palenius et al., 2006). Reduction of ethylene production in transgenic melon resulted in reduced fruit softening, aroma volatile production and rind yellowing while carotenoid content and sugar accumulation were not affected. Further analysis showed that shelf life was extended from days to a few weeks and 68-85% of total volatiles were reduced in the transgenic melons which greatly decreased the final quality of the resulting fruit (Bauchot et al., 1998; Flores et al., 2002). While the outcome of this effort did not yield marketable transgenic fruit, it did demonstrate the primary role of ethylene in regulating important post-harvest characteristics with additional demonstration of an ethylene requirement for the critical quality factor of ripe fruit aroma.

Several additional techniques including differential screening of cDNA libraries, suppressive subtractive hybridization analysis and homology cloning strategies based on genes defined in other systems, have also been used to identify genes associated with the ripening process of climacteric melon (Aggelis et al., 1997; Choi et al., 2004; Hadfield et al., 2000; Hadfield et al., 1998; Nishiyama et al., 2007b). A number of cDNAs whose abundance were much higher during ripening have been isolated, though due to the low regeneration rate of transformation technology in melon species, the biological functions of most of these candidate genes remain largely unknown (Nunez-Palenius et al., 2008).

II-1. Current Status of Melon Molecular Biology and Tools

Melon (*Cucumis melo*) is a diploid species that contains 2n = 24 chromosomes. The genome size of melon is 450Mb, which is close to rice (430Mb) and about three times that of Arabidopsis (125Mb). Prior to initiation of this project, a total 162 loci that control different phenotypes have been described (Pitrat michel, 2002 Gene List for Melon) with 96 of them having complete or partial sequence available in public databases. Genetic linkage maps using different types of molecular markers have been developed for melon (Oliver et al., 2001; Perin et al., 2002). QTLs for ethylene production, fruit softening, flesh color and sugar content have been found (Monforte et al., 2004; Morales et al., 2005; Moreno et al., 2008; Perchepied et al., 2005; Zalapa et al., 2007)

There is no genome sequence available for melon as of the writing of this document and few genomic tools were available until recently. A genome sequencing project on the related cucumber genome has been recently initiated in China and

which should impact melon efforts once fully available (Huang S. The Cucurbit Workshop of PAG, 2008). In 2005, and partly as a result of the data released to the public through this project, the International Cucurbit Genomics Initiative (ICuGI) project was initiated with the aim to generate genomics information and tools for the whole Cucurbit family using melon as a model species (Cucurbitaceae 2006 Proceedings. P180-183). 5531 high quality melon EST were generated at our lab under this project in collaboration with research groups in Israel resulting from sequencing inserts from 21 cDNA libraries mainly constructed from fruits of different developmental stages. These sequences were released to the public immediately and led to the recent release of 30,675 melon ESTs produced and previously held in confidence in Spain (Gonzalez-Ibeas et al., 2007). This combined data source yielded a total 16,128 unigenes for melon. A Cucurbit Genomic Database (CGD) has been developed by Dr. Fei at BTI in cooperation with our project and serves as the hub for the whole cucurbit community as per an agreement with members of the cucurbit genetics/genomics community (http://www.icugi.org). In addition to the melon ESTs described above, the CGD also include ESTs from cucumber and watermelon, genetic maps, BLAST (gene sequence) and DNA marker identification tools and is constantly upgraded for inclusion of additional melon and Curcurbit genomics resources. In addition and as a part of this project, a melon cDNA microarray containing 3066 unigenes spotted in triplicate has been made available to the public and is being used for identification of candidate genes especially related to fruit ripening and quality traits. Other tools for functional genomics studies of melon such as BAC libraries, EMS mutants and TILLING collections are available or are in progress (Ezura and Owino, 2008). Together these genomic resources will greatly facilitate molecular research on melon species and the whole cucurbit family.

III. Status of Molecular biology of Fruit Ripening with Emphasis on Pathways Important for Melon Fruit Development and Quality

As mentioned above, fruit ripening is a genetic and environmentally influenced process which requires the coordination of numerous biochemical and physiological changes to yield the overall ripe phenotype. Ripening pathways typically include synthesis of the photohormone ethylene (in climacteric fruit), pigment alterations, flavor and aroma development, sugar accumulation, and cell wall degradation. During ripening, the expression of many genes has been shown to be differentially regulated and in some cases functionally involved in fruit development and maturation providing insights toward understanding molecular control of fruit ripening. Following is a summary of the current state of ripening molecular biology with an emphasis on pathways especially important for melon fruit traits.

III-1. Ethylene biosynthesis

The hydrocarbon ethylene is the key hormone for regulating the ripening process of climacteric melon. In plants, ethylene is derived from the amino acid methionine and three sequential reactions catalyzed by SAM (S-adenosyl-L-methionine) synthetase, ACC (1-aminocyclopropane-1-carboxylic acid) synthase and ACC oxidase respectively. Methionine is first converted to SAM by SAM synthetase (SAMS), which is then converted to ACC by a reaction catalyzed by ACS. The later step is the first rate-limiting step in ethylene biosynthesis. The intermediate product methylthioadenine from this reaction enters to the Yang cycle to recycle its methyl group (Adams and Yang, 1979; Dong et al., 1992). Ethylene is the direct production of oxidization of ACC catalyzed by ACO which is the second rate-limiting reaction in

the ethylene biosynthesis pathway. The two enzymes, ACS and ACO were also the main targets for antisense suppression in a range of climacteric fruits species with the aim of manipulating ethylene production during fruit ripening.

In tomato, one of the best studied species regarding ethylene synthesis, ACS is encoded by a gene family of at least nine members (*LeACS1A, LeACS1B* and *LeACS2-8*) but only four of them show fruit specific expression (*LeACS1A, LeACS2, LeACS4* and *LeACS6*) (Barry et al., 2000). *LeACS 1A* and *LeACS6* are only expressed in green fruit while the transcript levels of *LeACS 2* and *LeACS4* are more abundant in ripening fruit indicating that the latter two genes are responsible for climacteric ethylene biosynthesis (Barry et al., 2000). A very recent *in vivo* binding study using ChIP (chromatin immunoprecipitation) and gel retardation assays showed that the RIN protein binds to the promoter region of *LeACS2* (Ito et al., 2008) indicating that RIN may initiate climacteric ethylene biosynthesis by controlling *Le ACS2* transcription.

In melon, ACS is encoded by a gene family of at least five members: *CM-ACS1-5*. The expression of *CM-ACS1* is induced in the mesocarp tissues of ripening and wounded melon fruit (Miki et al., 1995) and most likely is the major contributor for climacteric ethylene production during fruit ripening (Yamamoto et al., 1995). *CM-ACS2* and *CM-ACS3* are auxin-responsive genes and expressed primarily during the period when fruit is developing (from 2 or 3 days after pollination to 3 or 5 days after harvest) (Ishiki et al., 2000) except that the expression level of *CM-ACS3* is lower than that of *CM-ACS2* in fruit. The expression of *CM-ACS5* is also high in ripening fruit but its expression seemed independent of ethylene. Little is known about *CM-ACS4* (Li et al., 2006).

Five ACO genes have been identified in tomato (*LeACO1-5*) (Barry et al., 1996; Nakatsuka et al., 1998; Sell and Hehl, 2005). Except for *LeACO2* which is mainly expressed in anther cone tissue, the other four *LeACOs* accumulate in fruit. *LeACO1* is the main gene expressed in ripening tomato fruit. *LeACO3* transcripts transiently accumulate in fruit at the breaker stage at very low levels. *LeACO4* shows similar expression patterns with that of *LeACO1* in fruit by starting to accumulate at the immature green stage and increasing dramatically during ripening. *LeACO5* was isolated in an effort to identify candidate interacting factors with an anaerobically induced bZIP transcription factor in a yeast two-hybrid screen (Sell and Hehl, 2005). *LeACO5* transcription is mainly induced in fruits and at lower levels in leaves and may respond to ethylene production under stress.

Three *Cm-ACOs* have been cloned so far and showed differential expression during fruit ripening in cantaloupe melon. *Cm-ACO1* transcripts accumulate mainly in ripe fruit and also in response to wounding. *Cm-ACO2* is expressed at low levels in etiolated hypocotyls while *Cm-ACO3* is only expressed in flowers (Lasserre et al., 1996). The genomic sequences corresponding to these three different *Cm-ACOs* members have been determined. The coding region of *Cm-ACO1*contains four extrons separated by three introns. Both *Cm-ACO2* and *Cm-ACO3* contain two introns within the same region as *Cm-ACO1*. *Cm-ACO2* and *Cm-ACO3* share 59% and 75% sequence similarity respectively with *Cm-ACO1* in their coding regions.

Fruits of different melon species show different capabilities for ethylene biosynthesis during fruit ripening. Some cultivars such as Dulce behave like climacteric fruits producing higher amounts of ethylene during ripening. Fruits of

other melon varieties, for example Rochet, behave like non-climacteric fruits with no detectable ethylene when fruit ripen. Cultivars with intermediate ethylene production (and ripening phenotypes) also exist. Shiomi *et al*. first compared the expression patterns of *Cm-ACSs* and *Cm-ACOs* between two melon varieties with different production rates of ethylene during ripening and previously recognized as climacteric (Andes) and non-climacteric types (Earl's Favourite), respectively. He found that *CM-ACS1* mRNA levels and its corresponding enzyme activity were high only in the mesocarp and placenta of Andes (climacteric) fruit at 50 dpa (representing the commercial harvest maturity for this variety). At the same stage *Cm-ACO1* accumulated in the mesocarp and placenta of both cultivars. *Cm-ACS2* was constitutively expressed at low levels in mesocarp and placenta in both varieties. Shiomi et al. thus suggested that the different levels of ethylene production among melon fruits during ripening might be the result of differential expression of *Cm-ACS1* genes (Shiomi et al., 1999). It has been proposed that for the species that bear fruits of both climacteric and non-climacteric types such as melon, the non-climacteric fruits may be the result of mutation in ethylene biosynthesis or signaling genes, or in primary regulatory genes analogous to the *RIN, CNR* and *NOR* genes in tomato. This proposal was based on the observation of lack of abscission zones in the ripe fruit of non-climacteric melon fruit and supported by the experimental results from Perin C. *et al*. Suggesting that the differences in climacteric versus non-climacteric types could be tracked to one or a few genetic loci in melon (Giovannoni, 2004; Perin et al., 2002). By analysis of recombinant inbred lines generated from crossing of cantaloupe and a non-climacteric breeding line, Perin *et al*. showed that fruit abscission and ethylene production were controlled by only two independent loci named Abscission layer (AI)-3 and AI-4. As the roots of PI161375 responded to exogenous ethylene, it has been suggested that the non-climacteric behavior of PI 161375 might be due to a fruit

tissue specific effect (Barry and Giovannoni, 2007; Giovannoni, 2004; Perin et al., 2002).

III-2. Ethylene signaling

The steps and components of the ethylene signal transduction pathway have been well characterized in the model plant *Arabidopsis*. In *Arabidopsis*, ethylene is perceived by a family of membrane-localized receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) that are homologous to bacterial two-component histidine kinases. Downstream of the receptors is a MAP-kinase cascade that includes CTRs, the Raf family of Ser/Thr kinase, and MAPKs. EIN2, encoding a protein similar to the Nramp family of metal ion carriers and probably represents a pivot for multiple signaling systems. The ethylene signal transduction pathway also includes groups of transcription factors inside the nucleus that control the expression of ethylene responsive genes such as EIN3, EIL (EIN3-like) and ERFs (ethylene response factors). Ethylene receptors and CTRs are negative regulators. In the absence of the ethylene signal, ethylene receptor active CTRs and CTRs in turn repress the expression of downstream genes through inhibition of the activity of the MAP kinase cascade. Binding of ethylene inactive the receptors, resulting in the deactivation of CTRs and the inhibition of the MAP kinase cascade is relieved which allows EIN2 to mediate activation of the EIL family transcription factors. EILs likely initiate a TF cascade through activation of secondary TFs such as ERFs, which in turn active downstream ethylene responsive genes (Adams-Phillips et al., 2004; Wang et al., 2002).

Components of the ethylene signaling pathway have been isolated from other species based on homology to Arabidopsis. To date only three genes involved in

the ethylene signal transduction pathway, *Cm-ETR1*, *Cm-ETR2* and *Cm-ERS1*, were cloned and studied in melon. Both *Cm-ETR1* and *Cm-ERS1* contain three hydrophobic transmembrane domains and a histidine kinase domain, only *Cm-ETR1* has the receiver domain at the C terminus (Sato-Nara et al., 1999). *Cm-ETR2* was reported to have four transmembrane domains and its kinase domain lacks the catalytic sub-domain (Ezura and Owino, 2008). *Cm-ETR1* and *Cm-ERS1* are expressed in a stage and tissue specific manner. *Cm-ETR1* is highly expressed in the in the seed and placenta of developing to fully enlarged fruit, whereas its mRNA level increased again in the pericarp of fruit at the time when climacteric ethylene synthesis begins. The mRNA level of *Cm-ERS1* increased dramatically in the pericarp of young, expanding melon fruit and remained low during fruit ripening. *Cm-ERS1* protein showed a different expression pattern as anticipated by gene expression results suggesting a component of post-transcriptional regulation. Western blot analysis showed that *Cm-ERS1* protein only accumulated during early fruit development (up to 29 dpa) and was not detectable during ripening. This result indicates that the expression of CM-ERS1 is under post-transcriptional control and the function of CM-ERS1 may be responsible for early fruit development (Ezura and Owino, 2008; Sato-Nara et al., 1999). The expression of CM-ETR2 is ripening related and ethylene-dependent (Ezura and Owino, 2008)**.**

III-3 Cell Wall Metabolism

Softening rate is the main factor limiting fruit shelf life and contributing to post-harvest deterioration. The softening process of fruit is believed to be the result of degradation of cell wall polysaccharides which provide a network comprised of a cellulose matrix, cross-linked hemicellulose and highly hydrated pectins.

Solubilization of the pectin rich middle lamella, de-esterfication of pectins and depolymerization of the polysaccharides matrix are proposed to be the major events causing softening during fruit ripening including that of melon (Rose JKC. *et al*. 1998). This process is catalyzed by the interaction of different groups of cell wall modifying enzymes including polygalacturonase (PG), pectin methylesterase (PME), beta-galactosidase (GAL), xyloglucan endotransglycosylase (XTH) and expansin (EXP) (Nishiyama et al., 2007a; Rose et al., 1998).

The molecular and biochemical functions of many of these enzymes have been characterized in tomato. In tomato, PG is the major cell wall polyuronide-degrading enzyme (Alexander and Grierson, 2002). The accumulation of PG mRNA is regulated by ethylene and is paralleled with the increase in PG protein and enzyme activity during ripening and the solubilization of polyuronide (pectin). Interestingly, tomato fruits with suppressed PG were only slightly firmer than wild type controls (Langley et al., 1994; Smith et al., 1990a; Smith et al., 1990b), indicating that PG alone is not sufficient for fruit softening. It has been suggested that the substrates of PG are highly methyl-esterified homogalacturonans which must be de-esterified prior to PG's function, further supporting that the softening of fruit during ripening is the result of co-ordination of multiple enzymes.

PG activity has been detected in melon (Hadfield et al., 1998). Six melon PG genes (*Cm-PG1-6*) were isolated and three of them, *Cm-PG1*, *Cm-PG2* and *Cm-PG3,* are expressed in ripening melon fruit. *Cm-PG1* is the most abundant melon PG in ripening melon fruit. Heterologouse expression of *Cm-PG1* protein indicated that *Cm-PG1* is an endo-PG enzyme capable of depolymerizing pectin substrates. *Cm-PG1* is highly expressed in ripening stage fruit, peaks at 43dpa and starts to decrease at 46

dpa. This expression is blocked in ACO antisense and 1-MCP treated fruit (Hadfield et al., 1998). When exogenouse ethylene is applied, the expression of *Cm-PG1* is induced again in ACO antisense fruit. This result indicates that the expression of Cm-PG1 is ethylene dependent. The expression pattern of *Cm-PG2* and *Cm-PG3* in wild-type is similar to that of *Cm-PG1* except that the expression of *Cm-PG2* is induced a few days earlier. In ACO antisense fruit the expression pattern of *Cm-PG2* and *Cm-PG3* did not change compared with that in wild-type controls. Applying exogenous ethylene did not increase the mRNA level of *Cm-PG2* but greatly induced the expression of *Cm-PG3*. Thus the regulation of *Cm-PG2* expression is ethylene independent while the expression of *Cm-PG3* seems to be under both ethylene and ethylene–independent control. These data indicate that *Cm-PG1* is likely the major contributor for ethylene dependent pectin depoymerization in climacteic melon fruit (Nishiyama et al., 2007b).

Additional enzymes activities have also been detected in melon and may be involved in cell wall modification during ripening such as beta -D-galactosidase (*Cm-GAL1,Cm-GAL2* and *Cm-GAL3*), expansins (*Cm-EXP1*) and xyloglucan endotransglycosylase (*Cm-XTH1* and *Cm-XTH3*) (Nishiyama et al., 2007b). GAL is responsible for degradation of beta-galactan from cell wall polymers. In tomato, three isomers of GAL have been identified: GAL-I, GAL-II and GAL-III. Only GAL-II is up-regulated in ripening fruit and capable of deplomerization of galactan from tomato cell wall extracts. Suppression of GAL activity at early ripening stage rather than in later stages greatly reduced galactose loss and reduced softening by up to 40%. This result indicates that beta-galactan degradation by GALs may decrease the porosity of cell wall backbones which facilitate the entrance and activity of other enzymes. In melon fruit, GAL activity has been detected at all ripening stages supporting the role

of GAL in the modification of cell wall components in melon fruit. Three *Cm-GALs* show ripening related expression in melon. The expression of *Cm-Gal*1 is upregulated at the 4th day after ripening and the expression of *Cm-Gal*2 about 2 days earlier than *Cm-Gal*1.The overall level of *Cm-Gal*3 was lower than that of *Cm-Gal*1 and *Cm-Gal*2 but it is expressed through out fruit growth and ripening stages.

XTH can cleave the backbone of xyloglucans in cell wall polymers and transfer the newly formed glycosyl to a new xyloglucan acceptor. This enzyme is encoded by a large gene family containing over 20 genes and the function of each isozymer varys significantly in biochemical properties. In tomato, two XTH genes, *Le*EXGT1 and *Le*XETB1, have been extensively studied. The expression of *Le*EXGT1 was only detected in green fruit and its activity is related to final fruit size (Asada K. *et al.*1999). The mRNA of *Le*XETB1 increased in ripening fruit, but suppression of *Le*XETB1 had little effect on fruit softening (de Silva et al., 1994). It is possible that more than one XTH gene is involved in the softening process of tomato. In melon, two XTHs, *Cm-XTH1* and *Cm-XTH2* showed ripening related expression. *Cm-XTH1* was strongly expressed at 36 days after pollination and then decreased dramatically to a barely detected level in ripening fruit. The expression pattern of *Cm-XTH3* was similar with *Cm-XTH1* except that *CM-XTH3* mRNA accumulated to a great extent at 43 days after pollination (Nishiyama et al., 2007b).

Expansins can loosen cell walls non-enzymetically by disruption of the hydrogen bonds between microfibrils of the polysaccharide matrix and this resulting porosity may be necessary for pectin depolymerization by PG(Brummell and Harpster, 2001). In tomato, *Le* Exp1 is the most abundant expansin expressed in ripening fruit. Homologus of *Le* EXP1 have been identified in ripening fruit of other species. As it

was in tomato, melon Cm-Exp1 was up-regulated only in ripening fruit. Although the expression of these genes are ripening associated, analysis of ACO antisense Charentais melon with ethylene and 1-MCP treatment showed that the expression of those genes are differentially regulated by ethylene. In summary, the expression of *Cm-Gal1* seemed ethylene dependent as expression was totally blocked in ACO antisense and 1-MCP treated fruit. Application of exogenous ethylene to these fruit greatly induced the expression of *Cm-Gal1*. While in contrast, the expression of *Cm-Gal2* and *Cm-Gal3* appears to be largely ethylene-independent. Comparing with wild type, the mRNA level of *Cm-Gal2* and *Cm-Gal3* did not change very much in ACO antisense fruit and 1-MCP treatment was unable to repress their expression. The regulation of *Cm-EXP1, Cm-Egase1, Cm-XTH1* and *Cm-XTH3* are influenced by both ethylene dependent and ethylene-independent regulatory mechanisms. Together these data indicate that the softening process of melon may be regulated by multiple enzymes and by both ethylene-dependent and ethylene-independent regulatory pathways (Flores et al., 2001).

III-4 Carotenoid Biosynthesis

Carotenogenesis in ripening has been extensively studied in tomatowhich accumulate lycopene to high levels during ripening. In higher plants, carotenoids are synthesized from the MEP pathway in plastids. Two important enzymes catalyze the conversion of pyruvate and GAP (glyceraldehyde 3-phosphate) to IPP (Isopetenyl diphosphate): DXS (1-Deoxy-D-xylulose-5-phosphate synthase) and DXR (1-Deoxy-D-xylulose-5-phosphate reductoisomerase). GGPP (geranylgeranyl diphosphate) is formed by adding two molecules of IPP to its allylic isomer dimethylallyl diphosphate (DMAPP) catalyzed by GGPS (GGPP synthase). GGPP is the precursor of all

carotenoids. The condensation of two molecules of GGPP to form phytoene is catalyzed by phytoene synthase (PSY). ZDS (ζ-carotene desaturase) and PDS (phytoene desaturase) convert phytoene into lycopene through their sequential action, respectively. The cyclization of lycopene can be catalyzed by either lycopene β or ε-cyclase and then can lead to the creation of β - or α-carotene, respectively.

Although the main steps and key enzymes that are involved in carotenoid biosynthesis have been well characterized, how this pathway is regulated is poorly understood. The expression of many genes involved in carotenoid biosynthesis show positive or negative regulation when comparing tomato nearly isogenic carotenoid QTLs lines with their parental backcross line using microarray technology (R. McQuinn and J. Giovannoni. personal communication). Differential gene expression has also been observed during the ripening of tomato fruit including the up regulation of *DXS* (Lois et al., 2000), *PSY-1, PDS* (Corona et al., 1996; Giuliano et al., 1993; Pecker et al., 1992) and the down regulation of Lycopene cyclases (Lcy-b and –e) (Pecker et al., 1996; Ronen et al., 2000). These data indicate that transcriptional regulation is involved in the accumulation of lycopene during tomato ripening and that these genes are likely the points of regulatory control of the pathway. Recently, a transcription factor belonging to the APETALA2 (AP2)/ethylene-responsive element-binding protein family, *AtRAP2.2*, has been cloned in Arabidopsis and shown to regulate *PSY* and *PDS* transcription by binding to an ATCTA element in the promoter region of this two genes (Welsch et al., 2007). Accordingly, carotenoid levels were decreased in lines carrying mutations in this gene. Furthermore, analysis of tomato high pigment (*hp1and hp2*) mutants revealed that light signaling is a regulator of carotenoid pathway activity. The entire repertoire of fruit carotenoid is elevated in *hp1and hp2* (Cookson et al., 2003) but the expression of phytoene synthase and

phytoene desaturase did not change in mutant lines suggesting additional points of regulatory control. Both *HP1* and *HP2* encode proteins involved in the light signaling pathways of plants (Liu et al., 2004). Additional regulatory mechanisms of carotenoid accumulation have been proposed and include post-transcriptional regulation, end-product feedback inhibition and carotenoids themselves via degradation derived signaling molecules (Bramley, 2002; Giuliano et al., 2008).

Four flesh colors due to carotenoids can be observed in melon fruits: orange, light orange, green and white (Watanabe et al., 1991). The principle pigment in the orange and light orange flesh melons is beta-carotene, about 9.2-18.0 ug/g flesh weight (FW) in orange flesh varieties and 4.0 ug/g FW in light-orange flesh types (Seymour et al., 1993). Our collaborator Dr. N. Kanzir evaluated the carotenoid content in a pale-green flesh melon, "Rochet". They found that Rochet contained about 0.31(± 0.05) ug/g FW beta-carotenes and 0.1472 (±0.124) ug/g FW lutein. Other compounds detected in Rochet included gamma-tocopherole (1.04±0.24 ug/g FW) and alpha-tocopherole (0.226±0.076 ug/g FW). Trace amounts of beta-carotene have also been detected in the other types of light-green and white flesh melons (Seymour et al., 1993). Thus it is more likely that the light-green and white fresh color is due to the lack of carotenoid compounds and a reduction of chlorophyll in chromoplasts of these melon fruit.

In melon, phytoene synthase is the only enzyme involved in this pathway that has been cloned and characterized to date. *Cm-PSY* is homologous to tomato *PSY1*. Two *PSYs* have been identified in tomato: *PSY1* and *PSY2. PSY1* is responsible for carotenogenesis in ripening fruit while *PSY2* predominates in green tissues, such as leaves and green unripe fruits (Fraser et al., 1999). The expression of *CM-PSY* is

greatly induced during fruit ripening although its expression was also found in other tissues such as unripe fruit, leaves and roots (Karvouni et al., 1995).

III-5 Aroma volatile Biosynthesis

Aroma is one of the major parameters in determination of melon fruit quality. Aroma volatile metabolism has been extensively studied in melon fruit probably because of the unique aroma and flavor that melon fruit possess and the diversity of aroma volatiles synthesized by the range of melon varieties. The aroma volatiles of melon include a mixture of ester, alcohols and volatile aldehydes. More than 240 volatile compounds have been reported(Nunez-Palenius et al., 2008). Shalit M. *et al.* first measured the volatile profiles of a climacteric melon with high aroma, Arava, as compared to that of Rochet, a non-climacteric type with low aroma volatiles. They found that 83.3% of the total volatiles were esters in mature Arava melon fruit, only 0.4 % were aldehydes and 4.3% were alcohols. In mature Rochet fruit, 60% of the volatiles detected were alcohols, 20% were aldehydes and only 7% were esters (Shalit et al., 2001) and the total content was much lower than in the climacteric melon. In another experiment performed by Aubert C. and Bourger N, volatile composition of long shelf life (LSL) and mid shelf life (MSL) melons showed that 2-30 fold lower level of esters were found in LSL as compared to MSL. Volatile esters are the main contributors to the unique fragrance of melons and most important in this regard are acetate esters. In climacteric melons, the last two steps for ester biosynthesis have been characterized. Two enzymes have been verified to catalyze two sequential reactions: alcohol dehydrogenase (ADH) that reduces aldehydes to alcohol and alcohol acyltransferase (AAT) that catalyzes the esterification of alcohols to esters. Four AAT genes have been isolated from melon (*Cm-AAT1* to *Cm-AAT4*).

Cm-AAT1 shares 84%, 58% and 22% sequence identity with *Cm-AAT2*, *Cm-AAT3* and *Cm-AAT4* at the amino acid level, respectively (El-Sharkawy et al., 2005). *Cm-AAT1*, *Cm-AAT3* and *Cm-AAT4* exhibit alcohol acyl-transferase activity while *Cm-AAT2* does not. Site directed mutagenesis verified that the loss of enzyme activity of *Cm-AAT2* was because the threonine at the 268 amino acid position of the other three *Cm-AATs* was replaced by alanine in *Cm-AAT2* (El-Sharkawy et al., 2005). *Cm-AAT1* and *Cm-AAT3* are also capable of converting a wider range of substrates than *Cm-AAT4* to esters, though with different preferences between these two proteins when analyzed *in vitro* (El-Sharkawy et al., 2005; Lucchetta et al., 2007). The expression of all four genes is ripening related and ethylene regulated (El-Sharkawy et al., 2005; Yahyaoui et al., 2002).

ADH is encoded by a gene family consisting at least two members (*ADH1* and *ADH2*). *Cm-ADH1* and *Cm-ADH2* share 15% sequence similarity at the amino acid level. They are capable of interconversion of aldehydes and alcohols with a preference for producing alcohols. NAPDH and NADH are required for the function of *Cm-ADH1* and *Cm-ADH2*, respectively. The expression of both ADH genes is fruit specific and ripening related. The expression of both ADH genes and their enzyme activities were suppressed in ACO antisense (AS) and 1-MCP treated wild type fruit and ethylene exposed AS fruit induced expression of these genes indicating that both are ethylene dependent (Manriquez et al., 2006). Flores F, *et al*. studied the role of ethylene in regulation of aroma volatile biosynthesis in melon fruit using ACO antisense plants (Flores et al., 2002). They reported that in the ACO antisense fruit, the reduction of fatty acids and aldehydes were ethylene-dependent, while the last step of ester synthesis, alcohol acetylation, was influenced by both ethylene dependent and ethylene independent regulatory systems.

III-6 Sugar and Organic Acid metabolism

Sweetness is one of the important edible qualities for melon fruit. Melon species contain fruits that differ widely in ripe fruit sweetness. For example, fruits from the *Cantaloupensis* family usually taste sweet but the *Conomon* group produce fruit with almost no sucrose. Fruits belonging to the *Inodorus* cultivars contain both sweet and non-sweet types. Three types of sugars have been fund in the mesocarp of ripe melon fruits and account for the sweetness phenotype: sucrose, glucose and fructose. Among them, sucrose is generally considered as the most important component contributing to the sweetness of melon fruit (Stepansky et al., 1999). Two enzymes, acid invertase and sucrose phosphate synthase (SPS), have been identified as the key regulators for sucrose accumulation in melon fruit (Hubbard et al., 1989). SPS catalyzes the synthesis of sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate, while sucrose synthase and sucrose-phosphatase can use UDP-glucose and sucrose-6-phosphate as substrates, respectively, to synthesize sucrose, which in turn can be broken down to glucose and fructose by (acid or neutral) invertases. During the ripening process of melon fruit, high concentrations of sucrose are typically associated with high activity of SPS and low activity of acid invertase, while the activies of sucrose synthase and neutral invertase are low (Hubbard et al., 1989). Comapring the enzyme activities between sweet type and non-sweet type melons, SPS was much higher in sweet melon fruit. Thus the final concentration of sugars in melon fruit is the result of upregulated SPS activity and down regulated invertase (Stepansky et al., 1999).

The main organic acids found in melon fruit are citric acid and malic acid. Several lines of evidence indicated that the sweetness of melon is due to the balance between sugar and organic acid metabolism. Organic acids accumulate at very low levels in sweet types but are higher in non-sweet melon fruit. At young stages soluble sugar is low but acid levels are high. At the onset of ripening, sugar content starts to increase with a reduction in acid levels (Hubbard et al., 1989; Lingle and Dunlap, 1987) (Burger et al., 2003). How these two pathways are regulated during ripening of melon is poorly understood.

Organic acid metabolism has been extensively studied in grapes and Citrus fruits. Phosphopyruvate carboxylase and NAD-dependent malate dehydrogenase are two key enzymes for cytosolic malic acid synthesis, while mitochondrial citrate synthesis and mitochondrial aconitase are responsible for citric acid synthesis. Malic acid degadation is catalyzed by cytosolic NADP-dependent malic enzyme while both cytosolic aconitase and NADP-dependent isocitrate dehydrogenase are responsible for breaking down citric acid. Vacuolar storage also contributes to the accumulation of organic acids in the cell. Vacuolar H^+-ATPase and vacuolar H^+-pyrophosphatase are believed to play an important role in vacuolar acid storage (Etienne et al., 2002).

Genetic studies in melon indicate that high sugar content is controlled by a single recessive locus (*suc*) and high acid is governed by one locus called *So*.(Burger et al., 2003). The activity of both loci seems to be ethylene-independent (Nunez-Palenius et al., 2007; Silva et al., 2004). Characterization of the genes underlying these loci would be extremely important in elucidating the regulation of melon sugar and acid quality traits. Homolog of the genes described above may serve as interesting candidates for these loci.

Summary

Using tomato as a model system and taking advantages of the plethora of information from Arabidopsis, studies on fruit ripening have advanced to provide us important information for understanding the regulatory role of ethylene during climacteric fruit ripening. Though common features exist among fruit bearing plants, the molecular controls resulting in the diversity of mature fruit traits among varying species and cultivars are still poorly understood. As one of the important economic fruit crops in the Cucurbitecea family, melon is highly appreciated by consumers for its unique flavor and aroma. Improvement of melon fruit quality has long been the research focus of those interested in melon fruit ripening. In order to understand the molecular regulation of fruit quality control, additional genomic information and tools need to be created and exploited for the Cucumis family. In the following chapters of this thesis I relay activities performed in order to help create public genomic resources to spur genomic research in the melon community and to facilitate my own studies comparing the molecular biology of climacteric versus non-climacteric melon fruit ripening.

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CHAPTER II

GENERATION OF EST AND MICROARRY RESOURCES FOR FUNCTIONAL GENOMICS STUDIES ON *CUCUMIS MELO* (MELON) FRUIT

Abstract

Melon species include a wide variety of cultivars producing fruits differing in many fruit traits, including flesh color, sugar content, aroma volatiles shelf-life and nutrient content. Great efforts have been made trying to understand the genetic mechanisms underlying these traits, but, without genomic sequences, research in this area is limited. Here, we introduce to the melon research community new genomic tools and resources including ESTs and microarrays, to provide public genomics infrastructure to assist research at the molecular level for species of the Cucurbitaceae family. In summary, we generated 3737 high quality ESTs with an average read length of 463 bp, derived from the sequencing of 5040 individual cDNA clones from 21 cDNA libraries constructed from a range of developmental stages of melon fruits. These ESTs, in combination with an additional 1800 collaborator-contributed melon phloem ESTs and 118 published melon genes from GenBank, were assembled together into 2422 singletons and 847 overlapping contigs resulting in 3269 unigenes. 82% of these unigenes were annotated based on sequence similarity against NCBI GenBank using BLASTX with a cutoff e value at 10^{-5} and 61% were assigned to biological and/or molecular functions according to Gene Ontology (GO) terms using the InterPro2GO program. In addition, a melon cDNA microarray, consisting of 3068 independent sequence elements derived from these EST collections has also been generated and produced a very low false positive

hybridization rate (essentially equal to zero) using our statistical analysis package developed in part by bioinformaticians in our laboratory. A transcriptome comparison between two developmental stages of fruit maturation, 24 days post anthesis (DPA; immature) and mature, of Tam Dew variety cantaloupe type melon fruits was performed to validate the utility of our melon cDNA chips. Of the total 238 genes differentially expressed between these two stages, the transcript abundance of 10 genes has been confirmed by previous reports in the public literature. 5 more genes were then chosen for further qRT-PCR verification. All five genes showed similar expression patterns with that derived from microarray data. These results indicate that the melon cDNA microarray generated in this study produced highly reliable expression information. This array currently represents the only public microarray available to the melon/cucurbit community.

Introduction

Melon (*cucumis melo* L.) is one of the most intensively studied species in terms of fruit quality and ripening and is becoming increasingly more important as a member of the economically valuable fruit cash crops in the Cucurbitaceae family. During 2003-2005, the average global production of melon reached 60.4 billion pounds, ranking at the 16th position and representing 1.3 % of the world production of fruits and vegetables (FAOStat database, 05/2006). The United States is one of the leading producers of melons (cantaloupe and a variety of related melons) world wide, only behind China and Turkey, with an average farm value of approximately one half billion dollars per year (Vegetables and Melons Outlook/VGS-279/June 20,2003). In addition, melon is also one of Americans favorite fruits for dessert and salad use because of its unique flavors and palatable aromas. During the past few

years (2000-2006), the estimated average U.S. per capital consumption exceeded 12 pounds per year, an 8 % rise from 1990-1999 (Vegetables and Melons outlook/VGS-320/April 19, 2007. Economic Research Service, USDA). The expanding economic role and increasing consumer demand in the market for melons including those with unique and new characteristics require broadening our knowledge accordingly on their molecular genetic make up in order to improve the depth and range of beneficial agronomic traits which can be bred into production varieties of melon fruit to meet the demands of both the US and the world market place.

Melon (*cucumis melo* L.) is a diploid species that contains 2n = 24 chromosomes. Even though the genome size of melon is relatively small (450Mb), which is close to rice (430Mb) and about three times that of Arabidopsis (125Mb), its genetic architecture seems more complex. *Cucumis melo* L. includes a very wide variety of cultivars producing fruits showing great phenotypic diversity for flesh color, sugar content, aroma, fruit shape and size and thus melon is considered one of the most genetically diverse species in the Cucurbitaceae family. For example, fruits in *C.melo* var. *reticulatus* group, such as the cultivar Dulce, usually contain a rough warty (often referred to as "netted") rind and sweet orange flesh. In contrast, in the *C.melo* var. *inodorus* family, fruits are characterized by smooth skin and light green to white flesh, as represented by the Tam Dew and Rochet cultivars. In addition, these melon fruit types are generally grouped into two categories based on their ripening physiology, specifically termed, climacteric and non-climacteric ripening types.

The definition of climacteric refers to a significant increase in respiration at the onset of ripening (Biale, 1960) which is generally measured as increased CO2

evolution. Non-climacteric fruits accordingly lack this respiration rise. Typically associated with the increase of respiration in climacteric fruits is an increase in ethylene production that is generally also necessary for ripening (Biale, 1960; Burg and Burg, 1965). Climacteric melons produce a high peak of ethylene production upon ripening and exhibit typically strong aroma and flavor compared with the non-climacteric types. Typically associated with climacteric ripening in melon is a relatively rapid ripening process and a moderate to short shelf life (Liu et al., 2004; Zheng and Wolff, 2000).

Fruit quality is an increasingly important trait of economic interest (only behind yield and pathogen resistance) and among the first considerations for both farmers and especially for consumers. For melon, the quality of fruit is generally determined by a number of readily measured and quantifiable factors, such as sugar concentration, aroma volatiles, and organic acid content. Recently, many molecular and genetic studies have been carried out in order to better understand the genetic mechanisms underlying these traits (Ayub et al., 1996; Burger et al., 2003; El-Sharkawy et al., 2005; Flores et al., 2002; Hadfield et al., 2000; Hadfield et al., 1998; Ibdah et al., 2006; Lingle and Dunlap, 1987; Lucchetta et al., 2007; Manriquez et al., 2006; Nishiyama et al., 2007; Shalit et al., 2001; Yahyaoui et al., 2002), but because of a limitation in available public genomic information, research on *cucumis melo L* remains limited as compared to many other plant and fruit systems.

Many efforts have been made in recent years to develop genetic and more recently genomic resources for melon species with a primary aim to discover genes controlling fruit quality traits. These include the development of marker merged full linkage maps, near isogenic lines (NILs), QTLs mapping populations and SSR/SNP

markers (Danin-Poleg et al., 2000; Eduardo et al., 2005; Monforte et al., 2004; Moreno et al., 2008; Oliver et al., 2001; Perin et al., 2002; Silberstein et al., 2003; Zalapa et al., 2007). All of these are powerful tools for forward genetic dissection of the genetic basis of biologically interesting phenotypes, but the system as a whole remains limited in terms of the rate of new gene discovery.

Very recently the Cucumber Genome Initiative has been launched (Jan. 2008) in China with the aim to sequence the genome of cucumber (Huan S. et al. 2008 Plant and Animal Genome Conference, San Diego, CA). As was reported at the International Plant and Animal Genome Conference in San Diego (2008) approximately 400,000 ESTs have been generated using 454 pyro-sequencing. BAC end sequencing, genetic marker development and physical map construction are also under development. The initiation of this project will provide more information for our understanding of the genetic and molecular basis of major agronomical traits of the cucurbit family. While the cucumber sequence will likely prove to be a great resource for the entire Cucurbitaceae once fully analyzed and released to the public, at present access to this data is limited.

To date, there is limited genomic sequence available for melon species and thus for the gene sequences that contribute to their most important traits. ESTs (expressed sequence tags), which are created by large-scale, single-pass sequencing of randomly picked clones from cDNA libraries, are a way to capture sequence of many expressed genes without sequencing the whole genome and to gather expression information on the tissues from which they are derived. Recent high-throughput DNA sequencing technology, accompanied with sophisticated bioinformatics tools, has allowed for the generation and annotation of EST

sequences quickly and relatively cheaply. These technical advances have lead to EST sequencing projects for numerous different plant species of basic and agricultural interest and that currently lack full genomic sequence information - including but not limited to tomato (*Solanum lycopenium*), potato (*Solanum tuberosum*), grape, apple, peach and strawberry (Fei et al., 2004; Flinn et al., 2005; Horn et al., 2005; Jung et al., 2004; Peng et al., 2007; Ronning et al., 2003). Such EST repertoires provide comprehensive genomic resources for wide aspects of research such as molecular marker development, gene expression prediction, orthologous and gene family member comparisons, and especially for gene discovery. EST resources have proven especially useful as a fast and efficient way for novel gene identification, particularly when combined with suppressive subtractive hybridization methods that target acquisition of sequences particular to tissues, developmental stages or responses of interest. The SSH approach reduces the commonly expressed genes in two populations under comparison, thereby leaving the differentially expressed transcripts behind during enrichment, and thus greatly enhances the possibility of cloning uniquely expressed genes for tissues, stages, responses or due to genetic variation of interest. This approach can be very useful for studies of different traits related to melon fruit qualities, such as sweetness, aroma volatiles and flesh color.

ESTs also provide a large transcriptome resource for functional genomics research. One of the tools that can be derived from these resources is the necessary sequence infrastructure to construct microarrays. Microarray technology generates a platform for measuring the expression level of thousands of genes within a single experiment and provides a genomic approach to identify differentially regulated genes between two samples under a given condition (Chetverin and Kramer, 1994; DeRisi and Iyer, 1999; Gerhold et al., 1999). Verified transcripts of interest from

such an expression profiling experiment can be used as candidates for further functional identification through gene silencing, which provides insight into gene expression and regulation *in vivo* (Aharoni et al., 2000).

In order to generate resources for functional genomic studies of fruit biology and expanding the available set of novel genes potentially involved in fruit quality in melon, we report here the development of melon fruit ESTs through several approaches. Derived from these sequences our group has also developed a melon EST database and a melon cDNA microarray. 3737 high quality ESTs have been generated through this study after sequencing 5040 individual cDNA clones from 21 libraries constructed from a range of developmental stages of melon fruits. Those ESTs, in combination with an additional 1800 collaborator-contributed melon phloem ESTs and 118 published melon genes from GenBank, were assembled together into 3254 unigenes. 82% of these unigenes were annotated based on sequence similarity against NCBI GenBank using BLASTX at cutoff e value of 10^{-5}. The InterPro program, a protein signature based system for functional prediction of a given sequence, was also utilized in order to extend the functional classification of the resulting unigenes. The Cucurbit Genomics Database created by Dr. Zhangjun Fei at the Boyce Thompson Institute for Plant Research contains all of this information and is available to the research public at the web site: http://www.icugi.org.

A melon cDNA microarray, consisting of 3068 independent sequence elements derived from the unigenes in the melon EST database has also been generated and was utilized for an initial transcriptome comparison between two developmental stages of melon fruit, 24 days post anthesis (DPA; immature) and

mature, of Tam Dew non-climacteric melon fruits. 238 unigenes were identified as being differentially expressed between these 2 stages (FDR $\leq$ 0.05; fold $\geq$ 2). The transcript abundance of 5 genes was re-examined using real time RT-PCR analysis. All five showed identical expression patterns as compared to the microarray data and 10 additional genes were validated in terms of expression via the available literature. These results demonstrate the potential utility of the melon cDNA microarray for functional genomics studies and also provided valuable information pertaining to gene regulation during fruit development. The melon cDNA microarray is currently available at the Center for Gene Expression Profiling (CGEP), Boyce Thompson Institute for Plant Research, Cornell University (htty://www.bti.cornell.edu/CGEP/CGEP.html) and through our laboratory.

Results and Discussion

Library construction, EST sequencing and assemblies

21 cDNA libraries were constructed from a range of developmental stages of fruits with the goal of increasing the depth and representation of unique transcripts in melon (*cucumis.melo*.) fruit. These libraries include one melon young fruit non-normalized cDNA library, three melon mature fruit non-normalized cDNA libraries (Table 2.1) and seventeen melon suppressive subtractive hybridization (SSH) libraries (Table 2.2). The cultivars for constructing these libraries include climacteric types such as Dulce (*Cucumis melo* var.reticulatus.), Noy Yizre'el (*Cucumis melo* var.reticulatus.), Faqqous (*subsp. melo* var. flexuosus). Vedrantaise (*Cucumis.melo* var.cantalupensis Naud.), PI414723 (*subsp.melon* var.momordica) and non-climacteric types Tam Dew (*Cucumis melo* var.inodorus.) and Rochet

Table 2.1 Melon cDNA resources for EST generation.

Library Name	Cultivar	Tissue	Sequenced ESTs	Quality ESTs	Singletons
Melon Young Fruit cDNA Library (NY)	Noy-Yizre'el	mixture of fruits at 0,1,3,12 and 25 DAP	576	460 (79.9%)	233
Melon Mature Fruit cDNA Library (NY)	Noy-Yizre'el	mixture of mature green (35 DPA) and mature yellow (37DPA) fruits	576	379 (65.8%)	203
Melon Mature Fruit cDNA Library (Dul)	Dulce	mature fruits (?DPA)	1152	977 (84.8%)	485
Melon Mature Fruit cDNA Library (TamD)	Tam Dew	Mature fruits (?DPA)	1152	970 (84.2%)	467
Summary			3456	2786 (80.6%)	1388

Table 2.2 Suppressive Subtractive Hybridization (SSH) libraries used in ESTs generation

Library ID	Tester	Driver	Sequenced ESTs	Quality ESTs	Singletons
MSL1*	Noy-Yizre'el mixed stages of fruits	Vedrantais mixed stages of fruits	144	98 (68.1%)	40
MSL 2	Vedrantais mixed stages of fruits	Noy-Yizre'el mixed stages of fruits	240	161 (67.1%)	43
MSL 3	Noy-Yizre'el plant roots,stems and leaves	Noy-Yizre'el mixed stages of fruits	192	27 (14.1%)	13
MSL 4	Noy-Yizre'el mixed stages of fruits	Noy-Yizre'el plant roots,stems and leaves	137	50 (36.5%)	16
MSL 5	Dulce mixed stages of fruits	PI 414723 mixed stages of fruits	70	24 (34.3%)	5
MSL 6	PI 414723 mixed stages of fruits	Dulce mixed stages of fruits	115	58 (50.4%)	31
MSL 7	Roshet mixed stages of fruits	Noy-Yizre'el mixed stages of fruits	123	103 (83.7%)	50
MSL 8	Noy-Yizre'el mixed stages of fruits	Roshet mixed stages of fruits	157	149 (94.9%)	52
MSL 9	Dulce mixed stages of fruits	Noy-Yizre'el mixed stages of fruits	22	15 (68.2%)	2
MSL 10	Faqqous mixed stages of fruits	Noy-Yizre'el mixed stages of fruits	144	91 (63.2%)	12
MSL 11	Noy-Yizre'el mixed stages of fruits	Faqqous mixed stages of fruits	48	26 (54.2%)	10
MSL 12	Noy-Yizre'el mature fruits (47dpa) fresh	Noy-Yizre'el young fruits (25dpa) fresh	23	15 (65.2%)	3
MSL 13	Noy-Yizre'el young fruits (25dpa) fresh	Noy-Yizre'el mature fruits (47dpa) fresh	3	2 (66.7%)	2
MSL 14	Noy-Yizre'el mature fruits (47dpa) rind	Noy-Yizre'el young fruits (25dpa) rind	21	16 (76.2%)	6
MSL 15	Noy-Yizre'el young fruits (25dpa) rind	Noy-Yizre'el mature fruits (47dpa) rind	27	16 (59.3%)	5
MSL 16	F2 from NY* and FQ* high sucrose mature fruits	F2 from NY* and FQ* low sucrose mature fruits	39	31 (81.6%)	13
MSL 17	F2 from NY* and FQ* low sucrose mature fruits	F2 from NY* and FQ* high sucrose mature fruits	13	10 (76.9%)	1
Summary			1518	892 (58.8%)	304

*MSL: Melon Substraction Library
*NY: Noy-Yizre'el
*FQ: Faqqous

(*Cucumis.melo* var.inodorus). In addition, 41 melon carotenoid/organic acids related genes and 24 sugar metabolism related genes that were cloned based on homology to conserved sequences in other plant species were included through the generous contribution of Dr. Ari Schaffer (Volcani Inst., Israel) (Table 2.3 and Table 2.5) in order to enlarge the gene sequence repertoire associated with fruit quality.

Approximately 5040 cDNA clones were randomly selected from the libraries and gene collections mentioned above and sequenced using a single-pass sequencing strategy involving an in house ABI 3100 capillary DNA sequencer. After removing vector sequence and melon insert sequences of low quality by Phred (Ewing and Green, 1998), a total of 3737 (74.1% of the total) sequences were retained as high quality ESTs (Phred quality values greater than 20). The lengths of these ESTs ranges from 101 to 823 bp (100 bp cut-off for minimum acceptable), with the average of 463 bp. Approximately 36% are longer than 500 bp (Figure 2.1). The 3737 high quality ESTs, together with an additional 1800 collaborator-contributed melon phloem ESTs (Table 2.4) and 118 published melon genes (available at the time our ESTs were developed) from GenBank, were then assembled together with the expectation of identifying more unigenes. The assembly of the combined 5670 ESTs resulted in 2422 singletons and 847 overlapping contigs (designated MU1 ~ MU 847), giving 3269 unigenes in total. The number of ESTs in each contig is from 2 to 72 (Figure 2.2). 73% of those contigs were defined by 2 or 3 ESTs and 49 contigs contained more than 10 ESTs. The largest two contigs had 72 (MU1) and 62 (MU115) ESTs, respectively, and were derived entirely from the ESTs of the phloem libraries. The mean length of the contigs is 689 bp, with the longest assembled sequence reaching 3576 bp (MU188) and the shortest 105bp

Table 2.3 Carotenoid biosynthesis, organic acid metabolism and sugar metabolism related gene collection

	Cultivar	Tissue	Sequenced ESTs	Quality ESTs	Singletons
Carotenoid Biosynthesis & Organic Acid metabolism related genes	Noy-Yizre'el	fruit	41	37 (90.2%)	20
Sugar metabolism related genes	Noy-Yizre'el	fruit	24	22 (91.7%)	17

Table 2.4 Melon phloem libraries

	Cultivar	Tissue	Quality ESTs	Singletons
Melon Phloem Library F	Hales Best Jumbo	phloem sap stem collected between the 4th leaf and the 6th leaf from plants bearing fruits	1078	363
Melon Phloem Library UN	Hales Best Jumbo	phloem sap stem collected between the 4th leaf and the 6th leaf from plants not bearing fruits	722	272

Table 2.5 Melon Gene Pathway Collections

	Clone ID	GenBank Acc#	Annotation	E value
Carotenoid Biosynthesis related genes	SSH9H5	DV632337	Geranylgeranyl pyrophosphate synthase [Lupinus albus]	3e-43
	SSH9H7	DV632338	Geranylgeranyl hydrogenase [Glycine max]	1e-105
	FR13P16	DV633488	Geranylgeranyl reductase [Prunus persica]	5e-029
	SSH9H13	DV632328	Phytoene synthase [Momordica charantia var. abbreviata]	1e-122
	SSH9H20	ES597103	Phytoene desaturase [Momordica charantia var.abbreviata]	1e-127
	SSH9H19	ES597102	Zeta-carotene desaturase [Helianthus annuus]	1e-119
	MU254	DV632329	Lycopene-beta-cyclase [Citrus maxima]	4e-76
	MU238	DV632326	Beta-carotene hydroxylase 2 [Capsicum annuum]	5e-60
	FP12P13	DV633172	Zeaxanthin epoxidase [Thellungiella halophila]	1e-022
Organic Acid metabolism related genes	MU263	DV632335	malate dehydrogenase [Glycine max]	1e-119
	MU271	DV632312	malic enzyme/ oxidoreductase [Arabidopsis thaliana]	1e-117
	MU29	ES597096	Actin [Cucumis sativus]	1e-118
	MU300	DV632321	NADP-dependent malic enzyme (NADP-ME) [Vitis vinifera]	0
	MU493	DV632316	Phosphoenolpyruvate carboxykinase (PEPCK [Cucumis sativus]	1e-131
	MU501	DV632323	aconitase [Cucumis melo]	0
	MU612	DV632336	ATP citrate lyase b-subunit [Lupinus albus]	0
	MU666	DV632319	NAD-dependent malate dehydrogenase [Prunus persica]	1e-160
	SSH9G10	DV632310	mitochondrial citrate synthase precursor [Citrus junos]	7e-068
	SSH9G13	ES597095	Malate synthase [Cucumis sativus]	2e-56

Table 2.5 (Continued)

	Clone ID	GenBank Acc#	Annotation	E value
	SSH9G14	DV632313	Citrate synthase [Cucurbita maxima]	3e-029
	SSH9G17	DV632315	phosphoenolpyruvate carboxylase [Cucumis sativus]	2e-045
	SSH9G20	ES597097	Proton-translocating inorganic pyrophosphata [Cucurbita moschata]	1e-117
	SSH9G21	DV632318	malate dehydrogenase precursor [Medicago sativa]	2e-063
	SSH9G22	ES597098	phosphoenolpyruvate carboxylase [Cucumis sativus]	2e-35
	SSH9G24	ES597099	fumarate hydratase [Arabidopsis thaliana]	1e-133
	SSH9G9	DV632324	putative vacuolar assembling protein [Ipomoea trifida]	8e-011
	SSH9H1	DV632325	Inorganic diphosphatase [Flavobacterium johnsoniae UW101]	9e-050
	SSH9H11	DV632327	Vacuolar ATP synthase subunit E [Gossypium hirsutum]	2e-068
	SSH9H9	DV632339	ATP citrate lyase a-subunit [Lupinus albus]	3e-029
Sugar metabolism related genes	MU461	DV632341	acid invertase [Lagenaria siceraria]	1e-103
	MU64	ES597106	alkaline alpha galactosidase II [Cucumis melo]	1e-114
	MU817	ES597112	UDP-glucose 4-epimerase [Solanum tuberosum]	1e-50
	SSH9H24	DV632334	acid alpha galactosidase 1 [Cucumis sativus]	1e-094
	SSH9I1	DV632340	acid alpha galactosidase 2 [Cucumis sativus]	3e-099
	SSH9I10	ES597107	Sucrose-phosphate synthase 1 (UDP-glucose-fructose-phosphate glucosyltransferase 1)	8e-51
	SSH9I11	ES597108	putative sucrose-H+ symporter [Datisca glomerata]	4e-78
	SSH9I14	DV632343	putative alkaline/neutral invertase [Oryza sativa]	1e-100
	SSH9I15	DV632344	neutral invertase [Daucus carota]	1e-107

Table 2.5 (Continued)

	Clone ID	GenBank Acc#	Annotation	E value
	SSH9I16	ES597109	hexokinase [Citrus sinensis]	6e-47
	SSH9I18	DV632345	fructokinase 3 [Lycopersicon esculentum]	1e-101
	SSH9I19	DV632346	fructokinase [Citrus unshiu]	6e-072
	SSH9I2	ES597111	putative galactose kinase [Oryza sativa (japonica cultivar-group)]	3e-10
	SSH9I20	DV632347	UDP-glucose pyrophosphorylase [Populus tremula x Populus tremuloides]	5e-074
	SSH9I21	DV632348	sucrose-phosphate synthase [Actinidia deliciosa]	5e-063
	SSH9I6	ES597113	Phosphoglucomutase, cytoplasmic 2 (Glucose phosphomutase 2) (PGM 2)	8e-54
	SSH9I7	ES597114	plastidial phosphoglucomutase [Pisum sativun	1e-118
	SSH9I9	ES597115	glucose-6-phosphate isomerase [Lycopersicon esculentum]	1e-94

Note: cultivar: Noy-Yizre'el
Tissue: Fruit

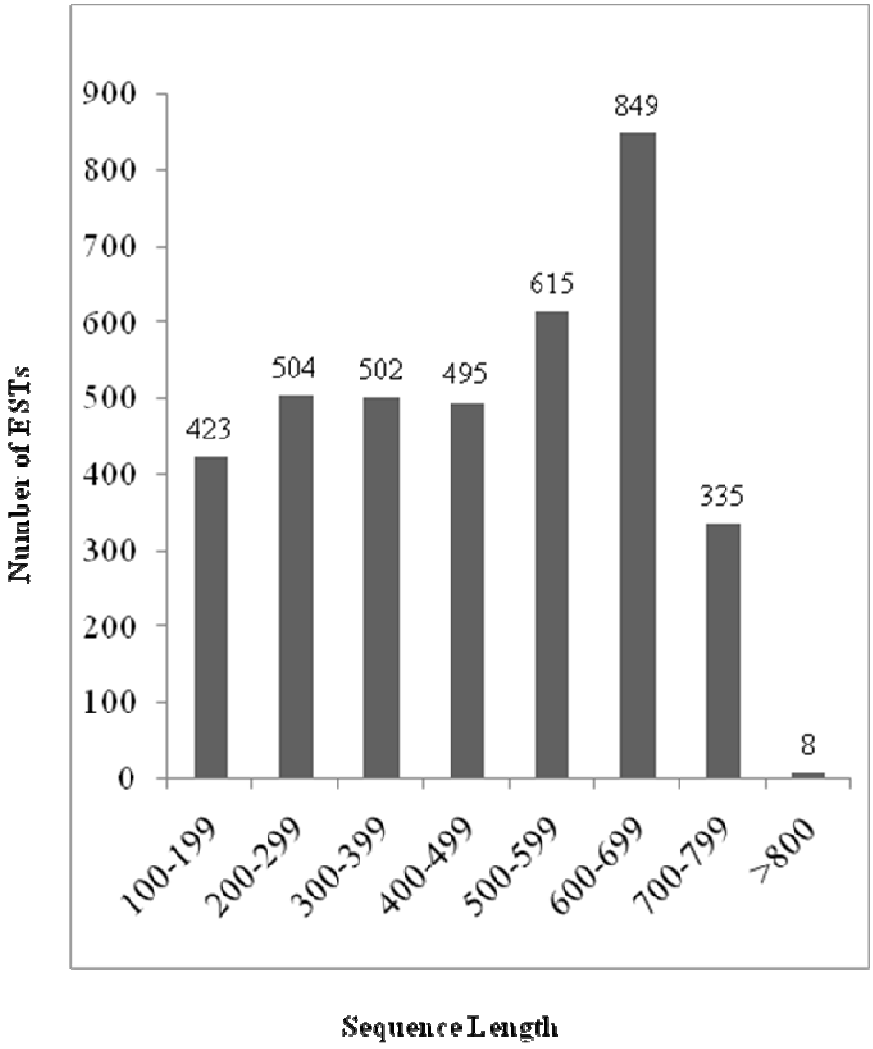


Figure 2.1 Sequence length distribution for melon ESTs

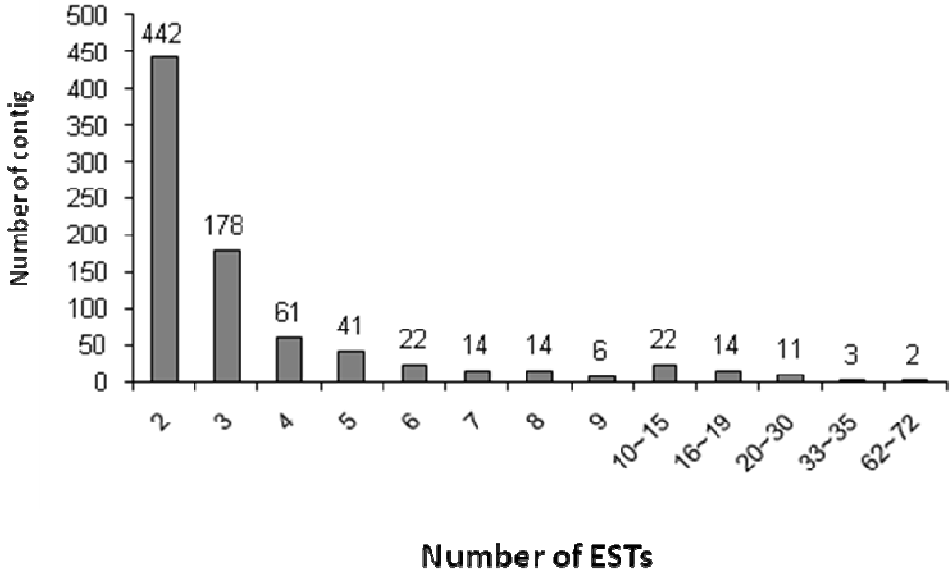


Figure 2.2 Number of ESTs in contigs

(Figure 2.3). The individual sequences of the 3737 high quality ESTs from melon fruits and the 1800 ESTs from melon phloem libraries have been deposited into GenBank. The assigned accession numbers are DV631406-DV635136 for the ESTs from melon fruit and EB714340-EB716139 for melon phloem ESTs. All the information pertaining to assembly contigs, singletons, sequence length, BLAST results and GenBank Accession numbers of the 3737 melon fruit ESTs and the 1800 melon phloem ESTs are stored in a public database and are available at http://www.icugi.org

Annotation and functional classification

The annotation of the unigene set was first performed based on sequence homology. A sequence similarity search was carried out for each of the unique sequences against the non-redundant (nr) gene sequence database at NCBI using the BLASTX program with the e-value cut off set at e $<10^{-5}$. Of the 3269 unique transcripts, 2576 (82%) were identified as having similar sequences at the nucleotide level with either known or unknown function in the database. The primary annotation for each of these unigenes was based on its homologue with the lowest e value (highest sequence similarity). The remaining 593 EST unigene sequences displayed no matches and account for 18% of the total.

InterProScan (Zdobnov and Apweiler, 2001) domain annotation, a protein signature based system for functional prediction of a given sequence, was then applied to all of these unique sequences in order to get meaningful inference of protein function for those that lacked BLASTX-based sequence-related biochemical

characterization and thus extend the degree of annotation. Functional categories were assigned to each of the 3269 unigenes according to Gene Ontology (GO) terms using the InterPro2GO program. Each deduced gene product derived from the unigene sequences was organized into one or more of the following three general ontologies: molecular function, cellular component or biological process. Of the 3269 unique sequences, 29% were assigned to "biological process", 35% to "molecular function" and 14% to "cellular component". The breakdown GO terms of each category are shown in Figure 2.4.

As indicated from the breakdown shown in Figure 2.4, unigenes generated in this study have a wide range of molecular functions and can be associated with a range of different biological processes, indicating the broad functional representation of our melon EST collection. Nevertheless, 36% of the total unigenes could not be functionally assigned and thus are annotated as "biological function unknown". Those, along with the 593 "no-hits-found" sequences, provide good candidates for further new gene identification and potential novel functions associated with melon fruit biology.

EST sequence representation and abundance

Analysis of the abundance of ESTs assembled into contigs assists in the understanding of the relative expression level of particular genes in a given tissue from which the library was constructed. In addition, organ or tissue-specific

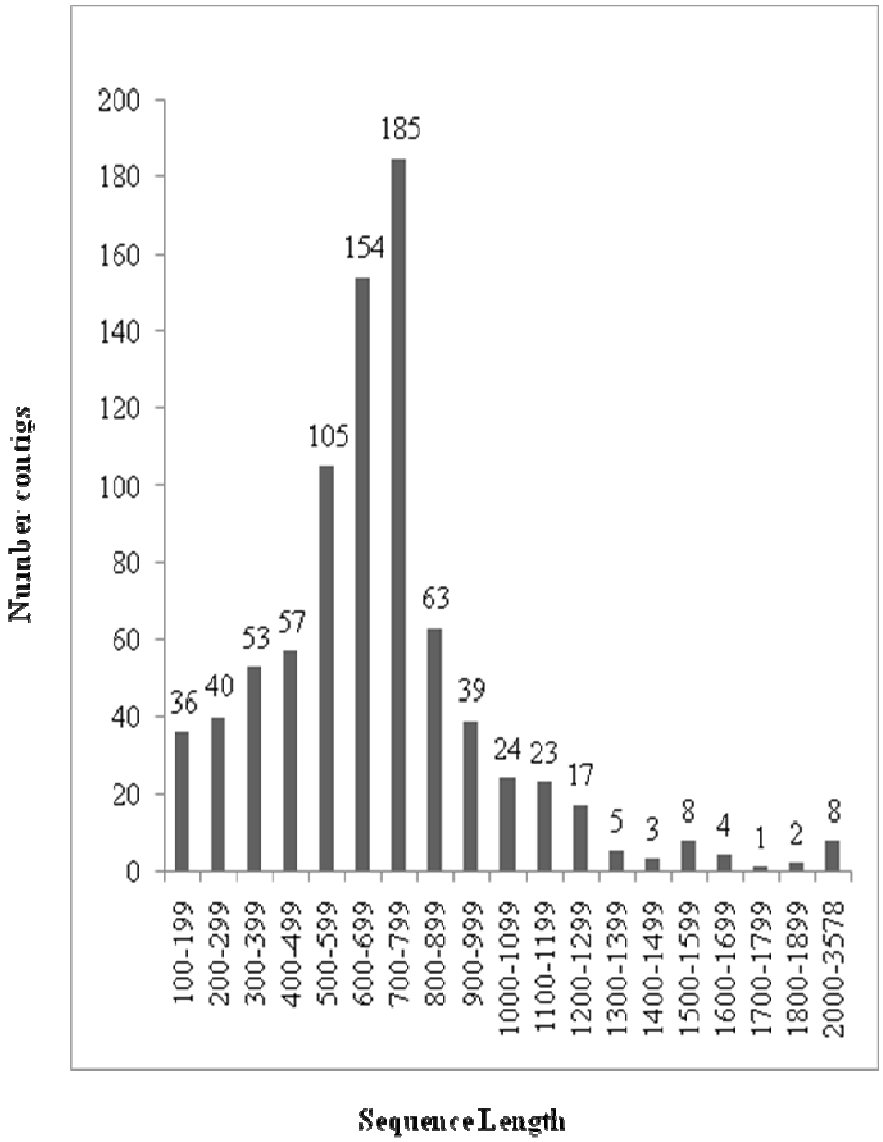


Figure 2.3 Sequence length distribution of the assembled melon contigs

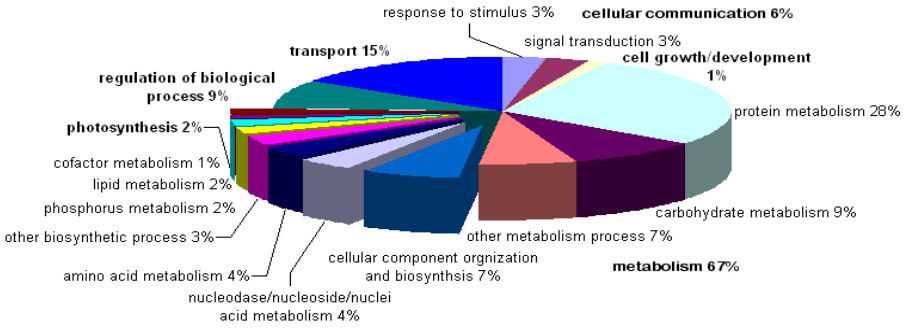
transcripts can be deduced by comparing the library sources from which the clustered ESTs are derived and can facilitate follow-up functional genomic studies.

Considering the relatively small size of the libraries, we set the threshold number of ESTs assembled into contigs and used for relative expression analysis as at least 10 ESTs. The total number of ESTs in each contig was used as the denominator and the number of ESTs contributed by each library was indicated as numerator. There are 49 contigs comprised of 10 or more ESTs (Table 2.6). 17 of the 49 are made up of ESTs that are entirely derived from phloem libraries and 7 were primarily (>90%) from phloem libraries (either bearing fruits or not). Five contigs did not find any matched sequences in the non-redundant (nr) protein database at NCBI with an e-value cut off at $<10^{-5}$ and 1 contig was annotated as "hypothetical protein" based on a sequence similarity search against NCBI.

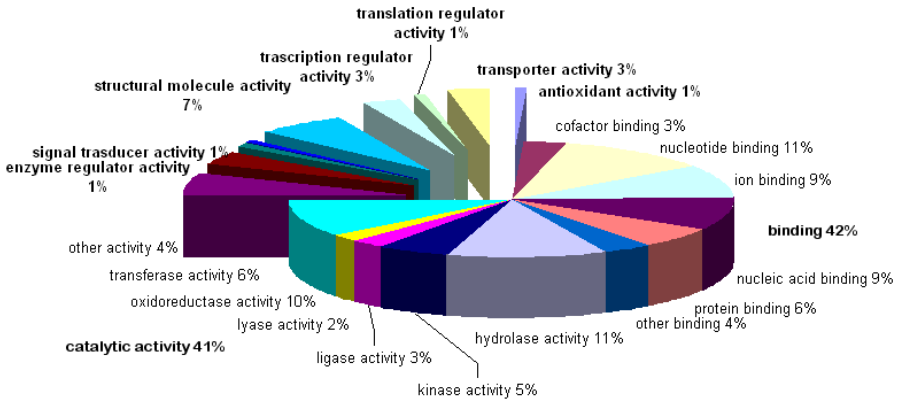
Of the 49 contigs, 16 comprised ESTs derived entirely from fruit cDNA libraries (standard, SSH or both) generated in this study. These contigs include proteins with annotations such as stearoyl-acyl carrier protein desaturase (MU13), aluminum-induced protein (MU60), branched-chain-amino-acid transaminase /catalytic (MU82), alcohol acetyltransferase (MU800), acetyl-CoA C-acetyltransferase (MU276), lipoxygenase (MU26), translation elongation factor 1-gamma (MU207), HMG-CoA reductase (MU332), 2OG-Fe (II) oxygenase (MU184), NADP-dependent malic enzyme (MU300), fructose-bisphosphate aldolase (MU411) and pyruvate decarboxylase (MU400). Among them, the catalytic properties of alcohol acetyltransferase(AAT) (MU800) in ester volatile biosynthesis has been well characterized in melon (El-Sharkawy et al., 2005; Lucchetta et al., 2007; Shalit et al., 2001). This enzyme catalyzes the last step of acetate volatile formation by acylation of alcohols and acetyl-CoA. It has been reported that the expression of MU800 is fruit specific as its mRNA has been reported to accumulate only in fruit tissues and

Figure 2.4 Gene Ontology (GO) mapping result for melon ESTs

Biological process



Molecular function



Cellular component

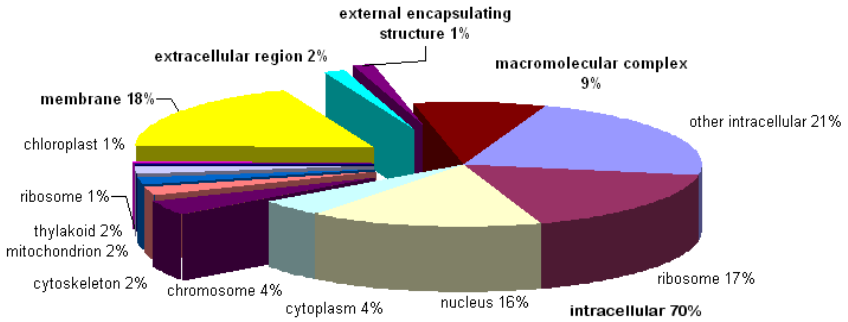


Table 2.6 High abundance EST sequences in the EST collections generated in this study

Contig ID	Total ETSs in cluster	Annotation	E value	EST contribution
MU1	72	Hypothetical protein [Arabidopsis thaliana]	3e-015	Phloem cDNA library (bearing fruit) (52/72) Phloem cDNA library (not bearing fruit) (20/72)
MU115	62	Phloem filament protein; phloem protein 1(PP1) [Cucurbita maxima]	3e-008	Phloem cDNA library (bearing fruit) (39/62) Phloem cDNA library (not bearing fruit) (22/ Young fruit cDNA library (NY) (1/62)
MU343	35	Type 1 metallothionein [Vigna radiata var. radiata]	2e-007	Phloem cDNA library (bearing fruit) (24/35) Phloem cDNA library (not bearing fruit) (10/35) Young fruit cDNA library (NY) (1/35)
MU567	35	Phloem filament protein; phloem protein 1(PP1) [Cucurbita maxima]	3e-024	Phloem cDNA library (bearing fruit) (27/35) Phloem cDNA library (not bearing fruit) (7/35) Young fruit cDNA library (NY) (1/35)
MU455	33	Polyubiquitin UBQ10 [Arabidopsis thaliana]	0	Mature fruit cDNA library (NY) (4/33) Mature fruit cDNA library (Dul) (8/33) Mature fruit cDNA library (TamD) (13/33) Phloem cDNA library (bearing fruit) (1/33) MSL4 (1/33), MSL5 (1/33), MSL7 (2/33), MSL10 (1/33), GenBank (2/33)
MU835	30	Cystein proteinase inhibitor [Cucumis sativus]	6e-011	Phloem cDNA library (bearing fruit) (14/30) Phloem cDNA library (not bearing fruit) (14/30) Young fruit cDNA library (NY) (1/30) Mature fruit cDNA library (NY) (1/40)
MU2	27	Phloem filament protein; phloem protein 1(PP1) [Cucurbita maxima]	1e-025	Phloem cDNA library (bearing fruit) (19/27) Phloem cDNA library (not bearing fruit) (8/27)
MU13	26	Stearoyl-acyl carrier protein desaturase [Cucumis sativus]	0	Mature fruit cDNA library (Dul) (7/26) Mature fruit cDNA library (TamD) (11/26) MSL2 (1/26) MSL4 (3/26), MSL5 (2/26), MSL11 (1/26), MSL14 (1/26)
MU38	23	NTGP4 [Nicotiana tabacum]	1e-054	Phloem cDNA library (bearing fruit) (15/23) Phloem cDNA library (not bearing fruit) (8/23)
MU60	22	Aluminum-induced protein [Codonopsis lanceolata]	1e-109	Young fruit cDNA library (NY) (1/22) Mature fruit cDNA library (NY) (2/22) Mature fruit cDNA library (Dul) (16/22) Mature fruit cDNA library (TamD) (3/22)
MU71	22	Putative major latex protein [Momordica charantia]	2e-037	Mature fruit cDNA library (NY) (5/22) Mature fruit cDNA library (Dul) (5/22) Phloem cDNA library (bearing fruit) (1/22) MSL4 (2/22), MSL5 (4/22), MSL8 (3/22), MSL12 (1/22), GenBank (1/22)
MU49	22	NTGP4 [Nicotiana tabacum]	2e-048	Phloem cDNA library (bearing fruit) (13/22) Phloem cDNA library (not bearing fruit) (9/22)
MU82	20	Branched-chain-amino-acid transaminase/catalytic [Arabidopsis thaliana]	1e-142	Mature fruit cDNA library (NY) (1/20) Mature fruit cDNA library (Dul) (14/20) Mature fruit cDNA library (TamD) (5/20)

Table 2.6 (Continued)

Contig ID	Total ETSs in cluster	Annotation	E value	EST contribution
MU800	20	Alcohol acetyltransferase [Cucumis melo]	0	Mature fruit cDNA library (Dul) (7/20) Mature fruit cDNA library (TamD) (3/20) MSL2 (2/20), MSL5 (2/20), MSL8 (1/20), MSL12 (2/20), GenBank (1/20), other (1/20)
MU241	20	Hypothetical protein [Macaca fascicularis]	4e-013	Young fruit cDNA library (NY) (1/20) Mature fruit cDNA library (Dul) (5/20) Mature fruit cDNA library (TamD) (12/20) Phloem cDNA library (not bearing fruit) (2/20)
MU104	20	26 kDa phloem lectin [Cucumis melo]	1e-137	Young fruit cDNA library (NY) (1/20) Phloem cDNA library (bearing fruit) (13/20) Phloem cDNA library (not bearing fruit) (5/20) GenBank (1/20)
MU26	19	Lipoxygenase [Cucumis sativus]	3e-046	MSL10 (19/19)
MU116	19	Not hits found		Phloem cDNA library (bearing fruit) (11/19) Phloem cDNA library (not bearing fruit) (7/19) MSL6 (1/19)
MU127	19	Not hits found		Phloem cDNA library (bearing fruit) (13/19) Phloem cDNA library (not bearing fruit) (6/19)
MU161	19	Actin depolymerizing factor-like protein [Arachis hypogaea]	1e-065	Mature fruit cDNA library (NY) (1/19) Mature fruit cDNA library (Dul) (3/19) Mature fruit cDNA library (TamD) (2/19) Phloem cDNA library (bearing fruit) (6/19) Phloem cDNA library (not bearing fruit) (7/19)
MU150	18	Type-2 metallothionein [Citrullus lanatus]	1e-022	Young fruit cDNA library (NY) (2/18) Mature fruit cDNA library (NY) (4/18) Mature fruit cDNA library (Dul) (2/18) Mature fruit cDNA library (TamD) (5/18) Phloem cDNA library (bearing fruit) (2/18) Phloem cDNA library (not bearing fruit) (3/18)
MU172	18	Protein binding / ubiquitin-protein ligase / zinc ion binding [Arabidopsis thaliana]	1e-024	Phloem cDNA library (bearing fruit) (12/18) Phloem cDNA library (not bearing fruit) (6/18)
MU93	17	ATPP2-A9 [Arabidopsis thaliana]	2e-026	Phloem cDNA library (bearing fruit) (8/17) Phloem cDNA library (not bearing fruit) (9/17)
MU207	16	Translation elongation factor 1-gamma [Prunus avium]	1e-035	MSL10 (16/16)
MU218	16	HOG1; adenosylhomocysteinase [Arabidopsis thaliana]	0	Mature fruit cDNA library (Dul) (7/16) Mature fruit cDNA library (TamD) (2/16) MSL1 (1/16), MSL5 (1/16), MSL8 (1/16), MSL10(2/16), MSL14 (1/16), GenBank (1/16)
MU139	16	Translation elongation factor 1A-2 [Gossypium hirsutum]	0	Mature fruit cDNA library (Dul) (1/16) Mature fruit cDNA library (TamD) (2/16) Phloem cDNA library (bearing fruit) (7/16) Phloem cDNA library (not bearing fruit) (3/16) MSL2 (1/16), MSL8 (1/16), MSL10 (1/16)

Table 2.6 (Continued)

Contig ID	Total ETSs in cluster	Annotation	E value	EST contribution
MU229	16	Unknown protein	6e-031	Phloem cDNA library (bearing fruit) (10/16) Phloem cDNA library (not bearing fruit) (6/16)
MU184	14	2OG-Fe(II) oxygenase [Medicago truncatula]	1e-086	Mature fruit cDNA library (Dul) (3/14) Mature fruit cDNA library (TamD) (3/14) MSL2 (3/14), MSL4 (1/14), MSL12 (3/14), other (1/14)
MU240	13	Polyprotein [Zucchini yellow mosaic virus]	1e-100	Young fruit cDNA library (NY) (2/13) Mature fruit cDNA library (NY) (5/13) MSL1 (3/13), MSL8 (2/13), MSL9 (1/13)
MU276	13	Acetyl-CoA C-acetyltransferase [Arabidopsis thaliana]	0	Mature fruit cDNA library (NY) (2/13) Mature fruit cDNA library (Dul) (7/13) Mature fruit cDNA library (TamD) (1/13) MSL4 (1/13), MSL12 (1/130), other (1/13)
MU264	13	Hypothetical protein [Oryza sativa]	6e-007	Mature fruit cDNA library (NY) (1/13) Phloem cDNA library (bearing fruit) (7/13) Phloem cDNA library (not bearing fruit) (5/13)
MU287	13	Unknown protein [Arabidopsis thaliana]	1e-061	Phloem cDNA library (bearing fruit) (7/13) Phloem cDNA library (not bearing fruit) (6/13)
MU252	12	Putative mitochondrial dicarboxylate carrier protein [Trifolium pratense]	1e-006	Young fruit cDNA library(NY) (5/12) Mature fruit cDNA library (Dul) (2/12) Mature fruit cDNA library(TamD) (5/12)
MU310	12	No hits found		Phloem cDNA library (bearing fruit) (6/12) Phloem cDNA library (not bearing fruit) (6/12)
MU321	12	Phloem protein 2, PP2 [Cucurbita maxima, cv Big Ma	4e-033	Phloem cDNA library (bearing fruit) (7/12) Phloem cDNA library (not bearing fruit) (5/12)
MU677	12	17 kDa phloem lectin [Cucumis melo]	2e-083	Phloem cDNA library (bearing fruit) (7/12) Phloem cDNA library (not bearing fruit) (5/12)
MU678	12	17 kDa phloem lectin [Cucumis melo]	2e-088	Phloem cDNA library (bearing fruit) (6/12) Phloem cDNA library (not bearing fruit) (3/12) MSL7 (1/12), GenBank (2/12)
MU332	12	HMG-CoA reductase [Cucumis melo]	0	Young fruit cDNA library (NY) (1/12) Mature fruit cDNA library (NY) (1/12) Mature fruit cDNA library (Dul) (6/12) MSL4 (1/12), MSL15 (1/12), MSL16 (1/12), GenBank (1/12)
MU344	11	Not hits found		Mature fruit cDNA library (NY) (1/11) Young fruit cDNA library (NY) (2/11) Mature fruit cDNA library (TamD) (7/11) GenBank (1/11)
MU300	11	NADP-dependent malic enzyme (NADP-ME) [Vitis vinifera]	0	Mature fruit cDNA library (NY) (1/11) Young fruit cDNA library (NY) (1/11) MSL1 (4/11), MSL5 (1/11), MSL8 (2/11), Other (2/11)

Table 2.6 (Continued)

Contig ID	Total ETSs in cluster	Annotation	E value	EST contribution
MU355	11	Unknown protein [Arabidopsis thaliana]	1e-052	Phloem cDNA library (bearing fruit) (8/11) Phloem cDNA library (not bearing fruit) (3/11)
MU377	11	Putative senescence-associated protein [Pisum sativum]	8e-094	Mature fruit cDNA library (NY) (2/11) Mature fruit cDNA library (TamD) (4/11) Phloem cDNA library (bearing fruit) (2/11) Phloem cDNA library (not bearing fruit) (3/11)
MU411	10	Fructose-bisphosphate aldolase [Persea americana]	0	Young fruit cDNA library (NY) (1/10) Mature fruit cDNA library (Duc) (4/10) Mature fruit cDNA library (TamD) (2/10) MSL2 (1/10), MSL9 (2/10)
MU366	10	Unknown protein [Arabidopsis thaliana]	2e-021	Phloem cDNA library (bearing fruit) (4/10) Phloem cDNA library (not bearing fruit) (6/10)
MU400	10	Pyruvate decarboxylase [Fragaria x ananassa]	1e-165	Mature fruit cDNA library (Dul) (9/10) GenBank (1/10)
MU422	10	No hits found		Phloem cDNA library (bearing fruit) (5/10) Phloem cDNA library (not bearing fruit) (5/10)
MU433	10	Putative histone H2A [Oryza sativa]	3e-042	Young fruit cDNA library (NY) (1/10) Mature fruit cDNA library (Duc) (7/10) Phloem cDNA library (bearing fruit) (2/10)
MU444	10	Unknown protein [Arabidopsis thaliana]	2e-006	Phloem cDNA library (bearing fruit) (7/10) Phloem cDNA library (not bearing fruit) (3/10)
MU456	10	No hits found		Phloem cDNA library (bearing fruit) (5/10) Phloem cDNA library (not bearing fruit) (5/10)

in concert with maturaton (Aggelis et al., 1997). This result is in agreement with our EST resource based expression analysis. MU800 was derived entirely from ESTs derived from libraries constructed with fruit tissues in support of a fruit-specific expression pattern.

The number of contigs consisting of ESTs that were restricted to a single fruit library was low. Only two different contigs, MU26 (lipoxygenase) and MU207 (translation elongation factor1-gamma), contained ESTs all from the same fruit library (MSL 10). MSL 10 is a subtractive library that was constructed by substracting mixed stages of fruits tissue from Noy-Yizre'el (*Cucumis melo* var.reticulatus.), a cultivar having fruits with high sugar content, from that of Faqqous (*subsp. melo* var. flexuosus), a cultivar whose fruits produce low sucrose. This data suggests that the transcripts of these two genes are more abundant in Faqqous fruits compared with Noy-Yizre'el. Whether or not either may be related to sugar phenotypes remains unknown but may be an interesting avenue of study.

Few abundant ESTs show a high degree of similarity with previously characterized cDNA sequences isolated from melon fruits by other research groups (primarily due to the fact that relatively few melon genes have been characterized to date). For example, MU139 (16 ESTs), a elongation factor-1 alpha, has 98% sequence identity (e value: 0.0) with Cmf-123 (GenBank# AT007014). Cmf-123 was obtained from a 9 DPA Charentais melon fruit (*Cucumis melo* L cv. Reticulatus) cDNA library subtracted by ovary tissue (Choi et al., 2004). RT-PCR data showed that the expression of this gene increased during early fruit development (from 0 DPA to 27 DPA) and decreased dramatically at 36DP. Cmf-123 mRNA was also detected at moderate level in leaves. In our study, the MU139 contig is assembled by

ESTs both from fruit and phloem cDNA libraries, consistent with the notion that the expression of MU139/Cmf-123 is not limited to fruit.

MEL7 and MEL 2 are the longest ESTs in the MU7 (22 ESTs, a major latex protein) and MU800 (20 ESTs, an alcohol acetyltransferase) contigs, respectively. MEL7 and MEL2 were initially identified together as ripening-related genes by differential screening of a melon (*Cucumis melon* L.cv. Cantaloup charentais) ripe fruit cDNA library (Aggelis et al., 1997). Both of these sequences were highly induced during the ripening of cantaloup melon fruit and it has been reported that MU71/ MEL 7 is expressed in different organs of melon plants, including roots, stems, seeds, leaves, ovary and petals (Aggelis et al., 1997). On the other hand, the expression of MU800 / MEL2 is more fruit specific as its mRNA has been reported to accumulate only in fruit (Aggelis et al., 1997). This result is consistent with what we observed in that MU800 was assembled by ESTs all from fruit cDNA libraries while the MU71/MEL7 contig contains ESTs from only fruit and phloem libraries.

The largest contig, MU1, contains 72 ESTs all derived from phloem libraries and is annotated as a Hsp20/alpha crystallin family protein based on sequence similarity searches. Proteins in this family are characterized by the presence of an alpha crystallin domain at their C-terminus (de Jong et al., 1998; Kato et al., 1994). Hsp20/alpha crystalline family proteins exist in almost all organisms and have been found to have diverse functions ranging from cytoskeleton protection to stress response (Bruey et al., 2000; Caspers et al., 1995; Liang et al., 2008). One important function of Hsp20/alpha crystallin proteins is as molecular chaperones, assisting in the folding of denatured protein (Studer and Narberhaus, 2000). For the

contigs that were constructed entirely of ESTs derived from phloem libraries, phloem structural proteins, such as phloem filament protein 1 (PP1), phloem filament protein 2 (PP2) and the 17 KD phloem lectin (homologous to PP2), were dominant, indicating both the quality of the library and the abundance of these proteins in melon phloem.

Characterization of melon ESTs from suppressive subtractive hybridization libraries (MSL)

The suppressive subtractive hybridization library has long been used as a powerful tool for identifying low abundance differentially expressed and tissue/stage-specific genes. With the aim of discovering important genes controlling the major metabolism components associated with melon fruit quality, such as aromatic volatiles, sugar content and shelf life, as well as increasing the gene sequences repertoire associated with fruit development, a series of SSH libraries were constructed by using fruit tissues from various genotypes and comprising a range of developmental stages. These melons included varieties belonging to both climacteric (e.g. Vedrantaise, Noy Yizre'el, Dulce) and non-climacteric types (Rochet). Varieties with high and low sugar content (Noy yizre'el versus Faqqous) and varieties having orange or greenish flesh (Vedrantaise versus Noy Yizre'el and Dulce versus Rochet) were also targeted as possessing important traits for commercial melon production. Clones with smaller insert sizes (<400 bp) were typically found from SSH as compared to standard cDNA libraries when the PCR process was performed to amplify and characterize inserts from the SSH libraries. This is to be expected as the SSH process generally results in shorter cDNA fragments. These EST sequences together with the putative singletons in each SSH

library (labeled from MSL1 to MSL17) are summarized in Table 2.2. The individual unigene ID, clone ID, GenBank accession numbers, plus the annotation and e value for the ESTs in each MSL are listed in supplemental tables 1 to 17(data not shown).

Melon cDNA Microarray and Validation

In total 3068 unigenes (78% are from fruit cDNA libraries, 22% are from phloem libraries) was available following amplification of unigenes by PCR. Note that this is slightly less than the total number of available unigenes due to the fact we did not have access to all cloned sequences deposited in GENBANK by other researchers and some clones failed to yield amplifiable PCR products. The PCR products were assessed using gel electrophoresis and then purified and along with 4 negative controls (printing buffer only), printed in triplicate on glass sides coated with γ-amino-propyl-silane using a MicroGrid Pro arrayer (BioRobotica inc., Boston, MA, USA). The resulting cDNA microarray thus contains a total of 9216 spots (3072 x 3).

The false-positive rate of the melon cDNA microarray was first assessed using "self-self" hybridizations. Specifically, 3 total RNA samples representing 3 different biological replicates isolated from the over ripe stage of Dulce (*Cucumis melo* var. *reticulatus*) fruit tissues were reverse-transcribed independently. The synthesized cDNA probes from the same biological replicate were aliquoted into two batches, labeled with either Cy3 or Cy5, and then directly compared on the same slide. As the Cy3/Cy5 labeled probes were from the same RNA source, we anticipated no significant differentially expressed genes in this comparison if all conditions were optimal. Nevertheless, in any array hybridization experiment, due to

cross-hybridization between homologous gene family members or other genes that share a significant degree of sequence similarity (or due to experimental variation introduced by technical and/or biological variation during hybridization), a false discovery rate (FDR) of differentially expressed genes is found in even the most rigorously controlled situations (Higdon et al., 2008; Pawitan et al., 2005). The FDR can be minimized using additional stringency criteria such as adjusting confidence levels and incorporating a minimum fold-change cutoff for accepting data for further analysis. Generally the acceptable FDR is less than 0.05 for microarray experiments (Higdon et al., 2008) which means for every 100 differentially expressed genes we identified, there is a chance that less than five of them were false (i.e. caused by reasons other than their true biological variation).

Following hybridization in our self-self test, the resulting raw data were normalized and analyzed using the statistical methods reported in Alba et al (2005). The number of differentially expressed genes identified at different confidence levels is shown in Table 2.7 to demonstrate the utility of adjusting confidence levels in creating data subsets with higher reliability. As the confidence level increased, the number of differentially expressed genes in the self-self comparison experiment decreased as expected. When the level of confidence is at or greater than 0.90, the FDR is equal to zero without addition of any fold-change cutoff. The same results were obtained when the hybridizations were repeated using different stages of fruits from other varieties (data not show). This result demonstrated that the microarray design, combined with the related experimental procedures and the subsequent statistical methods employed for our analysis generates very low FDR for the melon arrays and procedures used in this and subsequent experiments.

Transcriptome analysis of immature and mature melon fruit

In an initial transcriptome analysis application of the melon cDNA microarray we attempted to identify genes differentially expressed in 24DPA (immature) vs mature ripe stage fruits of non-climacteric Tam Dew melon fruit. Tam Dew melon fruit are typified by greenish flesh and a smooth rind. Fruit ripening behavior lies between typical climacteric and non-climacteric melon types in that trace amounts of ethylene can be detected at the onset of ripening (data not shown). Temdew fruit at 24 DPA basically reaches its final size. The fruit is firm with bright rind color, white flesh and no aroma. At the mature ripe stage, the rind color of TemDew fruit turns to white yellow and the flesh becomes white-green. Though the mature fruit is still firm, it is not as hard as at 24 DPA (Choi et al., 2004; Portnoy et al., 2008). Total RNA was isolated from the two stages mentioned above with 3 biological replicates with pairs of replicate cDNA probes hybridized together on the slides for a total of 3 biological replicates with three more technical replicas in the form of dye-swaps. The resulting data were analyzed using statistical tools summarized in Alba et al. (2005). The resulting data indicated that 7.8% of the genes on the array (representing 238 unigenes) were differentially expressed (stringency criteria set at FDR $\leq$ 0.05, fold difference $\geq$ 2). Of the total 238 differentially expressed unigenes, 152 were induced at the mature stage and 86 were up-regulated in the immature fruit.

For the 86 genes that were induced during the immature stage (24 DPA), 41% were annotated as “biological function unknown”. The remainder fell into a wide variety of functional categories which include photosynthesis (14%), phloem proteins (7%), binding (such as GTP binding, calcium binding and protein binding)

Table 2.7 False Positive Rate (FDR) test results

Confidence rate	Number of up regulated genes	Number of down regulated genes
0.5	2162	1945
0.55	1970	1700
0.6	1750	1420
0.65	1582	1005
0.7	907	508
0.75	487	29
0.8	51	5
0.85	2	2
0.9	0	0
0.95	0	0

(7%), transcription factors (6%), biotic/abiotic stress response and carbohydrate metabolism (5%).

Five transcription distinct factors were up-regulated at 24 dpa. They include homeodomain Hfi22, IAA7 (AUXIN RESISTANT 2), GATA transcription factor 1 (GATA-1), putative ethylene-responsive element binding protein (EREBP) and a bZIP transcription factor, bZIP_1. The homeodomain protein Hfi22 is a homeodomain leucine zipper (HD-Zip) family transcription factor which contains a leucine zipper motif adjacent to the homeodomain. Proteins belonging to this family exhibit diverse functions in plant development including meristem developmental regulation, organ and vascular development and hormone action mediation (Ariel et al., 2007). bZIP transcription factors contain a basic region that binds to DNA and a leucine zipper dimerization motif. Members of the family have been shown to be involved in the regulation of biological processes including pathogen defense, light and stress signaling, seed maturation and flower development (Jakoby et al., 2002).

Arabidopsis HY5 (LONG HYPOCOTY 5) is one of the most extensively studied bZIP family transcription factors in terms of plant development. HY5 is a positive regulator of light signaling that works down stream of photoreceptors for promoting photomorphogenesis (Ang et al., 1998; Oyama et al., 1997; Ulm et al., 2004). With regards to fruit development and quality, the tomato LeHY5 homolog regulates fruit color formation during ripening by manipulating carotenoid biosynthesis through light signaling pathways (Liu et al., 2004). GATA transcription factor 1 (GATA-1) binds to the GATA motif found in the promoter regions of many light and circadian responsive genes. It has been reported that GATA transcription factors are involved in regulating the expressions of light responsive genes, seed

germination (Liu et al., 2004), chlorophyll synthesis, glucose sensitivity (Bi et al., 2005), shoot apical meristem determination and flower development (Zhao et al., 2004). IAA7 is a member of the *IAA* family of auxin-inducible genes. IAA7 mutation in Arabidopsis causes gravitropic root and shoot growth, short hypocotyls and stems, and auxin-resistant root growth, indicating IAA7 influences many aspects of development in light-grown seedlings (Nagpal et al., 2000). Finaly, EREBPs are involved in ethylene hormone signaling. In summary, the transcription factors showing induction at 24 dpa relative to mature fruit are associated with diverse roles in plant development.

Even though TamDew melon has been traditionally classified as a non-climacteric fruit in terms of its ripening behavior, an average of 4 PPM/Kg/Hr ethylene was detected at the horticulture maturity stage. At the equivalent stage, the more typical climacteric type fruit, Dulce, produced an average of 80 PPM/Kg/Hr ethylene (data not shown). For the 152 unigenes that were up regulated in mature ripe Tam Dew fruit, 38% were annotated as biological function unknown, 10% (16 genes) have been associated with ripening related fruit development. Additional functional categories include stress response (15%) and protein metabolism (8%). The ripening related genes that were up-regulated at the mature stage relative to immature fruit include ACC Oxidase (ACO), ACC synthase (ACS), homocysteine S-methyltransferase and methionine synthase which are all involved in ethylene biosynthesis and regulation (Cara and Giovannoni, 2008). Xyloglucan endotransglycosylase, beta-D-galactosidase and polygalacturonase play important roles in fruit cell wall metabolism and have been associated with ripening-related textural changes (Brummell and Harpster, 2001) and alcohol acetyltransferase encodes the rate-limiting enzyme catalyzing the final step of aroma ester biosynthesis

in melon fruit (El-Sharkawy et al., 2005; Lucchetta et al., 2007; Shalit et al., 2001). The expression patterns of 10 genes including those described above have been documented in published studies from a number of different research groups (Table 2.8) and correlate with our array results.

Four transcription factors were shown to be up-regulated at the mature stage in Tam Dew and include the following: a NAC4 protein, a bZIP transcription factor (AtZIP60), a GRAS transcription factor and an EREBP. The NAC family of proteins represent plant specific transcription factors that has been previously reported to play important roles in embryonic, floral and vegetative development, lateral root formation and auxin signaling (Olsen et al., 2005). The tomato NOR protein belongs to the NAC family and the tomato *nor* mutant displays inhibition of all major ripening phenotypes including fruit softening, post-harvest pathogen susceptibility, accumulation of color pigments and synthesis of flavor and aroma volatiles indicating that the normal NOR protein represents a global regulator of fruit ripening processes (Vrebalov J and Giovannoni JJ. unpublished data). Tomato SENU5 (Senescence up-regulated) is a senescence related NAC family protein whose mRNA accumulated during leaf senescence (John et al., 1997). The expression of 20 NAC transcription factors have also recently been shown to be differentially expressed during leaf senescence (Guo and Gan, 2005). One of them, AtNAP, has been identified as a key negative regulator of leaf senescence (Guo and Gan, 2006). As senescence is considered to be a component of the final phase of fruit ripening, it is very interesting to note the function of the senescence related NAC domain proteins in fruit development.

Table 2.8 Genes whose expression patterns have been confirmed in the literature

Clone ID	Sequence homology	E value	Data from microarray	Varified method	Varified in cultivar	Literatures published
FR13N15 FR15J17	Cm-ACO1	3.00E-83	up in mature stage	Northern	*Cucumis melo L.cv* AMS	Miki T. et al. (1995)
SSH1N10	ACS	2E-33	up in mature stage	Northern	TamDew	Katzir N. (unpublished data)
SSH1L18	Cm-AAT1	0	up in mature stage	qRT-PCR Norther	Cantaloup (*C.melo* var.*Cantalupensis, Naud cv, Vedrantais*)	EI-Sharkawy, I. et al.(2005) Yahyaoui, F. et al. (2002)
FR14L3	Cm-AAT2	9.00E-113	up in mature stage	qRT-PCR Norther	Cantaloup (*C.melo* var.*Cantalupensis, Naud cv, Vedrantais*)	EI-Sharkawy, I. et al.(2005) Yahyaoui, F. et al.(2002)
FR14B12	MPG1	9.00E-285	up in mature stage	Northern	Cantaloup (*C.melo* var.*Cantalupensis, Naud cv, Vedrantais*)	Nishiyama K. et al.(2007)
SSH9B1	Cm-EXP1	6.00E-167	up in mature stage	Northern	Cantaloup (*C.melo* var.*Cantalupensis, Naud cv, Vedrantais*)	Nishiyama K. et al.(2007)
SSH1C21	RM13	2E-124	up in mature stage	Northern	Cantaloup (*C.melo* var.*Cantalupensis, Naud cv, Vedrantais*)	Hadfield KA. et al.(2000)
FR17M11	Cm-CCD	2E-148	Equally expressed	qRT-PCR	TamDew (*C.melo* var. *inodorus)*	Ibdah, M. et al.(2006)
FR15D13	Cm-ETR1	0	Equally expressed	Northern	Muskmelon (*C.melo* L. *reticulatus*)	Sato-Nara, K. et al.(1999)

The bZIP transcription factor AtZIP60 was predicted to be involved in endoplasmic reticulum (ER) mediated stress response by activating the expression of chaperone genes, including *BiP* and Hsp70 (Iwata and Koizumi, 2005). The GRAS family of transcription factors are named as an acronym representing the first three genes cloned and characterized: GIBBERELLINE-ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA) and SCARECROW (SCR) (Bolle, 2004; Di Laurenzio et al., 1996; Paine et al., 2005; Peng et al., 1997). The importance of the GRAS gene family in plant biology has been established by functional analyses of SCR, GAI and RGA. GAI and RGA are involved in the gibberelline signal transduction pathway (Dill et al., 2001; Peng et al., 1997) . SCR is involved in root patterning (Pysh et al., 1999). The high expression of these transcription factors at the mature stage in Tem Dew fruit make them good candidates for regulation of fruit ripening.

Validation of microarray results via quantitative real-time RT-PCR (qRT-PCR)

In order to verify our microarray results beyond the collection of genes whose expression patterns are consistent with our array results as has been reported in the literature, five additional genes were selected at random for validation via real-time RT-PCR analysis (Table 2.9). Based on the array data, three of the five genes were up regulated in the mature stage fruit and 2 were more highly expressed in the 24 dpa tissue (stringency criteria of fold change $\geq$ 2.0 and FDR $\leq$ 0.05). Gene-specific primers for the five genes were designed using Primer Express software

Table 2.9 Genes tested for qRT-PCR conformation

Clone ID	Sequence homology	E value	Folde change	P value
Up in 24 dpa stage				
FR17C10	Homeodomain protein Hfi22 (Nicotiana tabacum)	6.00E-58	4.4	0.029
PH4B8	Auxin resistant 2 (Arabidopsis thaliana)	3.00E-102	3.1	0.029
Up in mature stage				
FR11B10	Sugar transporter (Arabidopsis thaliana)	3.00E-100	2.4	0.03
FR15F22	ANC4 protein (Glycine Max)	6.00E-110	8.3	0.029
FR17E5	Auxin and ethylene cross-talk marker (Nicotiana tabacum)	2.00E-72	5.8	0.031

(Applied Biosystems Inc., Foster city, CA, USA) and their sequences are shown in "Methods and Materials" section.

The results from the real-time RT-PCR analysis showed similar expression patterns to that obtained by microarray for all five genes validated. For example, the homeodomain protein Hfi22, auxin resistant 2 (IAA7) and NAC4 protein homologs showed basically the same expression patterns and fold changes for both microarray and real-time RT-PCR results. While there was greater variation for the sugar transporter gene and the auxin-ethylene cross-talk gene (AER) homolog, the real-time RT-PCR data displayed the same expression patterns for both genes (up regulated in TamDew fruits at the mature stage), but the fold changes were variable (see Figure 5). Specifically, the fold changes of all 3 copies of the AER gene spotted on the microarray had significant variation with one showing a mature to immature fruit expression ratio of 1.71 ($p = 0.681$), the second 5.81 ($p = 0.031$) and the third yielded no data due to failure to pass the statistical significance test. As a result, the mean fold change for the AER homolog was 3.76 with a standard deviation of 2.90. In comparison, the fold change generated by real-time PCR was 1.62 ± 0.08. The fold change by real-time PCR was 7.18 ± 0.5 as compared to 2.4 ± 0.15 from the microarray for the sugar transporter gene. Real-time RT-PCR is more sensitive than microarray methodology which is known to yield results in which fold changes in expression are generally compressed in comparison to data derived from real time RT-PCR and RNA gel-blot analysis (Alba et al., 2004).

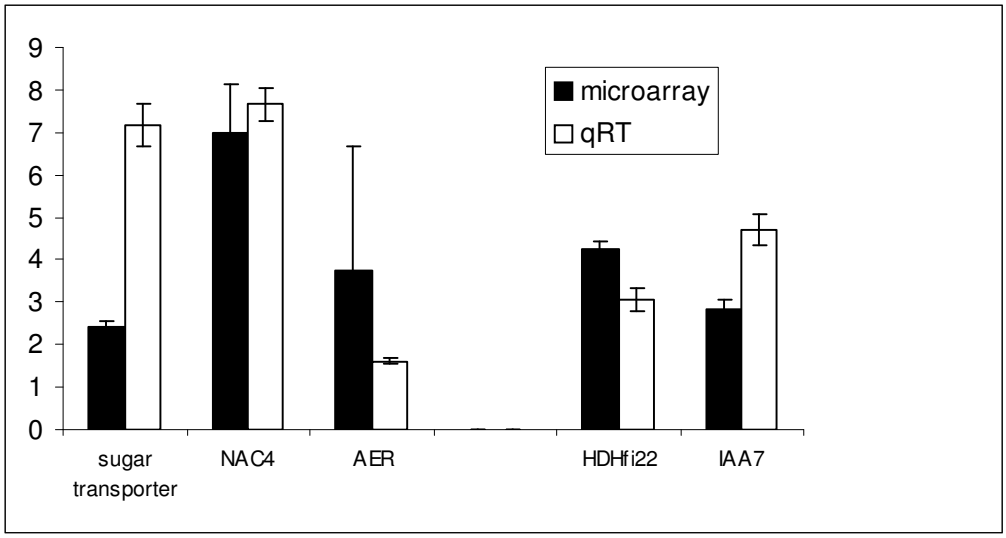


Figure 2.5 qRT-PCT comfirmation of differentially expressed genes from the melon cDNA microarray

Conclusions

Here we report the generation and use of two important genomic tools for the melon research community, melon ESTs and a validated cDNA microarray. These tools add to the repertoire of resources available to melon researchers and those working with members of the broader Cucurbitaceae family. A total of 3269 unigenes have been developed in this project adding approximately 30-fold gene sequence depth to the collection of melon gene sequences previously available to the research public. 82% of these unigenes were annotated based on sequence similarity against NCBI GenBank by BLASTX and 61% were assigned to biological and/or molecular functions according to Gene Ontology (GO) terms using InterPro2GO. The Melon EST Database contains all of this information in addition to additional sequences and mapping data available from others and is accessible at the web site: http://www.icugi.org. Based on this EST resource, a public melon cDNA microarray, consisting of 3068 independent unigenes derived from these ESTs, has also been created. Experiments comparing immature and mature melon fruit mRNA populations validated the integrity of the array in that array results were consistent with available data found in the literature and with real-time RT-PCR assays performed on a small number of random genes. In short, we demonstrated the utility of the public melon cDNA microarray for functional genomic studies in this important fruit species. The melon cDNA microarray is currently available to the research public at the Center for Gene Expression Profiling (CGEP), Boyce Thompson institute for Plant Research, Cornell University (htty://www.bti.cornell.edu/CGEP/CGEP.html).

Methods and Materials

Plant material

Plants were grown in the Newe Ya'ar Research Center in Israel under standard field conditions. The cultivars include: Tam Dew (*C.melo* var.*inodorus*), Dulce (*C.melo* var.*reticulates*), Noy Yizre'el (*C.melo* var.*reticulates*), Vedrantaise (*C.melo* var.*cantalupensis*), Roshet (*C.melo*.var.*inodorus*), Faqqous (*C.melo* var.*flexuosus)* and PI414723 (subsp.*melo* var.*momordica*). Fruits from different developmental stages were sampled based on days post anthesis (DPA). (1) Young fruits: Noy Yizre'el (25DPA). (2) Mature green fruits: Noy Yizre'el (35DPA); (3) mature yellow fruits: Noy Yizre'el (37DPA); (4) mature ripe fruits: (47dpa) (5) mixed stages of fruits: Noy Yizre'el; Vedrantais;Dulce; Roshet; Faqqous;(6) Dulce mature fruits (40DPA);Tam Dew mature fruits (36 DPA). Plant roots, stems and leaves were also sampled from Noy Yizre'el (*C.melo* var.*reticulates*) in order to make the suppressive subtractive hybridization libraries. Tissues were harvested in Israel, frozen in liquid nitrogen and shipped to Ithaca on dry ice.

Library construction

Melon EST libraries were provided by our collaborators in the Katzir laboratory in New Ya'ar, Israel (Volcani Institute).

EST sequencing

cDNA clones were randomly picked and inoculated into 96-well cell culture plates containing150 ul LB + 100ug/ml Ampicillin (for the SSH libraries) or 50 ug/ ml Kanamicin (for the standard cDNA libraries) and incubate one overnight at 37°C. 1-2 ul of cultured cells were used as template in a final volume of 100 ul for PCR . The primers for amplifying clones from SSH libraries were M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GGAAACAGCTATGACCATG-3'). T3 primer (5'-CGAAATTAACCCTCACTAA-3') and T7 primers (5'-GTAATACGACTCACTATAGGC-3') were used for amplifying the clones from the standard cDNA libraries. The PCR reactions were performed in a 96-well plate format and the PCR program included 95 °C 5 min, 95 °C 1 min, 54 °C 2 min, 66 °C 3 min for 35 cycles and a final extension at 66 °C for10 min. The quality of the PCR products was assessed by gel electrophoresis. The resulting PCR product was used as template for sequencing reactions using the ABI BigDye®Terminator sequencing kit Ver 3.1 (Applied Biosystem, Foster City, CA). For each sequencing reaction, 1ul of PCR product was used in a total 10ul reaction volume. M13R and T3 primers were used for sequencing clones from SSH libraries and standard cDNA libraries respectively. The sequencing program was subjected to 96°C 30s, 96 °C 15s, 50 °C 30s, 60 °C 3 min for35 cycles. After the sequencing reactions, the products were purified by adding 26 ul of water and 64 ul of 100% ethanol and then were incubated for 30 min at room temperature. After spinning 30min at 13,000 rpm and washing with 100ul 75% of Ethanol, samples were dried at room temperature for 10 min and were run on an ABI 3100 genetic Analyzer using procedures and reagents from the vendor.

EST processing and assembly

Sequence traces obtained from automatic sequencing were analyzed using a chromatogram base-calling program *Phred* (Ewing and Green.1998; Ewing et al., 1998). Low quality sequences and contaminating sequences, such as linkers, adaptors and primers that were commonly used in constructing cDNA libraries, were removed by the LUCY (Chou and Holmes. 2001) and SeqClean program (http://www.tigr.org/tdb/tgi/software). The processed sequenced were then compared with the UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) and *E.coli* whole genome sequence in order to eliminate nucleotides that maybe of bacterial or vector origin. The resulting sequences that had > 100 bases after trimming were considered as "high quality" sequences. Those sequences were first clustered using the TGICL program (Pertea *et al*., 2003) and then were assembled into contigs using CAP3 (Huang and Madam. 1999). Sequences that could not be assembled into contigs were termed "singleton".

Functional annotation of unique sequences

Functional annotation was first conduced based on sequence similarity. All qualified sequences generated in this experiment were blast searched against the NCBI non-redundant protein (nr) database using BLASTX with an arbitrary cut-off E-value at 10^{-5}. InterProScan and HMM search(Nucleic Acids Research,2006,34:D247-D251) were then carried out in order to detect the protein motifs and to get meaningful inference regarding possible protein functions for those that lacked biochemical characterization. Functional classification was based on

Gene Ontology (GO) terms. Assignment of GO terms to each of the unique sequence (cotigs and singletons) was conduced using InterPro2GO and pFam2Go programs.

Microarray construction

The cDNA clones for constructing the microarray were derived from the melon unigene set build. In totall 3062 unigenes were picked up from the master plates and rearranged into 8 x 384-well plates. The cDNA inserts were obtained by PCR amplification as described above. The PCR products were purified by adding 1/10 volume of 3M Sodium Acetate (PH 5.2) and 2.5 volume of 100% ethanol and then resuspended in 50 ul of spotting buffer (3 x SSC, 1.5 M Betaine). Each probe, including 4 negative controls that contain only printing buffer without any cDNAs, were printed in triplicate onto Corning UltraGAPS glass slides (25.3 x 75.5 mm) coated with γ-amino-propyl-silane using a MicroGrid Pro arrayer (BioRobotics Inc.) that has 32 MicroSpot2500 printing pins. The temperature inside the arrayer was kept at 18-21 ° C and the relative humidity was 35-45% RH. After printing, the slides were crosslinked at 300 mJ of UV irradiation and incubated at 85 °C for 2 h, followed by washing in 0.2% SDS for 2 min, rinsing in Milli-Q® water 3 times and dipping in 90% ethanol for few seconds. Ethanol was removed immediately by spinning at 500rpm for 2 min. Slides were then stored in a plexiglass chamber. The resulting melon cDNA microarrays contain 8 metal rows and 4 metal columns with 18 columns and 16 rows in each sub-grid.

RNA Isolation

For use in creating cDNA probes we employed a modified version of the

protocol reported by Chang et al (1993), which was designed for use with pine tissues that are rich in carbohydrates and secondary metabolites. Modifications include purification of total RNA from 3.5 g of frozen tissue powder using 17 mL of extraction buffer, additional chloroform:IAA extraction steps prior to the LiCl precipitation, and elimination of the chloroform:IAA extraction steps after the LiCl precipitation. When mRNA is desired, the EtOH-washed total RNA is brought to 0.75 ug/ul with 10 mM Tris-Cl (pH 7.5) and the mRNA is purified with oligo d(T)25 Dynabeads™ (catalog #610.05; Dynal Inc., NY), as per the manufacturer's protocol. We find that fluorescent cDNA targets prepared from mRNA usually result in stronger and more consistent signal across the array and reduced background fluorescence.

Microarray hybridization, scanning and data analysis

Total RNAs were isolated using the protocol mentioned above. The first-strand cDNA synthesis, cDNA labeling and purification were performed using SuperScript™Indirect cDNA Labeling System (Invitrogen™ Crop., Carlsbad, CA, USA). 15 ug of total RNA was used as templates for first strand cDNA synthesis. The efficiency of the labeling procedure was evaluated as described in "5.5 Analysis of labeling reaction" in TIGR's Standard Operating Proceedure #M004 (http://pga.tigr.org/sop/M004_1a.pdf).

A total of 200 pmol of Cy3™ coupled and an equivalent amount of Cy5™ coupled cDNA were combined and used to hybridize the slides. First the combined dye-coupled cDNA was dried using VacumFuge™ evaporator and re-suspended in 50 ul of hybridization solution [33 µl Milli-Q® H_2O, 30 µl de-ionized formamide (SIGMA, catalog no.9037), 25 µl 20x SSC, 10 µl 50x Denhardt's Soln (Invitrogen;

catalog no.50-018), 1 µl 10 µg/ µl PolyA RNA (Amersham Biosciences; catalog #27-4110) and 1 µl 10% SDS]. After heating at 95°C for 5 min and spinning at 14,000rpm for 1 min, the cDNA probes were then applied to a slide that has been pre-hybridized at 43°C for 45 min in buffer containing 5 x SSC, 0.1% SDS and 1% BSA and covered with LifterSlip glass (50mm, catalog no. 22x501-2-4711; Erie Scientific, Portsmouth, NH, USA). The hybridization was performed at 43°C for 12-16h under dark. Following hybridization, 3 washes were applied to the slides in Coplin jars: Wash 1: 1 x SSC with 0.2% SDS, 10 min at 43°C; Wash 2: 0.1 x SSC with 0.2% SDS, 10 min at room temperature (RT); wash 3: 0.1 x SSC only, 10min at RT. Then slides were dried by spinning at 1,500 rpm for 30 seconds under dark.

Slides were scanned by ScanArray scanner (ScanArray 5000; GSI Lumonics, Billerica, MA, USA) at 10 um resolution. The 90 % laser power and different PMT value were used for each slide in order to balance Cy3/Cy5 intensity. ImaGene software (v5.6, BioDiscovery Inc., EI Segundo, CA.USA) was chose to convert obtained raw fluorescence image to numerical signal data. Only spots that showed fluorescence intensity at least two times the mean background value for both channels were retained. Log transformation, normalization and further statistic analysis were all carried out within the R statistical package (R2.4.0, Bioconductor package). Differential expressed genes were identified using the PaGE program.

Quantitative RT-PCR

The total RNA from the same resource for microarray experiments were reverse transcribed into cDNAs using polyA primers under the condition suggested by SuperScript™Indirect cDNA Labeling System (Invitrogen™ Crop., Carlsbad,

CA,USA). Gene specific primers were designed using Primer Express software (Applied Biosystems).The (two-step)quantitative RT-PCR were performed using SYBR Green PCR master kits (Applied Biosystems) under the Standard Curve method on ABI 7900 (Applied Biosciences).The reaction volume was 10 ul (cDNA template 2.5 ul, 2x SYBR 5 ul, ddH2O 1.3 ul, forward primer(5 nmol/ul) 0.6 ul, reverse primer(5 nmol/ul) 0.6 ul).The quantitative PCR conditions were: 50^{o}C 2 minutes, 95 ^{o}C 10 minutes followed by 40 cycles of 95 ^{o}C 15 seconds and 60 ^{o}C I minute. Three technical replicates were performed for each sample and Actin was designed as endogenous control (Forward primer: CTTCTACAATGAGCTTCGTGTTGCT; Reverse primer: GGGTTGAGTGGTGCTTCAGTAAG)

The primers for the five genes are: IAA7-F: GGGCCCAAGGAATGATAGACT; IAA7-R: TGGCACATACTCAGAGCTGTTCA; NAC4-F: GTAGCCGGTTCGGGTTACTG; NAC4-RGTTCTTCCCTTCCGACGAGAT; AER-F: ACCTTTCGCTCGACTCTCTTCTT; AER-R: GGCGAAAAATGCCCTGAA; HomeodomainHfi22-F: TTCCAAAACCGTAGAGCACGAT; HomeodomainHfi22-R: TGGCTTTGAGGAGGCCATAA; Sugar transporter- F: GATTTGGGAGCCGATTCTCTT; Sugar transporter-R: GACACTCCAAACTTCTCTTCCAAAG;

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CHAPTER III

TRANSCRIPTOME COMPARISON REVEALS GENES ASSPOCIATED WITH FRUIT QUALITY TRAITS OF CLIMACTERIC AND NON-CLIMACTERIC MELONS

Abstract

Melon varieties (*Cucumis melo* L.) differ in many fruit traits including general ripening physiologies. Climacteric and non-climacteric melons defined by increased and constant respiration associated with ripening, respectively, exist and both types represent important crops. Dulce (*C melo var.reticulatus* Naud) fruit are classified as members of the climacteric group of melons typified by high levels of aroma volatiles, orange flesh and a relatively quick softening process during ripening. The ripening behavior of Rochet (*C melo var.indorus* Naud) is typical of non-climacteric fruit in that ripening is accompanied by low aroma, pale-greenish flesh and relatively slow softening. Little is known about how the genes related to these fruit quality traits are differentially regulated between climacteric and non-climacteric cultivars. A transcriptome comparison of fruit maturation in climacteric and non-climacteric melons was performed using a melon cDNA microarray. Our goal was to shed light on molecular mechanisms that underlie ripening while simultaneously increasing the reservoir of ripening related genes for melon species. A total 183 genes showed differential expression between Dulce and Rochet at an immature unripe stage (25dpa) and 302, 293 and 480 were differentially expressed at mature, ripe and over-ripe stages respectively. By focusing our analysis on the expression patterns of genes that may participate in biological pathways related to fruit quality traits, we were able

to identify specific differences that were consistent with the variable fruit traits between these two varieties and including: fruit softening, aroma, flavor and carotenoid biosynthesis. Our results suggest that the quick softening phenotype of Dulce during ripening was mainly caused by the concomitant up regulation of isoforms of genes involved in cell wall degradation including *PGs, GALs, XTHs, EXP*s and *PME*. Multiple regulatory mechanisms may contribute to the orange color (beta-carotene) of Dulce flesh but their gene targets are clear in that transcriptional regulation of DXR and PDS appears to be highly consistent with the carotenoid accumulation profiles of Dulce versus Rochet. Aroma variation between Dulce and Rochet is likely due to reduced transcription and enzyme activity of AAT.

Introduction

Fruits are important agriculture commodities. Fleshy fruits have long been an indispensable component in our daily diets. They provide not only attractive colors to foods but also pleasant flavors, multiple essential nutrients that are important to human health (vitamins, minerals, antioxidants) and fiber and calories. Fruit ripening thus has been receiving considerable attention for scientific research with a primary aim to understand the molecular mechanisms behind and in order to optimize genetic systems associated with fruit quality, yield and post-harvest manipulation.

Melon is one of American's favorite fruits because of its unique flavor. Melon species (*Cucumis melo* L.) contain cultivars producing fruits deferring in many fruit traits including sugar content, aroma volatiles, carotenoids, softening and fruit texture. In addition, melon varieties include both climacteric and non-climacteric types. The climacteric melons, for example cantaloupe, have a high respiration rate and increased production of ethylene at the onset of ripening and are appreciated by consumers for their aroma, high sugar and deep flesh color (due to beta-carotene accumulation). However, the softening process of climacteric melons is generally quick (4-7 days) and results in short shelf life and post-harvest handling challenges. Non-climacteric varieties, such as honeydew melon (*C melo var.indorus* Naud), do not increase respiration nor ethylene synthesis during fruit maturation. The softening of these cultivars is relatively slow and they usually produce lower concentrations of aroma volatiles, have greenish flesh containing only trace amounts of beta-carotene though some genotypes can retain high levels of sugars.

Improvement of fruit quality has long been the focus of many melon researchers. Traditional breeding has produced numerous commercially viable cultivars with enhanced yield and fruit traits, disease resistance and environment tolerance. This cross-based method is effective but generally slow and limited by many factors, such as sexual compatibility and saturation of genetic potential (Niemirowiczszczytt and Kubicki, 1979; Nunez-Palenius et al., 2008; Perchepied et al., 2005). Increased understanding of genes that contribute to traits of interest can lead to development of molecular tools including markers and trait diagnostic strategies to assist selection and phenotypic characterization. Biotechnological methods can also be applied directly to create transgenic plants to either test gene function or engineer superior cultivars. Transgenic approaches have been applied to generate transgenic melons in order to test the potential for providing desirable agronomic traits as related to the softening process and to extend shelf-life. Several lines of transgenic Charentais melon suppressing the expression of the ACC oxidase ethylene biosynthesis gene using antisense technology have been generated (Ayub et al., 1996; Nunez-Palenius et al., 2007). While the desired decrease of softening was achieved, additional desirable fruit qualities were also impaired and in particular the production of volatile aroma compounds (Flores et al., 2002). Several other techniques including differential screening of cDNA libraries and suppressive subtractive hybridization, have been used to identify genes associated with the ripening process of melon (Choi et al., 2004; Hadfield et al., 2000; Nishiyama et al., 2007). A number of ripening related cDNAs were isolated but little remains known about how these genes influence ripening and whether they exhibit differential effects between climacteric and non-climacteric fruit. The coexistence of both climacteric and non-climacteric melons provide an excellent opportunity to study the regulatory mechanisms of

ripening process displaying different ripening physiologies while simultaneously providing the opportunity to identify genes for fruit quality improvement.

Gene expression microarray analysis is a high-throughput technology which allows for the analysis of the expression of thousands of genes within a single experiment. This technique has been widely used for transcriptome analysis and gene identification and annotation. Here we describe transcriptome comparisons between two melon varieties, Dulce (climacteric) and Rochet (non-climacteric) using the melon cDNA microarray described in chapter 2. Use of this tool resulted in both the expansion of the population of ripening related genes and helped define how genes are differentially regulated between climacteric and non-climacteric melons.

Results

Experimental design

Two melon (*Cucumis melo* L.) varieties were used for this experiment: Dulce (*C. melo* var. *reticulatus Naud.*) and Rochet (*C. melo* var. *inodorus Naud.*). As we mentioned above, fruits of these two cultivars show a number of different traits of interest. Dulce fruit has higher nutrition value because of the large amount of beta-carotene it contains, which makes its flesh orange. Dulce fruit also produces strong aroma but quick softening that causes difficulty for post-harvest handling. Rochet fruit contains trace amount of beta-carotene, weak aroma but slow softening that leads to a relatively longer shelf-life. Microarray technology was applied to study the transcriptional differences between these two varieties in order to understand the molecular regulation behind these traits, especially the ones related to fruit qualities

such as softening, aroma volatile biosynthesis and carotenoids accumulation. mRNAs were isolated from fruit flesh of four distinct developmental stages of these two cultivars, 25 DPA (day after anthesis), mature (Dule: 42dpa; Rochet: 45dpa) ripe (Dulce: 46dpa; Rochet: 47 dpa) and over-ripe (Dulce: 54dpa; Rochet: 55dpa), and their transcripts abundance were compared using a direct comparison strategy via the microarrays. For each stage compared, at least six microarray hybridizations were performed, representing three independent biological replicates and three technical repeats or dye-flips (Fig 3.1). The microarray data were analyzed using the statistical tools introduced in the "Material and Methods" section. The melon cDNA microarray we used contains 3068 unigenes, obtained mainly from fruit cDNA and SSH libraries, and with each unigene printed in triplicate. The fold difference values of the three replicates were averaged (mean fold difference, MFD) and then used for expression analysis in this experiment. The cut-off value for differentially expressed genes is PDF ≤ 0.05 and mean fold difference (MFD) ≥ 2.0. The differentialyl expressed genes at each stage for Dulce and Rochet are summarized in Table 3.1.

Genes differentially expressed at 25dpa

Molecular mechanisms underlying early fruit development are poorly understood in melon as very few molecular research efforts have been undertaken on early fruit development. Early fruit development is mainly characterized by cell division and cell expansion which is believed to also contribute to final fruit traits such as fruit size. By comparing genes differentially expressed at early developmental stages we provide candidate genes that might be involved in early fruit development, which will greatly facilitate later research on fruit development and will help us understand the molecular biology of early fruit development. For the 3068 unigenes on

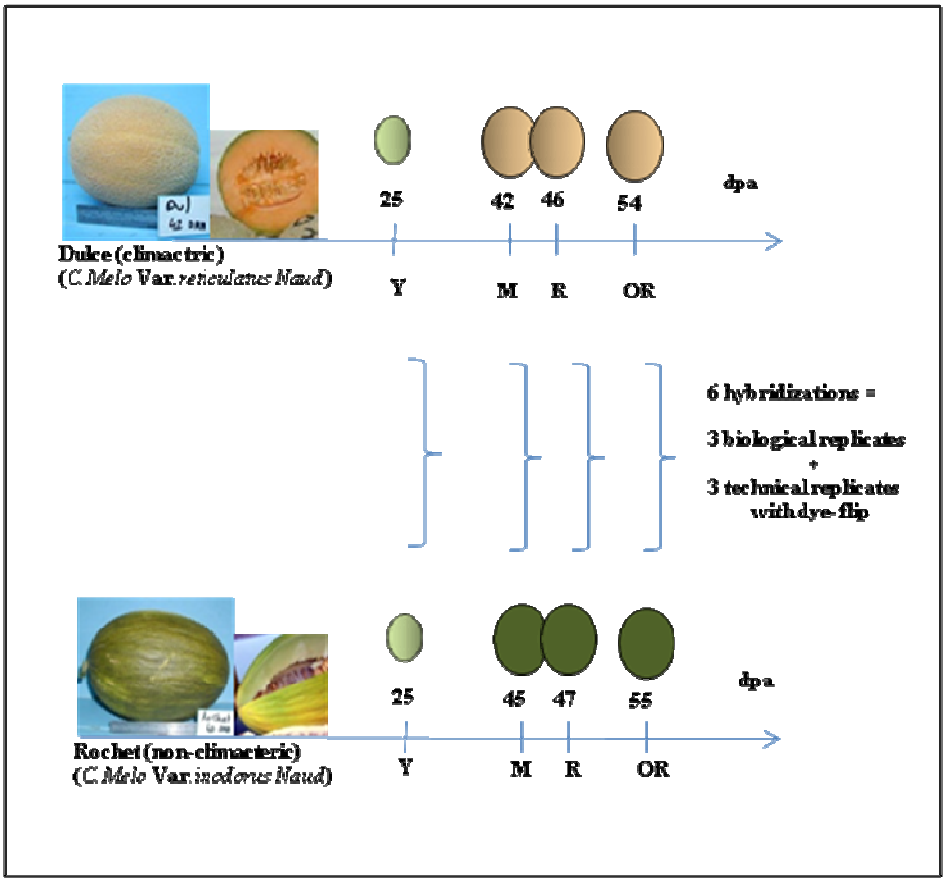


Figure 3.1 Experiment Design

Two melon varieties were used for this experiment: Climacteric Dulce that has orange flesh, high aroma and fast softening process during ripening, and non-climacteric type Rochet that contains pale-green flash, low aroma and slow softening process. Four stages representing four important developmental points were compared (young, mature, ripe and over-ripe). Six hybridizations were applied for each stage compared which represent three biological replicates and three dye-swap.

Table 3.1 General transcriptional alteration in Dulce and Rochet at each stage compared

		Ripening Stages		
	25dpa	**Mature**	**Ripe**	**Over-ripe**
Total unigenes differential expressed	183	302	293	480
Dulce				
Up-regulated	63	176	197	287
(FDR ≤ 0.05, MFD ≥ 2.0)	(2.1%)*	(5.7%)	(6.4%)	(9.4%)
Rochet				
Up-regulated	120	126	96	193
(FDR ≤ 0.05, MFD ≥2.0)	(3.9%)	(4.1%)	(3.1%)	(6.3%)

* relative to the 3068 spots on the chip

the Melon cDNA array, 183 showed differential expression at 25dpa. Among them, 63 were highly expressed in Duce fruits while 120 were up regulated in Rochet (MFD ≥ 2.0, FDR ≤ 0.05).

Of the 120 unigenes that were up regulated in Rochet, 26 (22%) were identified as biological function unknown. This subset includes genes that were annotated as unknown proteins, hypothetical proteins and genes that have no hits when BLASTX searched against GenBank. The remaining 95 include genes involved in widely varied functions such as amino acid metabolism (6%), stress response (8%) and carbohydrate metabolism (4%). Genes whose mRNAs were most abundant in Rochet at this stage include polygalacturonase (two unigenes: FR16G14 and FR14B12), branched amino acid aminotransferase (two unigenes FR15E23 and FR17D11), cytochrome P450 monooxygenase, sugar transporter and a dienelactone hydrolase family protein (Table 3.2). The two polygalacturonases (PGs) showed 95% (FR14B12, e = 2e-219, score = 765) and 82% (FR16G14, e = 3e-17, score = 92) sequence identity at the amino acid level with *Cm-PG1*, respectively. *Cm-PG1* is one of the most important enzymes involved in cell wall degradation during fruit ripening. The expression of *Cm-PG1* has been extensively studied in climacteric fruits (Charentais melon). During the ripening of Charentais melon, *Cm-PG1* increased expression at 40 dpa at very low levels and increased dramatically at 43 dpa remaining high to 46 dpa (Hadfield et al., 1998; Nishiyama et al., 2007). In the present analysis, the high MFD value of these two *Cm-PG1*s in Rochet at 25 dpa suggested that *Cm-PG1* was expressed at relative high levels in non-climacteric Rochet compared with Dulce. We then followed the expression patterns of these two genes at M (mature), R (ripe) and OR (over-ripe) stages between Dulce and Rochet. Both genes were highly expressed in Dulce as compared to Rochet at all ripening stages (mature, ripe and

Table 3.2 Up regulated genes in Rochet at 25 dpa

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR16G14	DV634293	polygalacturonase [Cucumis melo]	1.00E-29	39.7±9.9	0.011
FR14B12	DV633521	polygalacturonase precursor [Cucumis melo]	9.00E-285	32.4±2.8	0.011
FR15E23	DV633934	branched-chain amino acid aminotransferase [Arabidopsis thaliana]	4.00E-55	19.5±1.9	0.012
FR13D3	DV633254	cytochrome P450 monooxygenase CYP92A29 [Medicago truncatula]	5.00E-89	19.1±2.1	0.01
FR11B10	DV632631	sugar transporter, putative [Arabidopsis thaliana]	3.00E-100	16±2	0.011
FR13B7	DV633218	Upf2 [Volvox carteri f. nagariensis]	1.00E-12	14.7±2	0.012
FR17D11	DV634543	ATBCAT-2; branched-chain-amino-acid transaminase/ catalytic [Arabidopsis thaliana]	1.00E-183	12.5±1.9	0.012
FR13I12	DV633336	dienelactone hydrolase family protein [Arabidopsis thaliana]	1.00E-116	10.6±0.8	0.012
SSH1C5	DV631451	no hit fund		7.9±0.2	0.012
FR11B17	DV632634	Ribosomal protein S7 [Medicago truncatula]	9.00E-18	7.1±0.7	0.013
FR14A19	DV633510	heat shock protein 101 [Vitis vinifera]	4.00E-85	6.1±0.1	0.012
FR17M4	DV634742	no hit fund		5.7±1.0	0.012
FR13N18	DV633446	putative translation initiation factor eIF-1A-like [Solanum tuberosum]	1.00E-78	5.4*	0.021
FR14M24	DV633770	no hit fund		5.3±0.5	0.013
FR14A18	DV633509	17.6 kD class I small heat shock protein [Lycopersicon esculentum]	5.00E-33	5.2*	0.032
FR13C3	DV633233	NAM-like protein [Prunus persica]	3.00E-141	4.9±0.5	0.013
PH9H6	EB715181	DHS (DHS) [Arabidopsis thaliana]	3.00E-124	4.7±0.2	0.012
FR10H5	DV632457	no hit fund		4.7±0.1	0.011

Table 3.2 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR16G24	DV634301	GAST-like gene product [Fragaria x ananassa]	7.00E-43	4.6±0.8	0.012
FR16N23	DV634438	NADP dependent malic enzyme [Phaseolus vulgaris]	3.00E-184	4.6±0.6	0.013
FR16P3	DV634477	short-chain dehydrogenase/ reductase (SDR) family protein [Arabidopsis thaliana]	8.00E-81	4.5±0.4	0.012
FR11C19	DV632643	glutathione S-transferase [Cucurbita maxima]	3.00E-104	4.3±0.8	0.013
PH11E2	EB715327	no hit fund		4.3±0.1	0.012
SSH1E21	DV631477	no hit fund		4.3±0.2	0.013
FR17C16	DV634528	heat shock protein 101 [Vitis vinifera]	4.00E-136	4.2±0.6	0.013
FR12O16	DV633157	Protein kinase; NAF [Medicago truncatula]	3.00E-07	4.2±0.3	0.013
SSH4O4	DV631850	cystein proteinase inhibitor [Cucumis sativus]	3.00E-27	4±0.1	0.014
SSH4E14	DV631721	no hit fund		4±0.2	0.01
FR17E5	DV634580	AER [Nicotiana tabacum]	2.00E-72	3.8±0.4	0.014
SSH6A2	DV631884	low molecular weight heat-shock protein [Nicotiana tabacum]	3.00E-79	3.8±0.4	0.042
FR18M8	DV635072	haloacid dehalogenase-like hydrolase family protein [Arabidopsis thaliana]	5.00E-92	3.7±0.2	0.011
SSH4P23	DV631867	heat shock protein 101 [Oryza sativa]	2.00E-36	3.7±0.7	0.013
FR10N18	DV632570	Nthsp18p [Nicotiana tabacum]	2.00E-80	3.7±0.3	0.039
FR14L3	DV633750	putative acyltransferase [Cucumis melo]	9.00E-113	3.7±0.2	0.014
FR16L3	DV634400	stearoyl-acyl carrier protein desaturase	2.00E-281	3.6±0.2	0.011
FR15A22	DV633860	F-box family protein [Arabidopsis thaliana]	3.00E-19	3.5±0.1	0.014
FR16F20	DV634280	plasma membrane H+-ATPase [Sesbania rostrata]	8.00E-70	3.5±0.2	0.012
SSH1B3	DV631432	seed maturation protein PM40 [Glycine max]	1.00E-14	3.5±0.5	0.012

Table 3.2 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR17A20	DV634494	ACAT2/EMB1276 acetyl-CoA C-acetyltransferase [Arabidopsis thaliana]	7.00E-241	3.4±0.7	0.011
FR17K20	DV634698	DNAJ heat shock N-terminal domain-containing protein-like [Oryza sativa (japonica cultivar-group)]	5.00E-37	3.4±1.2	0.042
SSH1F1	DV631486	NADP-dependent malic protein [Ricinus communis]	5.00E-86	3.4±0.2	0.012
FR18N6	DV635093	hypothetical protein [Vitis vinifera]	7.00E-34	3.3±0.01	0.013
SSH9A6	DV632219	no hit fund		3.3±0.3	0.012
PH1H5	EB714423	no hit fund		3.2±0.3	0.013
FR18P23	DV635128	wound-responsive protein, putative [Arabidopsis thaliana]	1.00E-27	3.1±0.3	0.013
FR14F5	DV633621	arginosuccinate synthase family [Arabidopsis thaliana]	6.00E-127	3*	0.034
FR17G12	DV634611	CYP71A10 [Glycine max]	5.00E-81	3±0.1	0.01
FR13L1	DV633396	Zinc transporter 4, chloroplast precursor (ZRT/IRT-like protein 4)	2.00E-77	3*	0.011
SSH1A20	DV631413	C2 domain-containing protein (sytC) [Arabidopsis thaliana]	2.00E-34	2.9±0.3	0.012
FR10K6	DV632521	cytochrome P-450LXXIA1 [similarity] - avocado	4.00E-60	2.9±0.2	0.012
SSH7G17	DV632202	seed specific protein Bn15D1B [Brassica napus]	9.00E-26	2.9±0.2	0.013
FR11K18	DV632777	unknown [Medicago truncatula]	9.00E-30	2.9±0.2	0.012
FR14D20	DV633574	esterase [Cucumis melo]	2.00E-76	2.8±0.3	0.015
SSH1M17	DV631600	Uncharacterized Cys-rich domain [Medicago truncatula]	6.00E-33	2.8±0.2	0.012
SSH4F13	DV631736	2-cys peroxiredoxin-like protein [Hyacinthus orientalis]	1.00E-99	2.7±0.1	0.011
SSH4F11	DV631735	aspartic proteinase [Cucumis sativus]	5.00E-42	2.7±0.2	0.012

Table 3.2 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR14G24	DV633641	Heat shock protein DnaJ [Medicago truncatula]	4.00E-90	2.7±0.2	0.012
FR16D11	DV634229	HSP20-like chaperone [Medicago truncatula]	2.00E-30	2.7±0.2	0.012
SSH6C13	DV631918	no hit fund		2.7±0.4	0.011
FR12I15	DV633040	auxin-repressed protein-like protein ARP1 [Manihot esculenta]	9.00E-46	2.6±0.3	0.014
FR15C12	DV633894	NAD(P) transhydrogenase, alpha subunit; Saccharopine dehydrogenase; [Medicago truncatula]	5.00E-50	2.6±0.3	0.013
FR18M9	DV635073	40S ribosomal protein SA (p40)	2.00E-122	2.5±0.2	0.013
FR18J11	DV634990	Bax inhibitor [Lycopersicon esculentum]	2.00E-111	2.5±0.4	0.015
FR11C2	DV632644	catechol O-methyltransferase [Nicotiana tabacum]	2.00E-84	2.5±0.1	0.014
FR11L23	DV632800	cytosolic class II low molecular weight heat shock protein [Prunus dulcis]	1.00E-74	2.5±0.2	0.013
FR13G23	DV633311	endochitinase MCHT-2 [Cucumis melo]	2.00E-220	2.5±0.6	0.014
SSH1A14	DV631409	Histone H3	3.00E-39	2.5±0.2	0.011
FR18J16	DV634995	homocysteine S-methyltransferase [Arabidopsis thaliana]	4.00E-65	2.5±0.1	0.017
FR14M14	DV633761	; UDP-glycosyltransferase/ transferase, transferring glycosyl groups [Arabidopsis thaliana]	2.00E-45	2.5±0.1	0.013
SSH1A13	DV631408	NADP-dependent malic protein [Ricinus communis]	2.00E-31	2.5±0.3	0.01
FR15P24	DV634159	putative alcohol dehydrogenases [Cucumis melo]	1.00E-181	2.5±0.3	0.013
FR17M19	DV634735	terpene cyclase/mutase-related [Arabidopsis thaliana]	8.00E-97	2.5±0.1	0.012
FR10M13	DV632544	universal stress protein (USP) family protein / early nodulin ENOD18 family protein [Arabidopsis thaliana]	1.00E-74	2.5±0.2	0.013
FR14O2	DV633813	no hit fund		2.5±0.02	0.012

Table 3.2 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR14I18	DV633679	3-ketoacyl-CoA thiolase; acetyl-CoA acyltransferase [Cucumis sativus]	4.00E-150	2.4±0.3	0.012
FR14A20	DV633512	protein phosphatase 2C, putative / PP2C, putative [Arabidopsis thaliana]	9.00E-51	2.4±0.1	0.013
FR15K19	DV634047	putative peptide transporter; [Arabidopsis thaliana]	3.00E-29	2.4±0.1	0.014
FR12M9	DV633130	SNF7 family protein [Arabidopsis thaliana]	2.00E-78	2.4±0.1	0.017
SSH1A22	DV631415	no hit fund		2.4±0.1	0.013
SSH1N14	DV631611	no hit fund		2.4±0.4	0.013
FR11L13	DV632791	histone H4	5.00E-62	2.3±0.1	0.014
FR14C24	DV633556	hypothetical protein [Vitis vinifera]	7.00E-52	2.3±0.4	0.017
FR12L18	DV633100	NADP-dependent malic enzyme	0.00E+00	2.3±0.1	0.017
FR17L1	DV634708	ubiquitin family protein [Arabidopsis thaliana]	2.00E-44	2.3±0.2	0.014
SSH4J1	DV631771	no hit fund		2.3±0.2	0.012
SSH4F19	DV631739	1-deoxy-D-xylulose 5-phosphate reductoisomerase precursor [Oryza sativa]	9.00E-136	2.2±0.1	0.012
FR12N8	DV633149	DnaJ-like protein [Glycine max]	9.00E-84	2.2±0.1	0.012
FR15G9	DV633979	EIN3-binding F-box protein 2 [Lycopersicon esculentum]	4.00E-55	2.2±0.1	0.013
SSH1F13	DV631490	glycosyl transferase family 1 protein [Arabidopsis thaliana]	2.00E-24	2.2±0.1	0.012
FR12B7	DV632915	H1 histone-like protein [Solanum lycopersicum]	3.00E-32	2.2±0.1	0.02
FR16K2	DV634374	Histone H1	1.00E-32	2.2±0.2	0.012
FR13I22	DV633345	oxidoreductase family protein [Arabidopsis thaliana]	5.00E-50	2.2±0.2	0.05
FR18L11	DV635032	pentatricopeptide (PPR) repeat-containing protein [Arabidopsis thaliana]	4.00E-59	2.2±0.1	0.022
FR17O20	DV634780	putative Signal peptidase [Oryza sativa]	2.00E-90	2.2±0.2	0.014

Table 3.2 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR16E22	DV634261	no hit fund		2.2±0.1	0.011
FR10N8	DV632580	ABC1 family protein, expressed [Oryza sativa (japonica cultivar-group)]	1.00E-46	2.1±0.2	0.014
SSH9E20	DV632281	Adenylate kinase [Medicago truncatula]	5.00E-56	2.1±0.04	0.013
FR10I6	DV632479	aluminum-induced protein [Codonopsis lanceolata]	1.00E-136	2.1±0.1	0.013
FR11G15	DV632704	glutathione S-transferase [Cucurbita maxima]	1.00E-08	2.1±0.1	0.017
FR17H13	DV634633	Guanylate kinase/L-type calcium channel region; Galactose oxidase, central [Medicago truncatula]	1.00E-49	2.1±0.2	0.013
SSH1N6	DV631623	Heat shock protein DnaJ [Medicago truncatula]	1.00E-42	2.1±0.3	0.013
FR10D16	DV632391	hypothetical protein [Vitis vinifera]	2.00E-37	2.1±1.2	0.013
FR15F22	DV633949	NAC4 protein [Glycine max]	6.00E-110	2.1±0.4	0.031
SSH6A1	DV631874	Phosphoenolpyruvate carboxykinase [ATP] 1	7.00E-48	2.1±0.2	0.013
FR13O21	DV633472	phosphofructokinase family protein [Arabidopsis thaliana]	2.00E-137	2.1*	0.012
FR17I9	DV634667	seed specific protein Bn15D17A [Brassica napus]	4.00E-40	2.1±0.2	0.013
PH7A12	EB714915	unknown protein [Arabidopsis thaliana]	7.00E-27	2.1±0.1	0.012
FR14I4	DV633686	no hit fund		2.1±0.2	0.013
FR16J13	DV634347	no hit fund		2.1±0.4	0.014
SSH1G4	DV631513	no hit fund		2.1±0.2	0.012
SSH2C16	DV631672	no hit fund		2.1±0.01	0.014
FR13M21	DV633427	(Cell division control protein 48 homolog E); ATPase [Arabidopsis thaliana]	5.00E-38	2*	0.013
FR18N3	DV635091	ferrous ion membrane transport protein DMT1 [Glycine max]	1.00E-79	2±0.1	0.015
FR11N23	DV632839	histone deacetylase HDAC2 [Triticum aestivum]	4.00E-49	2±0.1	0.014

over-ripe) which is consistent with Northern described elsewhere (Hadfield et al., 1998; Nishiyama et al., 2007) and also suggests that even though *Cm-PG1* expresses mainly during the ripening of climacteric fruit, its expression in non-climacteric fruit probably begins at young fruit stages and may play a role in early fruit development process such as cell expansion.

The mRNA levels of three transcription factors were highly accumulated in Rochet at 25dpa: homologs of a NAM-like protein, EIN3-binding F-box protein 2 and a NAC4 protein. Both the NAM like protein and NAC4 protein are members of the NAC family which are plant-specific transcription factors involved in many processes of plant development including formation of the shoot apical meristem, floral organs, defense and stress responses (Olsen et al., 2005). The tomato *NOR* gene encodes a NAC family transcription factor. The tomato *nor* mutant showed inhibition of all ripening phenomenon including fruit softening, pigment development, aroma flavor biosynthesis and climacteric ethylene production suggesting that NOR controls overall ripening processes in tomato (Giovannoni, 2001; Giovannoni, 2004). EIN3 transcription factors positively regulate the expression of ethylene responsive genes by activating the transcription of *ETHYLENE RESPONSE FACTOR* (ERF) genes in *A. thaliana*. The function of EIN3-binding F-box protein 2 (EBF2) was reported to be involved in EIN3 degradation through the ubiquitin/26S proteinsome pathway in the absence of ethylene (Binder et al., 2007; Gagne et al., 2004). The up-regulation of all these transcription factors in Rochet at 25 dpa indicates that they may participate in the control of expression of genes for early fruit development in Rochet.

Of the 63 unigenes that were highly expressed in Dulce at 25 dpa, 21 (33%) were annotated as hypothetical proteins, protein of unknown function or had no

homologs found. Others included genes that were involved in photosynthesis, carbon fixation and carbohydrate metabolism (11%). Genes whose transcripts were most abundant in Rochet at this stage include chloroplast nucleoid DNA-binding protein (two unigenes: FR11L11 and FR11O1), AG-motif binding protein, putative major latex protein, riboflavin synthase and methionine synthase (Table 3.3). Of the two unigenes encoding chloroplast nucleoid DNA-binding protein that were highly expressed in Dulce at 25 dpa, one (FR11O1) shares 92% sequence identity in 75 overlapping nucleotide residues (Score: 398.0, e value =1e-112) with Cmf120 (AT007009). Cmf120 is a melon chloroplast nucleoid DNA-binding protein encoding gene that was cloned from Charentaise melon (*Cucumis melo* cv. *Reticulatus*). Charantaise belongs to the same breeding family as Dulce and was characterized by Choi et al. (2004) in an effort to identify genes involved in early stages of melon fruit development using suppression subtractive hybridization. Northern blot analysis conformed that Cmf-120 is highly expressed at early stages of Charentaise melon fruit. Chloroplast nucleoid DNA-binding protein has been reported to regulate plastid gene expression by binding to the DNA sequence in plastid genomes which is organized into DNA-protein complexes (Nakano et al., 1997).

A putative major latex protein (FR10B2) that is also highly expressed in Dulce at this stage shared 100.0% sequence identity in 668 overlapping nucleotide residues (Score: 3648.0, e value = 0) with MEL7. MEL7 was identified as a ripening-related gene by differential screening a melon (*Cucumis melon* L.cv. Cantaloup charentais) ripe fruit cDNA library (Aggelis et al., 1997). Northen blot analysis showed that the expression of MEL7 was detected at low levels at 15 and 20 dpa, started to increase at 25 dpa and was highly induced during the ripening of Cantaloup melon fruit from 30dpa to 45dpa reaching the peak at 40dpa. We checked the

Table 3.3 Up regulated genes in Dulce at 25 dpa

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR11L11	DV632789	chloroplast nucleoid DNA-binding protein-related [Arabidopsis thaliana]	7.00E-38	19.7±1.9	0.02
SSH1M10	DV631595	No hit fund		17.3±2.0	0.02
FR11O1	DV632845	chloroplast nucleoid DNA-binding protein-related [Arabidopsis thaliana]	1.00E-62	16.4±1.1	0.02
SSH4E12	DV631719	No hit fund		15.7±0.9	0.02
FR13M11	DV633416	hypothetical protein [Vitis vinifera]	8.00E-43	12.0±1.8	0.02
SSH6D22	DV631950	No hit fund		7.5±0.6	0.02
FR12H23	DV633030	No hit fund		5.7±0.3	0.02
FR10C12	DV632376	AG-motif binding protein-1 [Nicotiana tabacum]	1.00E-36	5.6±0.7	0.02
SSH9D8	DV632269	proline rich protein [Malus x domestica]	1.00E-07	5.5±0.5	0.02
FR10B2	DV632367	putative major latex protein [Momordica charantia]	4.00E-48	5.2±0.4	0.02
FR11M2	DV632815	riboflavin synthase [synthetic construct]	6.00E-46	4.2±0.5	0.02
SSH1D6	DV631465	No hit fund		3.7±0.9	0.02
PH13H8	EB715544	No hit fund		3.7±0.2	0.022
FR16E10	DV634249	methionine synthase [Coffea arabica]	5.00E-92	3.7±0.3	0.02
FR12F17	DV632983	No hit fund		3.5±0.3	0.02
SSH1K9	DV631575	No hit fund		3.3±0.3	0.02
SSH1E18	DV631474	Peptidase A1, pepsin [Medicago truncatula]	9.00E-15	3.3±0.1	0.02
FR10N5	DV632578	No hit fund		3.2±0.1	0.02
SSH1L8	DV631593	chloride channel [Glycine max]	5.00E-41	3.2±0.5	0.02
PH16F1	EB715779	No hit fund		3.2±0.1	0.02
FR18F1	DV634909	potassium channel [Solanum lycopersicum]	3.00E-72	3.1±0.3	0.02

Table 3.3 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR11N10	DV632826	Protein of unknown function DUF250 [Medicago truncatula]	6.00E-64	3.1±0.01	0.02
FR18D19	DV634874	No hit fund		3.0±0.1	0.02
PH10E11	EB715243	Serpentine Receptor, class U family member (sru-8) [Caenorhabditis elegans]	2.00E-07	3.0±0.4	0.02
FR12J3	DV633068	26 kDa phloem lectin [Cucumis melo]	3.00E-172	3.0±0.3	0.02
PH3B4	EB714539	No hit fund		2.8±0.3	0.02
FR16G4	DV634303	S-adenosyl-L-methionine synthetase [Elaeagnus umbellata]	7.00E-150	2.7±0.2	0.02
FR12D22	DV632947	Aux/IAA protein [Cucumis sativus]	5.00E-109	2.7±0.3	0.02
PH1A7	EB714346	HAT22 (homeobox-leucine zipper protein 22); transcription factor [Arabidopsis thaliana]	3.00E-35	2.7±0.1	0.02
FR12E21	DV632967	molecular chaperone Hsp90-2 [Nicotiana benthamiana]	3.00E-90	2.7±0.1	0.02
SSH1J4	DV631562	DWARF1/DIMINUTO [Lycopersicon esculentum]	8.00E-47	2.5±0.1	0.02
SSH6E9	DV631983	BHLH093 (BETA HLH PROTEIN 93); DNA binding / transcription factor [Arabidopsis thaliana]	3.00E-21	2.5±0.1	0.02
SSH6E11	DV631962	Photosystem I P700 chlorophyll a apoprotein A1 (PsaA) (PSI-A)	4.00E-68	2.5±0.1	0.02
SSH4L7	DV631802	No hit fund		2.4±0.1	0.02
FR18G18	DV634939	UDP-glucose 6-dehydrogenase (UDP-Glc dehydrogenase) (UDP-GlcDH) (UDPGDH)	4.00E-108	2.4±0.2	0.021
FR12K12	DV633076	No hit fund		2.4±0.1	0.02
FR15J15	DV634024	heat shock protein binding [Arabidopsis thaliana]	6.00E-85	2.4±0.1	0.02
FR12G15	DV633002	Ribulose bisphosphate carboxylase small chain, chloroplast precursor (RuBisCO small subunit)	3.00E-115	2.4±0.2	0.02
FR12F14	DV632980	hypothetical protein [Medicago truncatula]	2.00E-31	2.3±0.5	0.026
PH3F6	EB714589	Peptidase S10, serine carboxypeptidase [Medicago truncatula]	2.00E-192	2.3±0.3	0.02

Table 3.3 (Continued)

Clone ID	GB #	Best hit in GenBank	e value	MFD	FDR
FR11E20	DV632676	anthocyanin acyltransferase-like protein [Arabidopsis thaliana]	3.00E-57	2.3±0.3	0.02
FR10O12	DV632584	putative 16kDa membrane protein [Nicotiana tabacum]	6.00E-72	2.3±0.1	0.024
FR16B5	DV634203	SCL3 (SCARECROW-LIKE 3); transcription factor [Arabidopsis thaliana]	2.00E-31	2.3±0.5	0.02
PH4B8	EB714638	IAA7 (AUXIN RESISTANT 2); transcription factor [Arabidopsis thaliana]	3.00E-102	2.3±0.1	0.043
PH4F9	EB714687	phloem filament protein; PP1; phloem protein 1 [Cucurbita maxima]	2.00E-34	2.3±0.1	0.043
FR12E17	DV632962	Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	3.00E-60	2.3±0.1	0.02
FR12E11	DV632956	cell wall glycine-rich protein [Cucumis sativus]	1.00E-05	2.3±0.03	0.02
FR15L15	DV634066	Response regulator, RegA/PrrA/ActR type [Medicago truncatula]	3.00E-49	2.3±0.3	0.02
FR11O11	DV632847	Phosphoethanolamine N-methyltransferase	5.00E-67	2.3±0.2	0.02
PH17D5	EB715846	No hit fund		2.2±0.1	0.02
FR12G2	DV633005	phospholipase D-alpha [Cucumis melo var. inodorus]	0	2.2±0.1	0.027
FR12C24	DV632931	Protein phosphatase 4 regulatory subunit 2-related protein CG2890-PA, isoform A [Drosophila melanogaster]	7.00E-08	2.2±0.02	0.024
FR11O24	DV632861	Tubulin alpha-3 chain	6.00E-121	2.2±0.2	0.02
FR10D21	DV632393	Protein of unknown function DUF248, methyltransferase putative [Medicago truncatula]	1.00E-10	2.1±0.9	0.02
FR10J3	DV632498	No hit fund		2.1±0.23	0.021
SSH2A5	DV631668	galactinol synthase [Cucumis sativus]	5.00E-27	2.1±0.1	0.02
PH2B11	EB714453	TatD-related deoxyribonuclease [Shewanella frigidimarina NCIMB 400]	1.00E-08	2.1±0.2	0.02
FR17I1	DV634649	unknown protein [Arabidopsis thaliana]	2.00E-06	2.1±0.3	0.023

expression pattern of this putative major latex protein (FR10B2) in the current microarray data. This protein was more highly expressed in Dulce than in Rochet through all four stages compared (25 dpa, mature, ripe and over-ripe) with the highest MFD value (MFD=23±1.6) at the mature stage (about 42 dpa). This expression patterns in Dulce are consistent with the Northen blot result mentioned above.

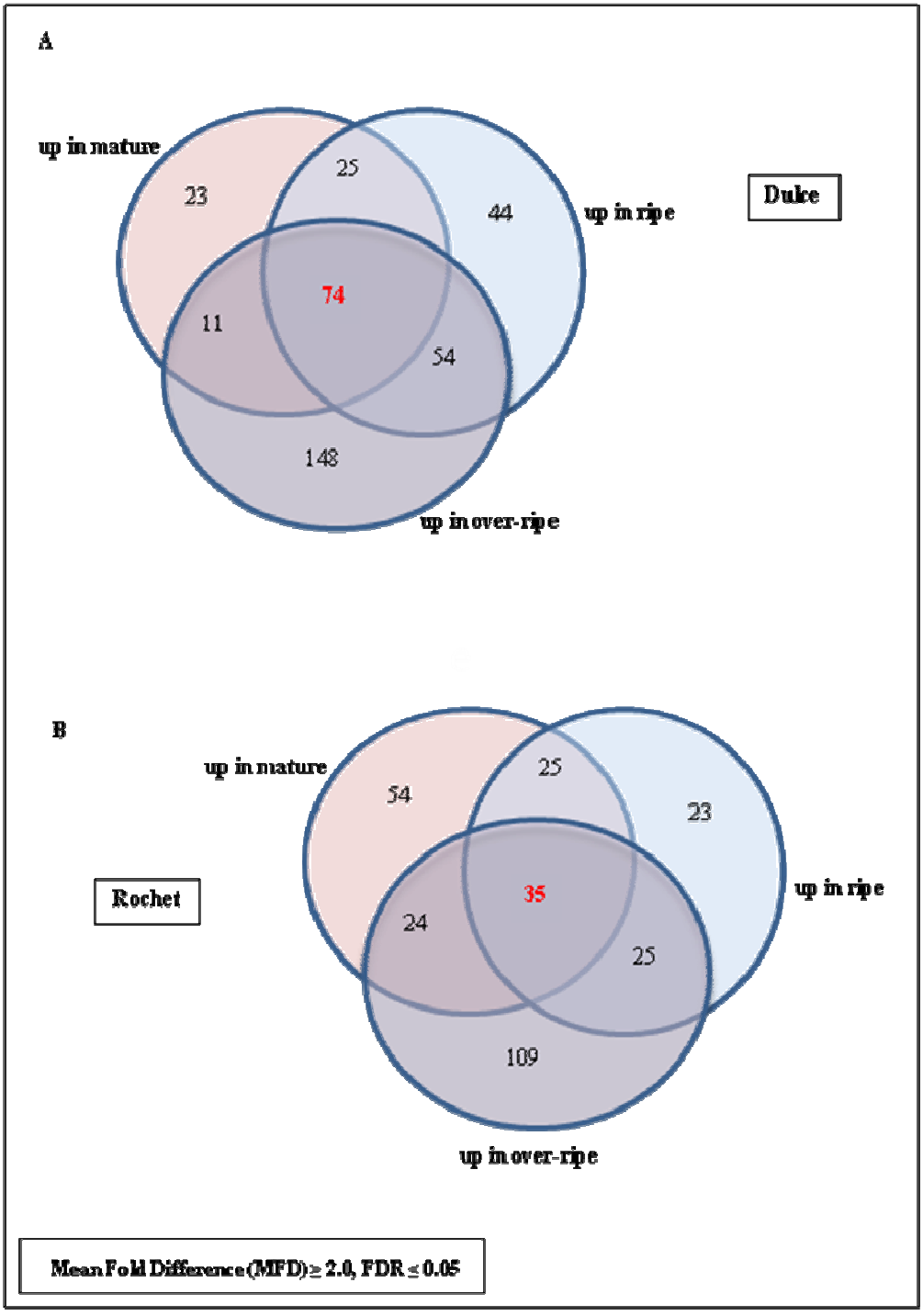
Four transcription factors showed up regulation in Dulce at 25 dpa. They were HAT22 (homeobox-leucine zipper protein 22, HDZip), BHLH093 (beta HLH protein 93), SCL3 (scarecrow-like 3) and IAA7 (auxin resistant 2, AXR2/IAA7). Both HDZip and beta HLH transcription factor families are large gene families in plants and mediate numerous aspects of plant growth and development such as cell elongation, cotyledon and leaf development, hormone response and seed germination. The SCL3 gene encodes a transcription factor that belongs to the GRAS family of proteins which was named based on the first three genes cloned: GIBBERELLINE-ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA) and SCARECROW (SCR). SCL3 is predominantly expressed in root and was suggested to regulate radical patterning of root in *A. thaliana* (Pysh et al., 1999). AXR2/IAA7 may control seedling and shoot development under light in *A. thaliana* (Nagpal et al., 2000).

Genes differentially expressed during ripening stages of climacteric versus non-climacteric melons

Figure 3.2 summarizes the differentially expressed unigenes for the two varieties at each stage and the overlapping up-regulated genes between stages. There were 74 genes up-regulated in all three ripening stages (mature, ripe and over-ripen) in

Figure 3.2 Summary of differentially expressed genes at ripening stages of Dulce and Rochet

(A) Genes differentially expressed in Dulce during ripening. Totally 74 genes were up regulated at all mature, ripe and over-ripe stage in Dulce. (B) Genes differentially expressed in Rochet during ripening. Totally 35 genes showed up regulation in Rochet at all three ripening stages. The cut-off value for differentially expressed genes was Mean Fold Difference (MFD) ≥ 2.0 and FDR ≤ 0.05.


A
up in mature
23
25
44
up in ripe
Duke
74
11
54
148
up in over-ripe
B
up in mature
54
25
23
Rochet
35
24
up in ripe
25
109
up in over-ripe
Mean Fold Difference (MFD) ≥ 2.0, FDR ≤ 0.05

Dulce and 35 in Rochet as showed in Tables 3.4 and 3.5, respectively. Of the 74 genes that were up-regulated in Dulce, 25% are annotated as unknown /hypothetical protein or have no homology. 14.7% are associated with fruit ripening modifications and include genes involved in ethylene biosynthesis (ACO and ACS), cell wall metabolism (polygalacturonase, beta-D-galactosidase and ripening-related expansin), aroma volatile biosynthesis (alcohol acetyltransferase, alcohol dehydrogenase) and sugar metabolism (starch phosphorylase type H, sugar transporter and fructose-1,6-bisphosphatase).

Of the 35 unigenes that were up regulated during ripening in Rochet, 20% were related to stress response such as low molecular weight heat shock proteins, a wound responsive protein and a hypersensitive reaction induced protein. Others include brassinosteroid -6-oxidase involved in brassinosteroid biosynthesis, phosphatidylinositol 3 (PI3P) and 4-kinase (PI4P) associated with lipid signaling and NADP-dependent malic enzyme. Aside from two additional genes, aldehyde dehydrogenase and alcohol dehydrogenase (FR12K20), no other genes previously related to ripening functions were up regulated at all stages in climacteric Rochet fruit. FR12K20 shows 88% sequence identity with *Cm-ADH1* (e = 6e-195, score = 684) at the amino acid level and is involved in ester volatile biosynthesis. *Cm-ADH1* is able to interconvert between alcohols and aldehydes. Aldehyde dehydrogenase (ALDH) catalyzes the oxidation of aldehydes. Both of these genes may be related to the stark differences in aroma volatile synthesis that differentiate climacteric versus non-climacteric melons.

Table 3.4 Genes upregulated in Dulce through all ripening stages

Clone ID	GB #	Best hit in GenBank	e value
FR13N20	DV633449	14-3-3-like protein [Gossypium hirsutum]	2.00E-71
FR15J17	DV634026	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1) (Ethylene-forming enzyme) (EFE)	3.00E-83
FR13N15	DV633444	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1) (Ethylene-forming enzyme) (EFE)	2.00E-219
FR17A20	DV634494	ACAT2/EMB1276 (ACETOACETYL-COA THIOLASE 2, EMBRYO DEFECTIVE 1276); acetyl-CoA C-acetyltransferase [Arabidopsis thaliana]	7.00E-241
SSH1N10	DV631608	ACC synthase [Musa acuminata]	2.00E-33
FR17E5	DV634580	AER [Nicotiana tabacum]	2.00E-72
PH6B3	EB714824	AGC2-1 (OXIDATIVE SIGNAL-INDUCIBLE1); kinase [Arabidopsis thaliana]	2.00E-66
SSH1L18	DV631584	alcohol acetyltransferase [Cucumis melo]	0
SSH6E12	DV631963	alcohol acetyltransferase [Cucumis melo]	4.00E-295
FR11E20	DV632676	anthocyanin acyltransferase-like protein [Arabidopsis thaliana]	3.00E-57
FR13N4	DV633455	AT4g10060/T5L19_190 [Arabidopsis thaliana]	3.00E-47
FR14F13	DV633607	At5g49900 [Oryza sativa (japonica cultivar-group)]	1.00E-106
SSH1G17	DV631510	ATBZIP60 (BASIC REGION/LEUCINE ZIPPER MOTIF 60); DNA binding / transcription factor [Arabidopsis thaliana]	6.00E-08
PH16F5	EB715783	auxin-responsive family protein [Arabidopsis thaliana]	3.00E-34
SSH1J24	DV631560	beta galactosidase [Vigna radiata]	8.00E-154
SSH1J16	DV631551	beta-D-galactosidase [Pyrus pyrifolia]	5.00E-68
FR15L5	DV634078	CCH (COPPER CHAPERONE) [Arabidopsis thaliana]	1.00E-39

Table 3.4 (Continued)

Clone ID	GB #	Best hit in GenBank	e value
FR15C21	DV633901	class III chitinase [Benincasa hispida]	2.00E-39
FR15B20	DV633880	clast3-related [Arabidopsis thaliana]	6.00E-53
FR13M24	DV633430	cold-induced glucosyl transferase [Solanum sogarandinum]	6.00E-41
FR15A10	DV633848	cyclin family protein [Arabidopsis thaliana]	7.00E-31
SSH6B3	DV631908	DELLA protein [Malus x domestica]	2.00E-80
FR15H19	DV633986	fatty acid 9-hydroperoxide lyase [Cucumis melo]	0
FR13C5	DV633235	fructose-1,6-bisphosphatase precursor [Solanum tuberosum]	7.00E-169
SSH1C21	DV631447	globulin-like protein [Cucumis melo]	2.00E-124
FR10K13	DV632507	glutaredoxin [Triticum aestivum]	3.00E-55
FR14D2	DV633573	glutathione transferase (EC 2.5.1.18) F1, Pugf - pumpkin	6.00E-101
FR15J16	DV634025	glycosyltransferase [Poncirus trifoliata]	5.00E-18
FR15M23	DV634096	GRAS10 [Solanum lycopersicum]	1.00E-33
FR15E24	DV633935	HMG-CoA reductase [Cucumis melo]	0
FR15I24	DV634011	homocysteine S-methyltransferase [Medicago sativa]	2.00E-70
FR16N3	DV634440	hydroxyproline-rich glycoprotein family protein [Arabidopsis thaliana]	2.00E-16
FR13M11	DV633416	hypothetical protein [Vitis vinifera]	8.00E-43
FR13P5	DV633498	hypothetical protein (japonica cultivar-group)]	3.00E-46
FR15N5	DV634120	isocitrate dehydrogenase (NAD+) [Nicotiana tabacum]	6.00E-61
SSH1B5	DV631434	isocitrate dehydrogenase, putative / NADP+ isocitrate dehydrogenase, [Arabidopsis thaliana]	7.00E-35

Table 3.4 (Continued)

Clone ID	GB #	Best hit in GenBank	e value
SSH4I8	DV631769	legumin-like protein [Zea mays]	1.00E-13
FR18B2	DV634836	lyase [Arabidopsis thaliana]	3.00E-44
SSH1H5	DV631526	Multicopper oxidase, type 1 [Medicago truncatula]	3.00E-26
FR15F22	DV633949	NAC4 protein [Glycine max]	6.00E-110
FR10P22	DV632610	Nitrate reductase [NADH] (NR)	7.00E-34
PH5D7	EB714757	Os04g0119000 [Oryza sativa (japonica cultivar-group)]	7.00E-08
FR18I15	DV634975	Os11g0242100 [Oryza sativa (japonica cultivar-group)]	3.00E-73
FR13A12	DV633192	polyamine oxidase [Nicotiana tabacum]	4.00E-119
FR13K13	DV633377	polygalacturonase precursor [Cucumis melo]	2.00E-113
FR15K20	DV634049	possible aldehyde decarbonylase [Arabidopsis thaliana]	6.00E-64
FR15P24	DV634159	putative alcohol dehydrogenases [Cucumis melo]	1.00E-181
SSH4G16	DV631745	putative ankyrin-repeat protein [Vitis aestivalis]	1.00E-76
FR10B2	DV632367	putative major latex protein [Momordica charantia]	4.00E-48
FR15K17	DV634045	putative pyrophosphate--fructose-6-phosphate 1-phosphotransferase [Arabidopsis thaliana]	1.00E-93
FR17O20	DV634780	putative Signal peptidase [Oryza sativa (japonica cultivar-group)]	2.00E-90
SSH9B1	DV632222	ripening-related expansin [Cucumis melo]	6.00E-167
FR10I16	DV632467	senescence-inducible chloroplast stay-green protein 1 [Lycopersicon esculentum]	8.00E-118
PH3C12	EB714559	SKP1 component [Medicago truncatula]	2.00E-86
FR15D18	DV633916	squalene monooxygenase 2 [Medicago truncatula]	4.00E-54

Table 3.4 (Continued)

Clone ID	GB #	Best hit in GenBank	e value
FR14O14	DV633807	starch phosphorylase type H [Citrus hybrid cultivar]	3.00E-133
FR11B10	DV632631	sugar transporter, putative [Arabidopsis thaliana]	3.00E-100
FR14K8	DV633732	THA1 (THREONINE ALDOLASE 1) [Arabidopsis thaliana]	8.00E-106
SSH6B1	DV631894	TTL4 (TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 4); binding [Arabidopsis thaliana]	2.00E-07
FR18K1	DV635011	UBC9 (UBIQUITIN CONJUGATING ENZYME 9); ubiquitin-protein ligase [Arabidopsis thaliana]	1.00E-100
PH2C11	EB714465	unknown protein [Arabidopsis thaliana]	1.00E-29
FR17C5	DV634538	unknown protein [Arabidopsis thaliana]	4.00E-24
SSH6D10	DV631937	unknown protein [Arabidopsis thaliana]	5.00E-30
FR15F3	DV633952	No hit fund	
FR10I3	DV632477	No hit fund	
FR14E16	DV633591	No hit fund	
PH3C4	EB714551	No hit fund	
SSH4C5	DV631714	No hit fund	
SSH1C24	DV631449	No hit fund	
PH11E2	EB715327	No hit fund	
FR10H5	DV632457	No hit fund	
SSH1N20	DV631617	No hit fund	
SSH4J7	DV631788	No hit fund	

Table 3.5 Genes upregulated in Rochet through all ripening stages

Clone ID	GB #	Best hit in GenBank	e value
FR17B16	DV634509	hypothetical protein OsI_013649 [Oryza sativa (indica cultivar-group)]	1.00E-22
PH6E2	EB714858	low molecular weight heat-shock protein [Nicotiana tabacum]	3.00E-73
SSH9B11	DV632224	Calmodulin-binding, plant [Medicago truncatula]	4.00E-15
SSH6A2	DV631884	low molecular weight heat-shock protein [Nicotiana tabacum]	3.00E-79
FR10F3	DV632428	Nthsp18p [Nicotiana tabacum]	3.00E-87
SSH4J3	DV631785	No hit fund	
FR18P23	DV635128	wound-responsive protein, putative [Arabidopsis thaliana]	1.00E-27
FR17C10	DV634524	homeodomain protein Hfi22 [Nicotiana tabacum]	6.00E-58
PH13E9	EB715511	X8 [Medicago truncatula]	5.00E-61
FR18C3	DV634861	EMB1417 (EMBRYO DEFECTIVE 1417) [Arabidopsis thaliana]	3.00E-92
SSH6C13	DV631918	No hit fund	
FR14E12	DV633588	senescence-associated protein-related [Arabidopsis thaliana]	3.00E-06
SSH6B14	DV631899	17 kDa phloem lectin Lec17-1 [Cucumis melo]	3.00E-119
FR17K24	DV634701	C2 [Medicago truncatula]	7.00E-51
FR17M21	DV634738	hypersensitive-induced reaction protein [Capsicum annuum]	1.00E-132
PH7H10	EB714996	C2 [Medicago truncatula]	1.00E-84
FR13L8	DV633412	brassinosteroid-6-oxidase [Vitis vinifera]	2.00E-123

Table 3.5 (Continued)

Clone ID	GB #	Best hit in GenBank	e value
FR12I18	DV633043	chloroplast drought-induced stress protein, putative [Arabidopsis thaliana]	2.00E-25
SSH6D22	DV631950	No hit fund	
FR17G15	DV634614	CYP81E8 [Medicago truncatula]	6.00E-72
SSH4J15	DV631777	aldehyde dehydrogenase [Vitis pseudoreticulata]	1.00E-54
FR12L18	DV633100	NADP-dependent malic enzyme (NADP-ME)	0.00E+00
FR12E7	DV632973	cystein proteinase inhibitor [Cucumis sativus]	3.00E-17
FR12I9	DV633054	TUB8 (tubulin beta-8) [Arabidopsis thaliana]	0.00E-01
FR12N13	DV633134	GATA transcription factor 1 (GATA-1) [Arabidopsis thaliana]	4.00E-18
PH3D7	EB714566	tetracycline transporter [Arabidopsis thaliana]	6.00E-38
SSH1A13	DV631408	NADP-dependent malic protein [Ricinus communis]	2.00E-31
SSH6J17	DV632080	putative glutamate decarboxylase [Glycine max]	3.00E-33
PH7G3	EB714977	phloem filament protein; PP1; phloem protein 1 [Cucurbita maxima]	4.00E-10
FR10B3	DV632370	No hit fund	
FR10O1	DV632582	Nucleic acid-binding, OB-fold [Medicago truncatula]	5.00E-44
FR18N19	DV635084	unknown protein [Arabidopsis thaliana]	1.00E-56
FR11O23	DV632860	Photosystem II 10 kDa polypeptide, chloroplast precursor (Light-inducible tissue-specific ST-LS1 protein)	3.00E-72
FR12K20	DV633085	putative alcohol dehydrogenases [Cucumis melo]	6.00E-272
SSH4P18	DV631863	Phosphatidylinositol 3- and 4-kinase, catalytic [Medicago truncatula]	1.00E-65

Four transcription factors were up regulated in non-climacteric Dulce through all ripening stages. They include homologs of NAC4, bZIP60, GRAS10 and DELLA proteins. A 14-3-3 like protein was also up regulated in Dulce in all mature fruit stages. 14-3-3s are a family of highly conserved proteins found in all eukaryotes. 14-3-3 proteins appear to play roles in many biological processes including signal transduction, cell cycle regulation, apoptosis, and stress responses (Janicka and Augustyniak, 2006). In a recent transcriptome comparison between wild type tomato and the ethylene-insensitive *Nr* ethylene receptor mutant, the mRNA of a 14-3-3 gene accumulated in ripening wild-type fruit in an ethylene dependent manner (Alba et al., 2005). This result is consistent with our melon array data and suggests that 14-3-3 proteins may play a role in climacteric ripening.

Two transcription factors were up regulated in Rochet during ripening: homeodomain Hfi22 and GATA transcription factor homologs. The homeodomain protein Hfi22 is a homeodomain leucine zipper (HD-Zip) family transcription factor which contains a leucine zipper motif adjacent to the homeodomain. Proteins belonging to this family exhibit diverse functions in plant development including meristem regulation, organ and vascular development and hormone signaling (Ariel et al., 2007). GATA transcription factor 1 (GATA-1) binds to the GATA motif found in the promoter regions of many light and circadian responsive genes. It has been reported that GATA transcription factors are involved in regulating expression of light responsive genes associated with seed germination (Liu et al., 2005), chlorophyll synthesis, glucose sensitivity (Bi et al., 2005) and shoot apical meristem and floral development (Zhao et al., 2004).

In an effort to better understand the distinct ripening behaviors of the non-climacteric Dulce and climacteric Rochet at the molecular level, we focused on genes in the following five major pathways important for melon fruit quality traits: ethylene biosynthesis and response, carotenoid biosynthesis, cell wall metabolism, aroma volatile compound biosynthesis and sugar and organic acid metabolism. MFD $\geq$ 1.5 and FDR $\leq$ 0.05 cut-off values for further consideration of the array data were employed for this analysis.

Ethylene biosynthesis and response

Ethylene is the key phytohormone regulating the ripening of climacteric fruits. Ethylene measurement showed that in Dulce fruit ethylene biosynthesis starts to increase at the mature stage (40-42 dpa, 74 PPM/KG/Hr), reached its peak at the ripe stage (45-46 dpa, 166 PPM/KG/Hr) and then decreased at the OR stage (over 50 dpa, 133 PPM/KG/Hr). Ethylene production did not rise above background during any of the ripening stages measured in Rochet (Figure 3.3). Accordingly, multiple genes related to ethylene biosynthesis and regulations were up regulated in Dulce fruit throughout ripening (Fig 3.4). *Cm-ACO1* (FR13N15 and FR15J17), and *ACS* (SSH1N10) which shows little sequence similarity with any melon ACS genes (*CMe-ACS1*=BAA93712; *CMe-ACS2*=BAA93713 and *pCMe-ACS3* =D68241) described to date, were highly induced in all ripening stages in Dulce. While both genes are induced in Dulce as compared to Rochet, their expression patterns are distinct (Fig 3.5). The transcripts of *Cm-ACO1* in Dulce fruit (compared to Rochet) are more abundant than *ACS*. In addition, the expression pattern of *ACS* (SSH1N10) in Dulce parallels ethylene production (increasing at M, peak at R and then decreased in OR).

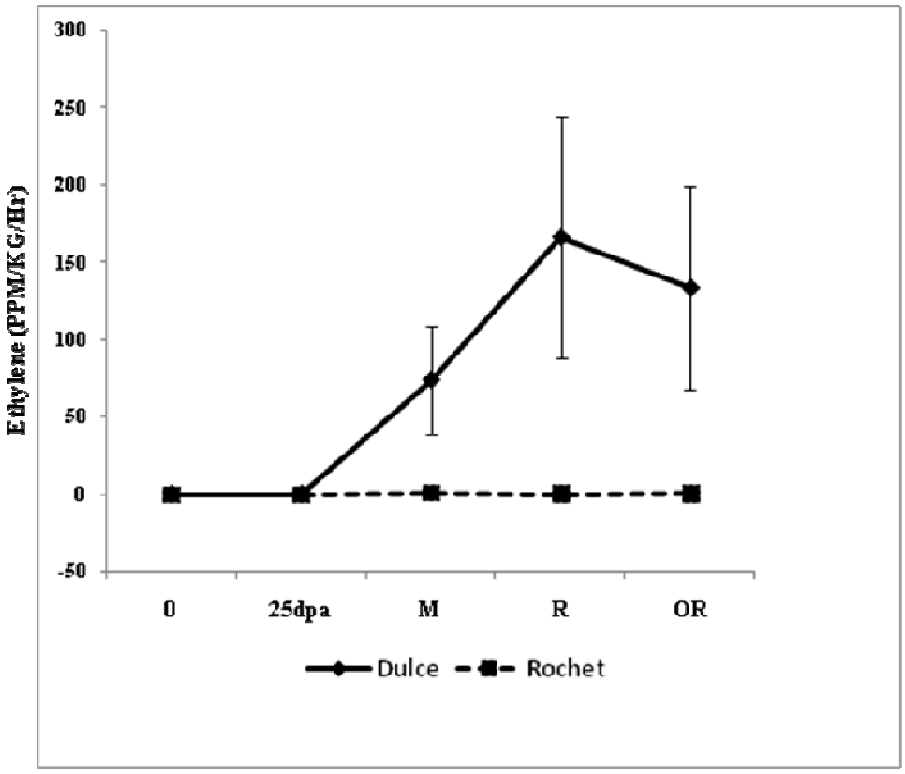


Figure 3.3 Ethylene production during fruit development of Dulce and Rochet

Ethylene production were measured in fruits from four developmental stages of Dulce and Rochet respectively. Stage 0 stand for the day right before pollination and the ethylene production at this stage was near zero. All fruits were harvested at the same season. The ethylene production of each stage were the mean value of the measurement of five individual fruit. During the ripening of Dulce fruit, ethylene started to increase at mature (M) stage and reached its highest level a ripe stage (R) then decreased, indicating that Dulce belongs to climacteric melon variety. The evolution of ethylene from Rochet was undetectable for all four stages measured that confirmed the non-climacteric ripening characteristic of Rochet.

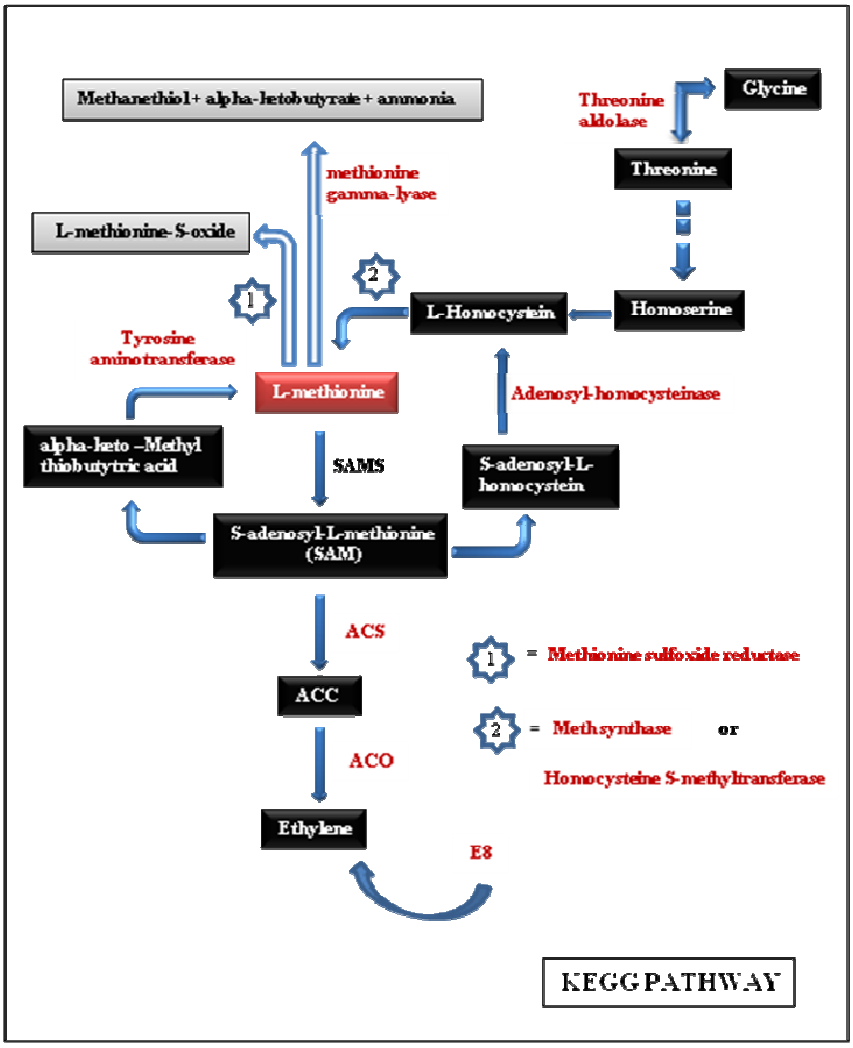


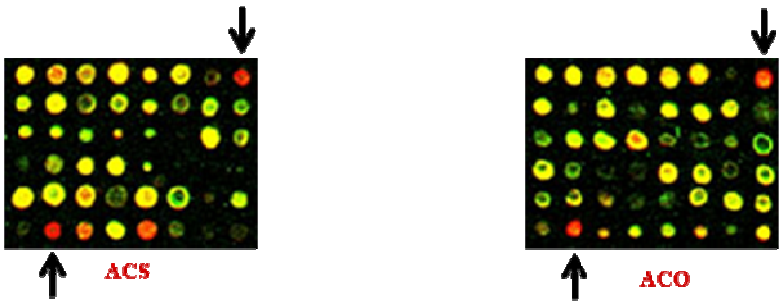
Figure 3.4 Ethylene Biosynthesis pathway

The sketch of ethylene biosynthesis pathway that was drawn based on the information from KEGG database. All the genes that were labeled in this pathway have been identified on the melon chip. The genes in red color showed differential expression during the ripening of Dulce fruit. While the ones in black color represented the genes that had no differential expression between Dulce and Rochet fruit during neither of the mature, ripe or over-ripe stage.

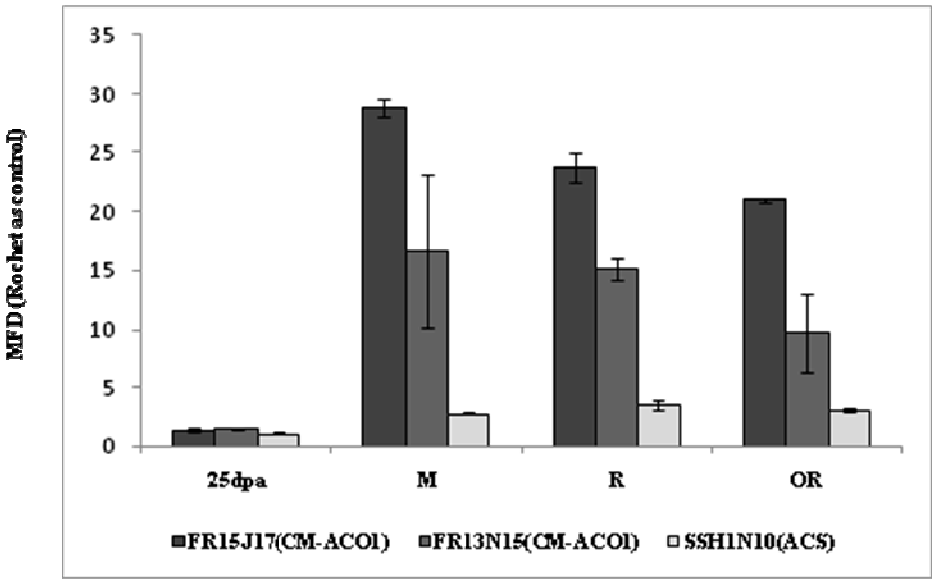
Figure 3.5 Expression patterns of ACO and ACS in Dulce

(A)The composite image of microarray hybridization on melon chips using tissue from fruit of mature stage. As the probes from Dulce was labeled with cy5 and probes from Rochet was labeled with cy3, the red color represented genes that were up regulated in Dulce, the spots with yellow color showed the genes that were equally expressed between these two species. While the green color stood for the genes that were highly expressed in Rochet at the stage compared.The two red spots pointed by the red and black arrows were two of the three replicates on melon arrays for ACS and ACO respectively.(B) Presented the expression pattern of ACO and ACS in Dulce fruit during ripening. The transcripts of *Cm-ACO*s increased dramatically at mature stage and then decreased gradually thereafter. The expression pattern of *Cm-ACO* was concomitant with the ethylene production in Dulce fruit during ripening. The cut-off value for differential expressed genes was MFD≤ 1.5 and FDR≥0.05.

A


ACS
ACO

B


MFD (Rochet as control)
35
30
25
20
15
10
5
0
25dpa
M
R
OR
FR15J17(CM-ACO1)
FR13N15(CM-ACO1)
SSH1N10(ACS)

In contrast, both *Cm-ACO1* unigenes reached their highest mRNA levels at the mature stage (i.e. prior to the climacteric ethylene peak) and gradually decrease during maturation. This pattern may reflect the previously documented nature of ACO activity in terms of being abundant in pre-ripening fruit and as such allowing ethylene biosynthesis once ACS activity becomes elevated during ripening (Alexander and Grierson, 2002; Barry et al., 1996; Barry et al., 2000; Liu et al., 1985). In addition, the expression patterns of *Cm-ACO1* and *ACS* (SSH1N10) in climacteric Dulce fruit are similar to tomato (Alba et al., 2005), indicating that the ACO and ACS activity may respond to similar regulatory mechanisms during climacteric fruit ripening.

In contrast, few genes involved in of the early pre-committed stages of the ethylene biosynthesis pathway showed differential expression between Dulce and Rochet during ripening, suggesting primary ethylene synthesis control occurs at the ACS and ACO steps. These earlier genes include those encoding homologs of homocysteine s-methyltransferase and methionine synthase, Adenosyl-homocysteinase, and tyrosine aminotransferase (Berger et al., 1998). Interestingly, transcript accumulation of these genes was increased in Dulce fruit through all ripening stages (MFD $\geq$ 1.5, FDR $\leq$ 0.05) (Fig 3.6). Among them, the mRNA levels of homocysteine s-methyltransferase and tyrosine aminotransferase were most dramatically elevated in Dulce suggesting ethylene dependent regulation of these two genes. Three genes with roles in methionine catabolism were also monitored: methionine gamma-lyase that converts methionine to methanethiol; threonine aldolase (two uigenes: FR14K8 and PH9F7) involved in threonine breakdown and L-methionine (S)-S-oxide reductase that interconverts L-methionine and L-methionine-S-oxide. Both methionine gamma-lyase and threonine aldolase were up regulated (MFD $\geq$ 1.5, FDR $\leq$ 0.05) in Dulce during all three ripening stages (Fig 3.7). These

results indicate that the lack of ethylene biosynthesis in non-climacteric melons may also be controlled in part at the substrate level by regulating the methionine supply balance via methionine catabolism.

An E8 gene homolog (SSH4B19) that showed 72% (e value: 1e-019) sequence identity with an Arabidopsis E8 gene at the amino acid level has also been characterized. E8 encodes a presumed oxidase that plays a role as a negative regulator of ethylene biosynthesis in tomato (Kneissl and Deikman, 1996). Expression of E8 increased dramatically during tomato ripening and is known to respond to both ethylene and non-ethylene ripening signals (Deikman and Fischer, 1988). SSH4B19 has only modest sequence similarity with tomato E8 at the nucleotide and amino acids level (data not shown). However, the expression of SSH4B19 was up regulated in Dulce at moderate levels (MFD: 1.5-1.9) at all three ripening stages and its expression pattern paralleled the level of ethylene in Dulce during ripening. While the DNA sequence was less convincing, the expression pattern of this melon E8 homolog suggests that this gene may indeed possess and E8-like role in melon ripening.

Few factors involved in ethylene signal perception and transduction have been identified in melon (Fig 3.8). Melon homologs of ethylene receptor *Cm-ETR1*, EIN3-binding–F-box protein 1 (*EIN3-1*), an EIN3-like protein (*Cm-EIL2*), *EIN2*, *Cm-ERF2* and *EREBP* homologs were monitored in our analysis. Only the expression of *Cm-ETR1*, *EIN3-1*, *Cm-EIL2* and *ERF5* were highly induced during fruit ripening in Dulce. *Cm-EIL2* and *ERF5* were increased only at the mature stage whereas *Cm-ETR1* and *EIN3-1* preferentially accumulated at the final stages of ripening (OR). It is important to keep in mind that ethylene signaling genes are also associated with

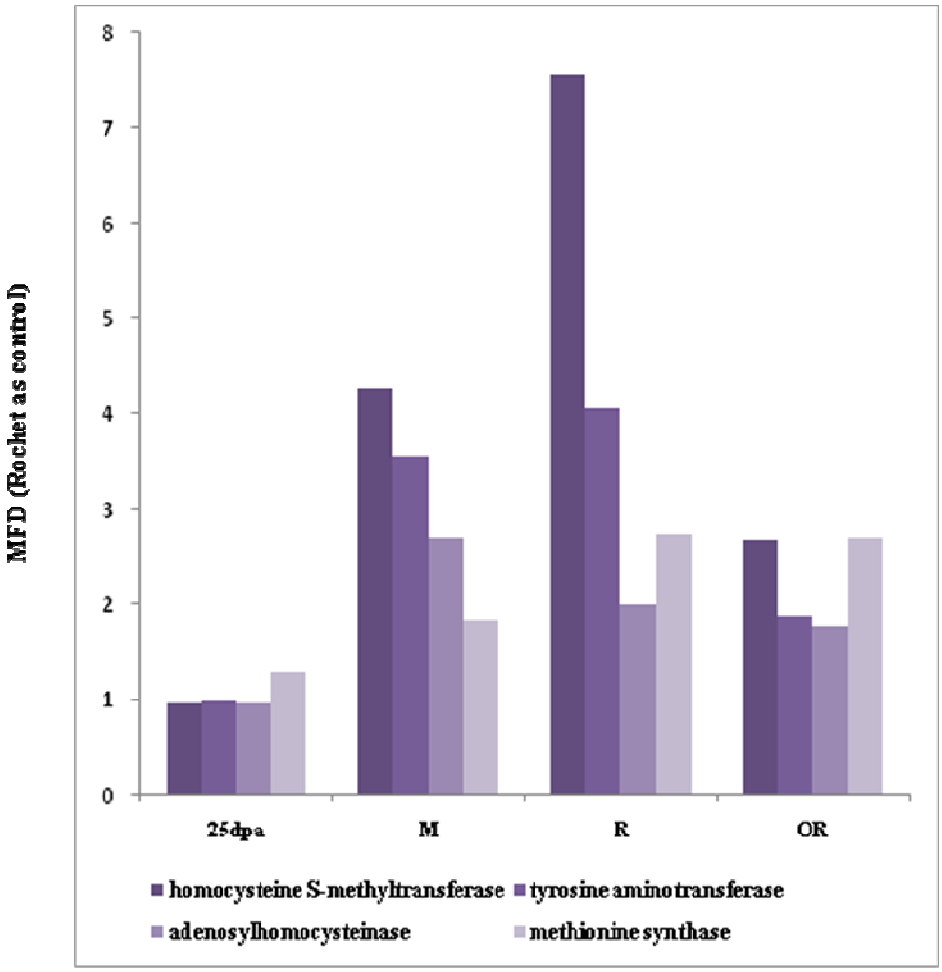


Figure 3.6 The expression patterns of genes involved in methionie biosynthesis in Dulce

All the four genes showed up-regulation in Dulce fruit during ripening but with different expression patterns. The mRNA level of homocysteine S-methyltransferase and tyrosine aminitransferase started to increase at mature stage, reached to highest level at ripe stage and then decreased which were similar with the ethylene evolution in Dulce fruit during ripening.The expression of adenosylhomocysteinase was greatly induced at mature stage and then decreased gradually .While the transcripts of methionine synthase were accumulated to higher level at ripe and over-ripe stage in Ducle.The cut-off value for differentially expressed genes was MFD $\leq$ 1.5 and FDR $\geq$ 0.05.

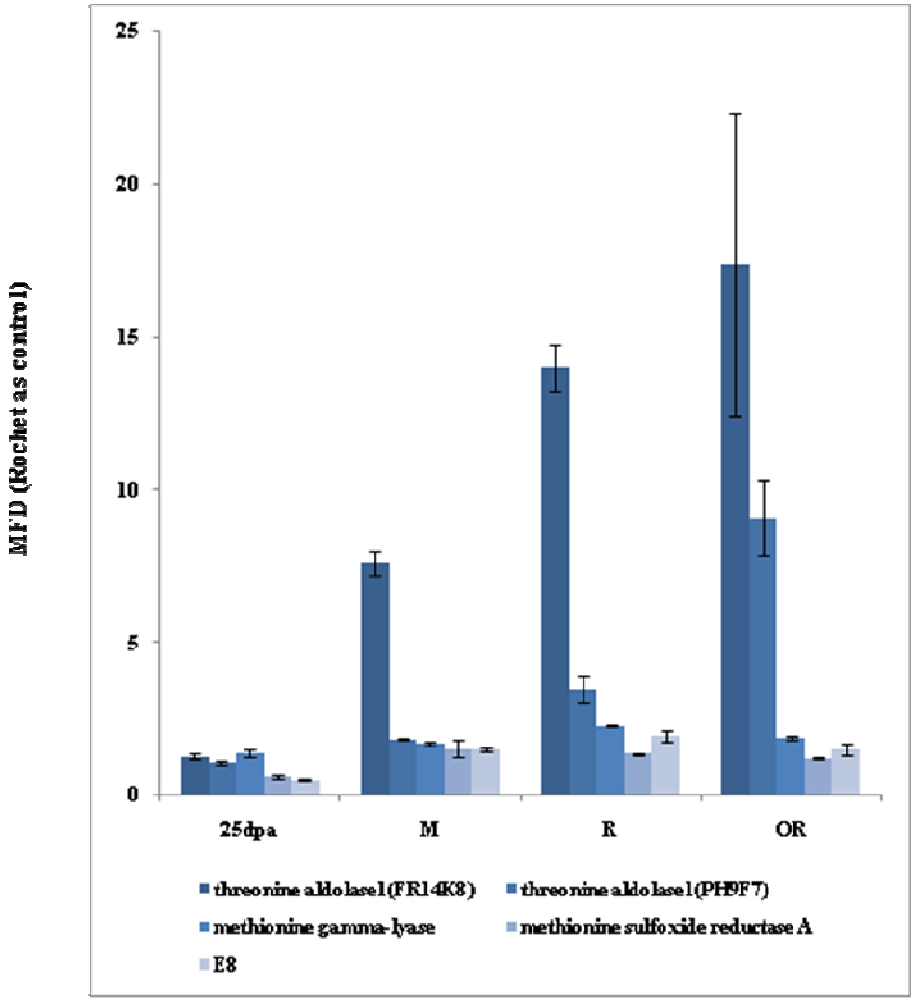
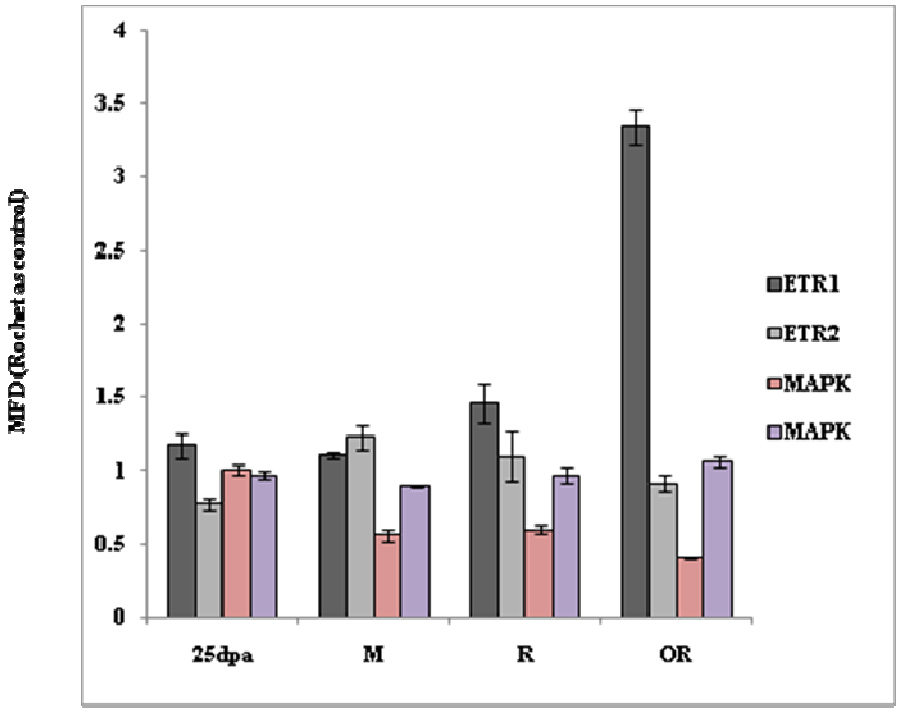


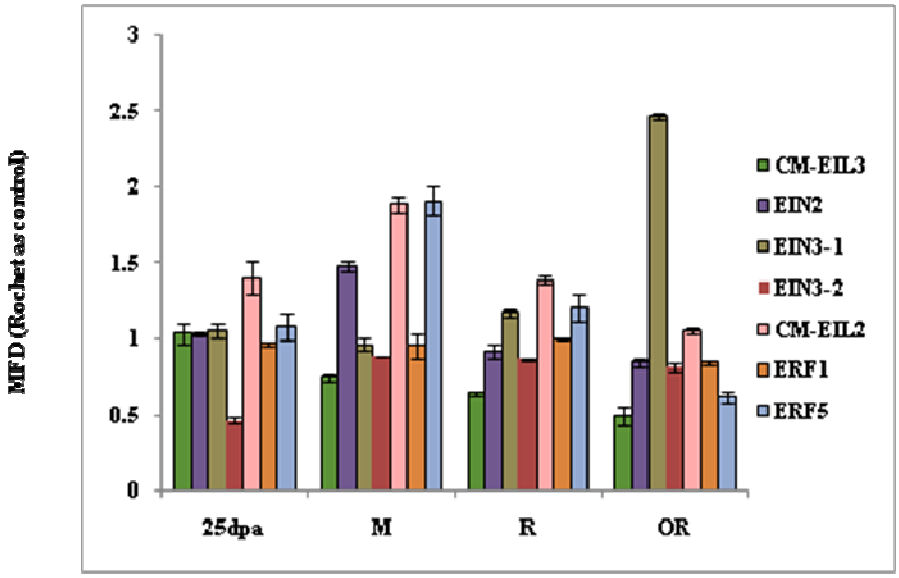
Figure 3.7 The expression patterns of genes involved in methionie catabolism and E8 in Dulce

The mRNA of theronine aldolase 1 (FR14K8) was the most abundance among the five genes during the ripening of Dulce fruit and the expressions of both theronine aldolase 1 genes were increased gradually during the ripening of Dulce fruit .The expression pattern of methionine gama-lyase and E8 mimicked that of ethylene evolution in Dulce during ripening.The methionine sulfoxidase reductase A was up regulated only at mature stage in Dulce.The cut-off value for differentially expressed genes was MFD≤ 1.5 and FDR≥0.05

Figure 3.8 The expression patterns of genes involved in ethylene signaling in Dulce

The expression patterns of the genes showing in the parenthesises in (A). Where Cm-EIL2 and ERF5 were up regulated only at mature stage.ETR1 and EIN3-1 were expressed at a higher level at over-ripe stage in Dulce. The cut-off value for differential expressed genes was MFD≤ 1.5 and FDR≥0.05


MFD (Rochet as control)
4
3.5
3
2.5
2
1.5
1
0.5
0
25dpa
M
R
OR
ETR1
ETR2
MAPK
MAPK


MFD (Rochet as control)
3
2.5
2
1.5
1
0.5
0
25dpa
M
R
OR
CM-EIL3
EIN2
EIN3-1
EIN3-2
CM-EIL2
ERF1
ERF5

pathogen response and as such, induction specifically at late ripening stages may reflect a response to increased fruit pathogen susceptibility. *CS-ETR2*, *ERF1* and *ERF2* were equally expressed in Dulce and Rochet for all the four stages and may be related to basal ethylene response in both varieties.

The role of ethylene on ripening in non-climacteric fruit is poorly understood. Some fruits such as citrus and grape do respond to exogenous ethylene by accelerating at least a subset of ripening phenotypes (e.g. pigment accumulation), it is not clear that ethylene is required for ripening in these species (Tesniere et al., 2004; Trebitsh et al., 1993). Strawberry is one of the few examples of non-climacteric ripening where ripening proceeds normally even in the presence of ethylene inhibitors (Bower et al., 2003; Tian et al., 2000). Even though ethylene is not required for the ripening of Rochet fruit, we observed four transcription factors involved in ethylene signal transduction that were up regulated: an ethylene response factor 2 (SSH6B7) and an ethylene-responsive element binding protein (FR12K1) were induced at the mature stage. The mRNA abundance of an EIN3 like protein (*Cm-EIL1*=FR18H24) was increased at the R and OR stages, and an *EREBP-4* homolog was elevated at the OR stage. It is plausible that the induction of these genes might reflect a mechanism to increase sensitivity to basal ethylene levels in maturing non-climacteric fruit or they may represent responses through the pathway to other signals. Interestingly, an Aux/IAA protein and a brassinosteroid biosynthesis enzyme, brassinosteroid-6-oxidase, accumulate in Rochet through out ripening and auxin and brassinosteroids have been shown to influence ripening of non-climacteric fruits (e.g. strawberry and grape) (Symons et al., 2006).

Cell wall metabolism

Climacteric melon such as Dulce, undergo a rapid decrease in fruit firmness during ripening. As shown in Figure 3.9 (data kindly provided by N. Katzir from the same fruit we used for microarray analysis), the internal and external firmness of Dulce fruit were lower than that of Rochet (T test p value < 0.05) when measured at the overripe stage, which indicates that Dulce is softer than Rochet at this stage. A range of cell wall modifying enzymes that contribute to the softening phenotype of fruit have been identified and characterized in the model fruit tomato, such as polygalacturonase (PG), pectin methylesterase (PME), beta-galactosidase (GAL), xyloglucan endotransglycosylase (XTH) and expansin (EXP). The melon cDNA microarray contains 4 fragments that were annotated as *Cm-PG1*, *Cm-PG2* and *Cm-PG3* respectively based on sequence similarity at the amino acid level. Two fragments on the melon chip were annotated as EXPs. One of them, SSH9B1 shared 100% sequence identity with *Cm-EXP1*. The other EXP, FR13C9, was similar to *Gossypium Hirsutum* EXP2. Our melon chip also contained 3 *PME*s (SSH1H5, FR12E14, FR12O19), 1 *RGase*, 6*XTH* [XTH (PH11F10), *XTH*1, 3, 29, 38, 3 9], 4 *GAL*s [*GAL*2, *GAL*6 and two other *GAL*s (SSH1J24, FR14M19)]. The best homology hits for these sequences in GenBank are shown in Table 6.

Expression patterns of these cell wall metabolism enzymes fall into 3 groups: 1) Up regulated in Dulce fruit during ripening (MFD ≥ 1.5 and FDR ≤ 0.05). Most fell into this group including *Cm-PG1*, *Cm-PG2*, *Cm-EXP1*, *EXP2, PME* (SSH1H5), *RGase*, *XTH3*, *XTH39, GAL6* and two *GALs* (SSH1J24, FR14M19). The mRNA level of *Cm-EXP1* and *Cm-PG2* increased the most in Dulce during ripening but showed distinct expression patterns. The expression pattern of *Cm-EXP1* followed

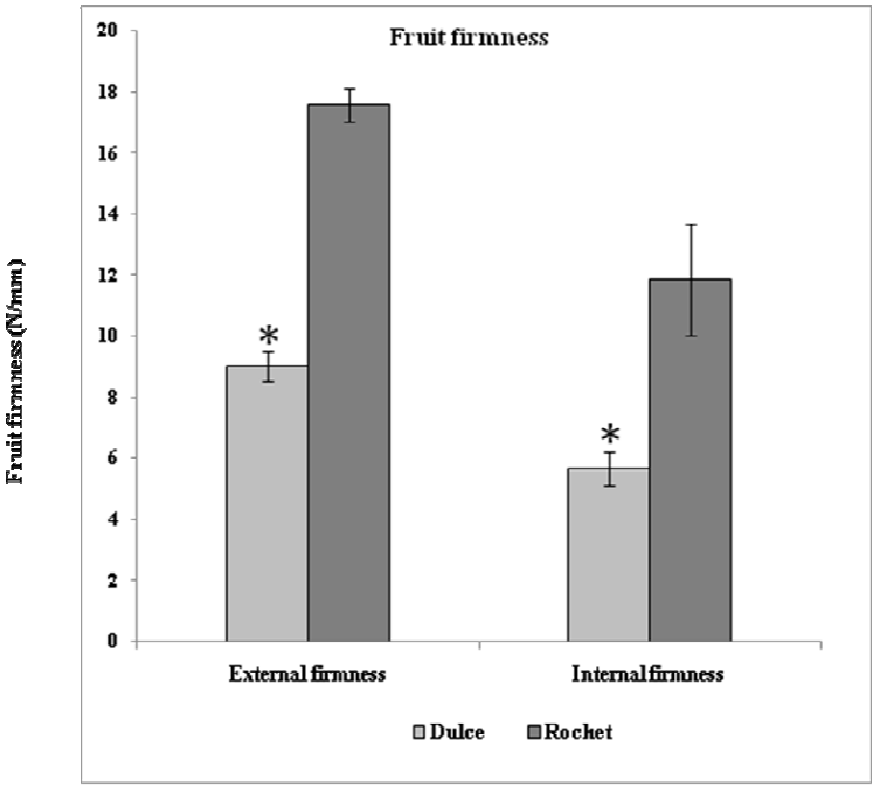


Figure 3.9 Fruit firmness of Dulce and Rochet during ripening

Both external and internal firmness were measured to compare the softness of Dulce and Rochet at over-ripe stage .T test result showed that Dulce was more softer than that of Rochet at ripe stage. ($p<0.05$).

Table 3.6 Differential expression of genes involved in cell wall metabolism in Dulce and Rochet during fruit ripenin

Enzyme	Clone ID	Best Hit in GenBank	E value	Up in Dulce	Equal	Up in Rochet
CM-PG1	FR14B12 FR16G14	polygalacturonase precursor [Cucumis melo]	0 2e-59	CM-PG1		
CM-PG2	FR13K13	polygalacturonase precursor [Cucumis melo]	4e-82	CM-PG2		
CM-PG3	FR14J7	polygalacturonase precursor [Cucumis melo]	0		CM-PG3	
CM-EXP1	SSH9B1	ripening-related expansin [Cucumis melo]	6E-167	CM-EXP1		
EXP2	FR13C9	alpha-expansin 2 [Gossypium hirsutum]	3E-135	EXP2		
PME	SSH1H5	Pectinesterase [Medicago truncatula]	3E-26	PME		
PME	FR12E14	pectinesterase family protein [Arabidopsis thaliana]	2E-57		PME	
PME	FR12O19	pectinesterase family protein [Arabidopsis thaliana]	3E-99			PME
RGase	FR18B2	lyase /Rhamnogalacturonate lyase family [Arabidopsis thaliana]	3E-44	RGase		
XTH1	SSH9C4	xyloglucan endotransglycosylase XET1 [Tropaeolum majus]	2E-25		XTH1	
XTH3	FR18A19	xyloglucan endotransglycosylase XTH-3 [Populus tremula]	2E-67	XTH3		
XTH29	PH8D8	xyloglucan:xyloglucosyl transferase XTH29 [Arabidopsis thaliana]	2E-166		XTH29	
XTH39	FR15H14 FR15O2	xyloglucan endotransglycosylase XTH-39 [Populus tremula]	5E-21 3E-69	XTH39		
XTH38	FR18O11	xyloglucan endotransglycosylase XTH-38 [Populus tremula]	5E-104			XTH38

Table 3.6 (Continued)

Enzyme	Clone ID	Best Hit in GenBank	E value	Up in Dulce	Equal	Up in Rochet
XTH	PH11F10	xyloglucan endotransglycosylase [Gossypium herbaceum]	3E-21		XTH	
GAL2	FR11L16	beta-D-galactosidase [Pyrus pyrifolia]PpGAL2	3E-34		GAL2	
GAL6	SSH1J16	beta-D-galactosidase [Pyrus pyrifolia]PpGAL6	5E-68	GAL6		
GAL	SSH1J24	beta galactosidase [Vigna radiata]	8E-154	GAL		
GAL	FR14M19	beta-galactosidase precursor [Carica papaya]	2E-138	GAL		

that of ethylene evolution increasing at the mature stage and peaking at ripe. Genes showing similar expression included *GAL6*. The accumulation of *Cm-PG2* transcripts was increased gradually from the mature stage through the over-ripe stage. *Cm-PG1* (FR16G14), *RGase* and *XTH39* all showed similar expression patterns. 2) Equally expressed between Dulce and Rochet at all ripening stages (MFD $\leq$ 1.5 and FDR $\leq$ 0.05) and which include *Cm-PG3*, one *PME* (FR12E14), *XTH* (PH11F10), *XTH1*, *XTH29* and *GAL2*. Finally, 3) highly expressed in Rochet during ripening (MFD $\geq$ 1.5 and FDR $\leq$ 0.05). There were only two genes up regulated in Rochet: *PME* (FR12O9) and *XTH* 38. The mRNA level of *PME* (FR12O9) increased in Rochet at the ripe stage and continued through the over-ripe stage, while *XTH* 38 was up regulated in the mature and ripe stages.

Carotenoid Biosynthesis

It has been reported that carotenoid profiles of orange flesh melons included: 84.7% beta-carotene, 6.8% delta-carotene, 2.4 % phytofluene, 1.5% phytoene, 1.2% alpha-carotene, and 1.0% lutein with trace amounts of additional carotenoids (Nunez-Palenius et al., 2008). Ibdah M, et al. reported that the predominant carotenoids in Dulce fruit are beta-carotene (95%) and lutine (5%) (Ibdah et al., 2006). Beta-carotene is the principle pigment of orange flesh melons ranging from 9.2 to 18.0 ug /g (Nunez-Palenius et al., 2008; Watanabe et al., 1991). The main carotenoids in the pale-green fleshed Rochet are beta-carotene and phytoene though net concentration of both is very low. Rochet fruit contain 0.31(± 0.05) ug /g fresh weight (FW) of beta-carotene with lutine at a similar level (Fig 3.10, data kindly provided by N. Katzir on the same fruit used in our microarray analysis).

Orange-fleshed Dulce :

total carotenoids : 45.4 ± 2.6 ug/ g FW

β - carotene : 95 % of total carotenoids

Lutine : 5 % of total carotenoids

(Ibdah M. et al. *Phytochemistry* 2005)

Pale-green fleshed Rochet :

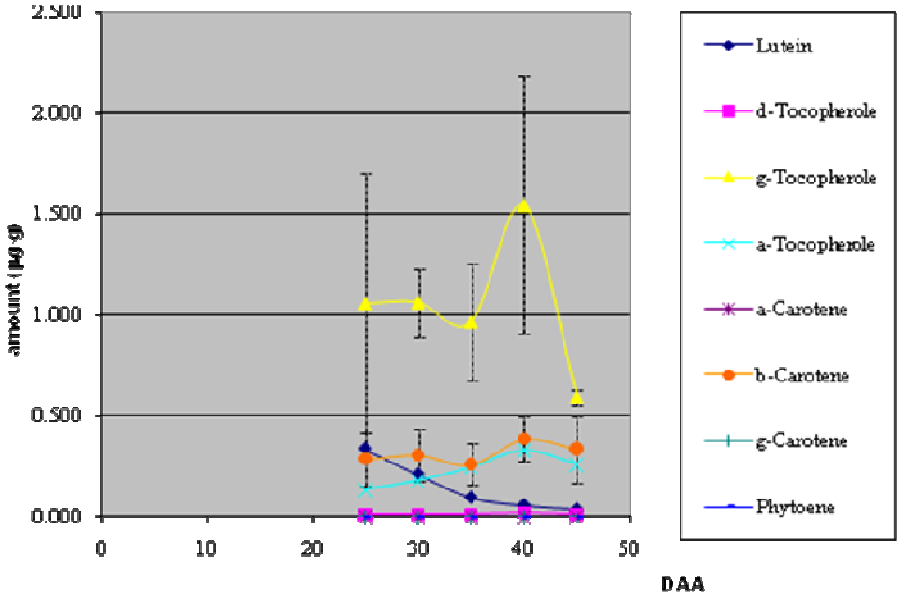


Figure 3.10 Carotenoids composition of Dulce and Rochet during ripening

The carotenoids composition data in Dulce fruit was derived from the paper of Ibdah M. et al. published in *Phytochemistry* 2005.There data showed that beta-carotene was the main carotenoids in Ducle fruit with the concentration up to 40 ug/ g FW. The main carotenoids compound in Rochet fruit during ripening were low level of beta-carotene (0.3 ug/g FW) and trace amount of phytoene.

The melon cDNA chip contains eight genes involved in carotenoid biosynthesis (Fig 3.11): DXR (1-deoxy-D-xylulose-5-phosphate-reductoisomerase),GGPS (geranylgeranyl pyrophosphate synthase), PSY (phytoene synthase), PDS (phytoene desaturase), ZDS (zeta-carotein desaturase), CHY-b (beta-carotene hydroxylase), b-LCY (lycopene beta-cyclase) and ZEP (zeaxanthin epoxidase). Most were cloned based on homology. Of the eight genes, only two showed differential expression between Dulce and Rochet during fruit ripening. DXR was up regulated in Dulce fruit through all ripening stages (M, R, OR). PDS mRNA started to accumulate more highly in Dulce at the ripe stage and remained at about the same level through the over-ripe stage. All the others were equally expressed within the criteria of our expression profiling (MFD $\leq$ 1.5 and FDR $\leq$ 0.05) suggesting that pathway regulation occurs primarily via expression of a few key pathway genes.

To better understand carotenoid accumulation in maturing melon fruit we also looked at the expression of genes involved in carotenoid catabolism. Four unigenes identified on the melon chip may play roles in carotenoid degradation or biosynthesis control: geranylgeranyl hydrogenase (GGHY), geranylgeranyl reductase (GGRD), carotenoid cleavage dioxygenase (CCD) and a chromoplast-specific carotenoid-associated protein. Both GGHY and GGRD are able to reduce free geranylgeranyl diphosphate (GGPP) to phytil diphosphate, which provides the side chain to chlorophylls, tocopherols, and plastoquinones. In our current microarray analysis, GGHY and GGRD were equally expressed between Dulce and Rochet during fruit ripening. CCD has been extensively studied because of its ability to generate varied apocarotenoid products from multiple carotenoid substrates (Auldridge et al., 2006; Ibdah et al., 2006; Vogel et al., 2008). One of the aprocarotenoids, beta -ionone, which is the product of cleavage of beta-carotene at the

9',10' position by CCD, contributes to the flavor of many fruits including melon and tomato (Auldridge et al., 2006; Ibdah et al., 2006). Chromoplast-specific carotenoid-associated protein has been reported to be involved in carotenoid sequestration within chromoplasts (Vishnevetsky et al., 1996; Vishnevetsky et al., 1997). The mRNA level of both CCD and chromoplast-specific carotenoid-associated protein were increased gradually in Dulce during ripening (Fig 3.12) and thus may contribute to total fruit carotenoid levels.

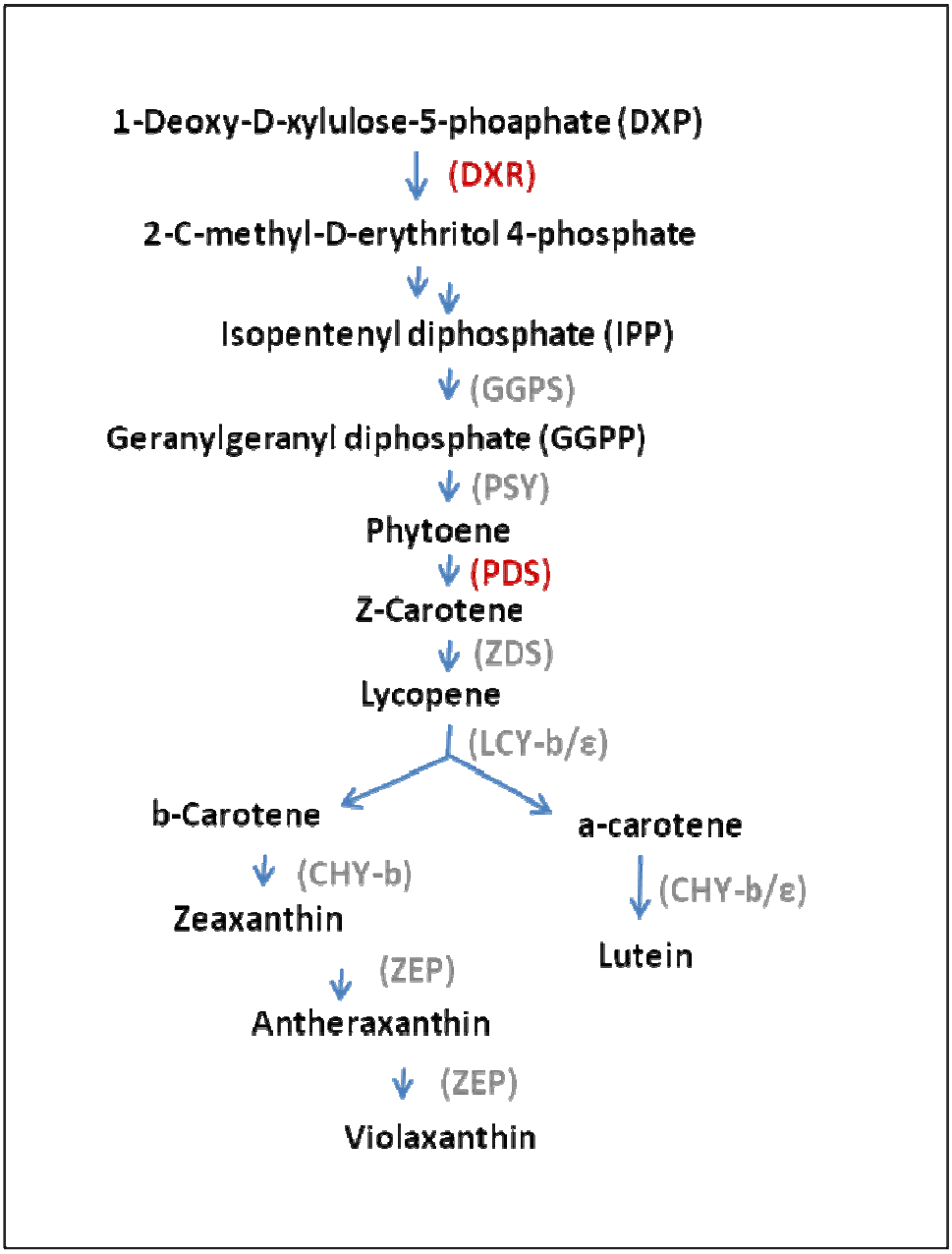
qRT-PCR analysis was employed to re-evaluate the expression patterns of nine genes involved in carotenoid metabolism (DXR, GGPS, GGHY, PSY, PDS, ZDS, CHY-b, b-LCY and ZED). Eight of them (all except b-LCY) showed similar expression patterns between Dulce and Rochet as compared to the microarray data (See below "**Microarray Data Validation "**).

Aroma volatile compound biosynthesis

The volatile profile of Rochet was first measured by Shalit M. in 2001 using Solid Phase Microextraction (SPME) (Shalit et al., 2001). In the ripe fruit of Rochet, 60% of the volatiles detected were alcohols, 20% s aldehydes and about 7% were esters. This profile did not change significantly from mature to over-ripe fruit. The volatile profile of a highly aromatic, orange-fleshed climacteric fruit Arava (*Cucumis melo* var.*inodorus*) at the unripe stage was mainly aldehydes (73.8%) and low levels of alcohols (13%). No esters were detected at this stage. During ripening, the volatile profile was greatly altered in Arava to predominantly esters (83.3%) with lesser amounts of alcohols (4.3%) and aldehydes (0.4%). As Dulce has breeding ancestry related to Arava and contains orange flesh and high aroma levels, we hypothesize that

Figure 3.11 Carotenoids biosynthesis pathway and related genes on melon chip

All the genes that have been labeled here in this pathway were identified on our melon chip. The genes in gray color showed no differential expression between Dulce and Rochet during ripening . While genes in red color were up regulated in Dulce at one or more ripening staged.


1-Deoxy-D-xylulose-5-phoaphate (DXP)
(DXR)
2-C-methyl-D-erythritol 4-phosphate
Isopentenyl diphosphate (IPP)
(GGPS)
Geranylgeranyl diphosphate (GGPP)
(PSY)
Phytoene
(PDS)
Z-Carotene
(ZDS)
Lycopene
(LCY-b/ε)
b-Carotene
a-carotene
(CHY-b)
(CHY-b/ε)
Zeaxanthin
Lutein
(ZEP)
Antheraxanthin
(ZEP)
Violaxanthin

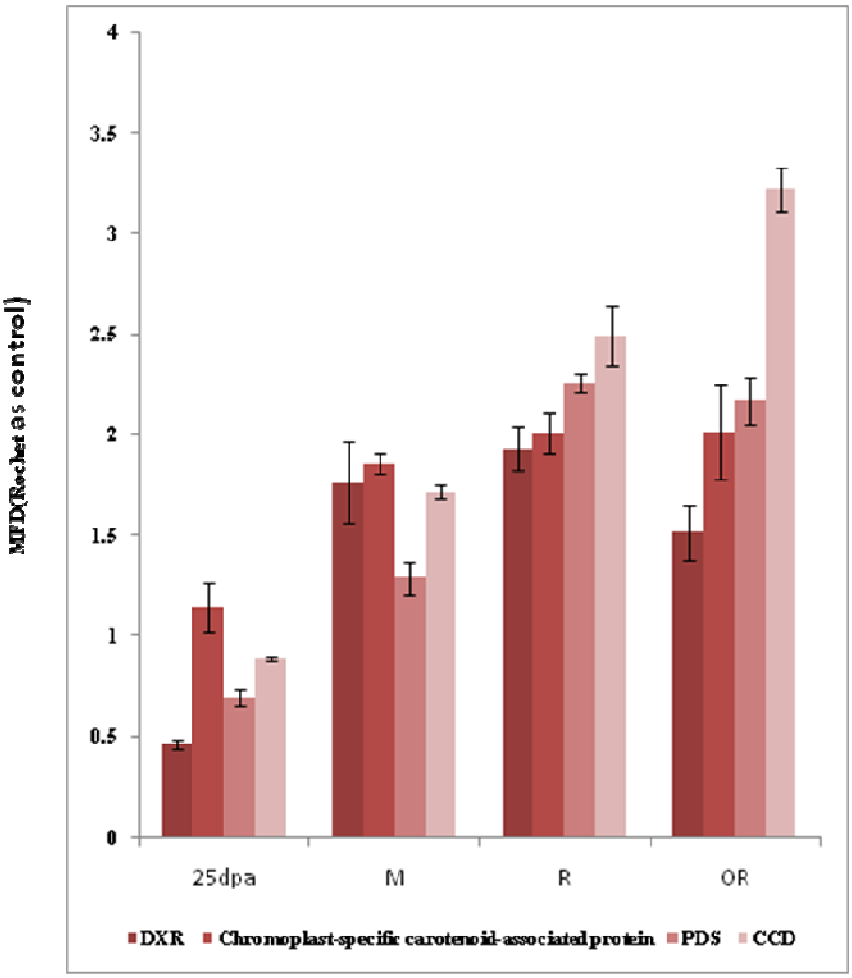


Figure 3.12 The expression patterns of genes involved in carotenoids metabolism in Dulce

DXR and PDS were involved in carotenoid biosynthesis. DXR were up regulated at all ripening stages in Dulce and its expression pattern followed that of ethylene production during the ripening of Ducle.PDS were up regulated at ripe and over-ripe stage. The chromoplast-specific carotenoid – associated protein that might involved in carotenoid sequestration in chromoplast were also up regulated through all mature, ripe and over-ripe stage in Ducle. The mRNA level of CCD (carotenoid cleavage dioxygenase) was gradually increased in Dulce during ripening.The cut-off value for differential expressed genes was MFD≤ 1.5 and FDR≥0.05

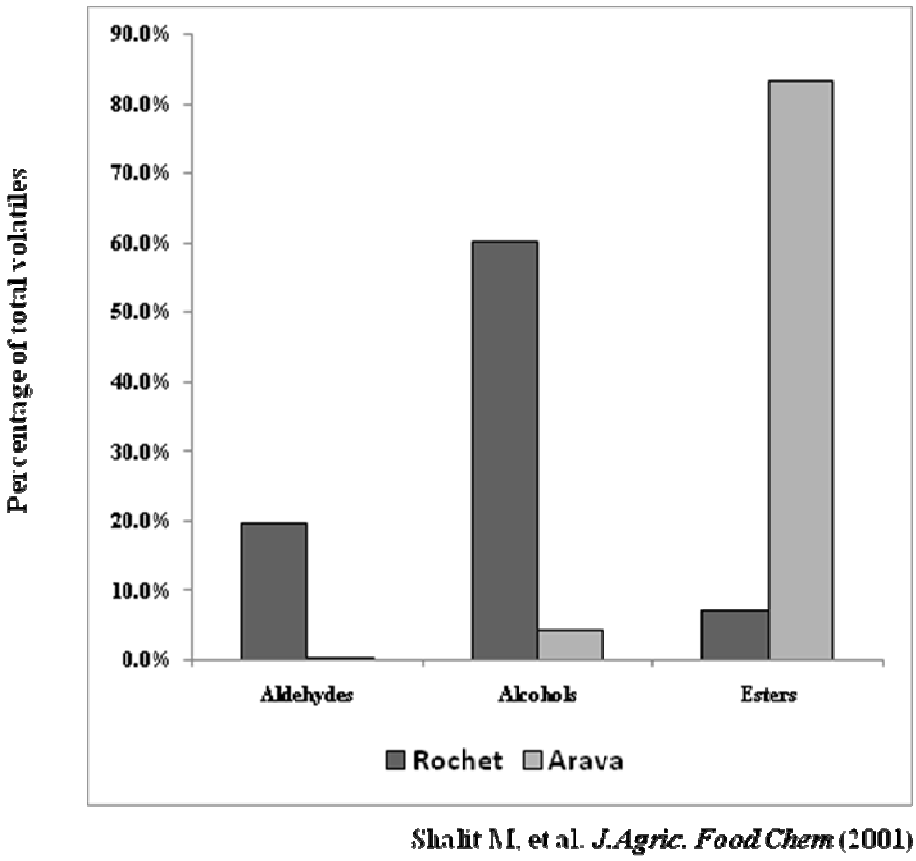


Figure 3.13 Main volatile components in ripe fruit of Rochet and Arava

The data presented here were derived from the paper of Shalit M. et al. published in *J.Agric. Food Chem* (2001). There data showed that during ripening, the main volatile compound in Rochet were alcohols (60%) and aldehydes (20%).The concentration of Ester was very low (7%). While in Dulce, the main volatile was Ester (83% of total volatile) and the concentration of alcohols (4%) and aldehydes (0.4%) were very low.

Dulce and Avara likely have similar volatile profiles during ripening (Fig 3.13). This remains to be confirmed via volatile analysis of Dulce.

An outline of aroma volatile biosynthesis in melon is shown in Fig 3.14. ADH and AAT are two enzymes shown to catalyze the last two steps of acetate ester biosynthesis in melon. In climacteric melon, acetate esters are the major volatile compounds and are synthesized by esterification of alcohols in the reaction catalyzed by alcohol acyltransferase (AAT). Alcohols are derived from the reduction of aldehydes through the action of alcohol dehydrogenase (ADH). Three microarray elements have high homology to melon *AAT*s (SSH6E12, SSH1L18 and FR14L3). SSH6E12 and SS1L18 share 97% (e value = 4E-295) and 96% (e value = 6e-256) sequence similarity with *Cm-AAT1* at the amino acid level, respectively, and thus were annotated as *Cm-AAT1*. FR14L3 shared 99% sequence similarity with *Cm-AAT2* and thus was annotated as *Cm-AAT2*.

Three fragments on the melon chip showed sequences similarity with melon *ADH* (FR17C2, FR12K20 and FR15P24). Both FR17C2 and FR12K20 belong to the same contig indicating that they represented the same unigene. MU100 shares 88% sequence similarity with *Cm-ADH1* at the amino acid level. FR15P24 shares 99% sequence similarity with *Cm-ADH2* at the amino acid level. Thus MU100 was annotated as *Cm-ADH1* and FR12P24 as *Cm-ADH2*.

Both *Cm-AAT1* and *Cm-AAT2* were up regulated in ripening stages of Dulce fruit (*Cm-AAT1* is one of the four genes that were up regulated in Dulce through all the four stages compared in this experiment as described above). However the two *ADH*s showed different expression patterns between these two varieties. The mRNA

level of *ADH*2 was consistently high in Dulce fruit during ripening, while *Cm-ADH*1 was accumulated preferentially in Rochet from the mature to the over-ripe stage. Meanwhile an *ALDH* (SSH4J15, aldehyde dehydrogenase) was also up regulated in Rochet at all ripening stages. ALDH catalyzes the oxidation of aldehyde to carboxylic acids (Kirch et al., 2004) but its role in melon volatile biosynthesis has not been defined.

The fatty acid 9-hydroperoxide lyase (FR15H19) was up regulated in Dulce fruit at all ripening stages. Most aldehydes are formed by degradation of fatty acids. Fatty acid 9-hydroperoxide lyase belongs to the plant cytochrome P450 CYP74 family of fatty acid hydroperoxide enzymes that produce aldehydes from linoleic and linolenic acids (Tijet et al., 2001).

In summary, of the likely genes encoding enzymes involved in volatile biosynthesis, fatty acid 9-hydroperoxide lyase, *Cm-ADH2*, *Cm-AAT1* and *Cm-AAT2* were highly expressed in climacteric Dulce fruit while only *Cm-ADH1* and *ALDH* showed relative up regulation in non-climacteric Rochet fruit during ripening (Fig 3.15).

Sugar and organic acid metabolism

Sugar is the major determinant of melon fruit sweetness. The main sugar content constituent in ripe melon fruit is sucrose. Both Dulce and Rochet belong to the sweet melon classification indicating both cultivars accumulate a certain minimum amount of sucrose during ripening (Burger et al., 2003). This character is verified by measuring total soluble solid (TSS) content during ripening. At the mature and

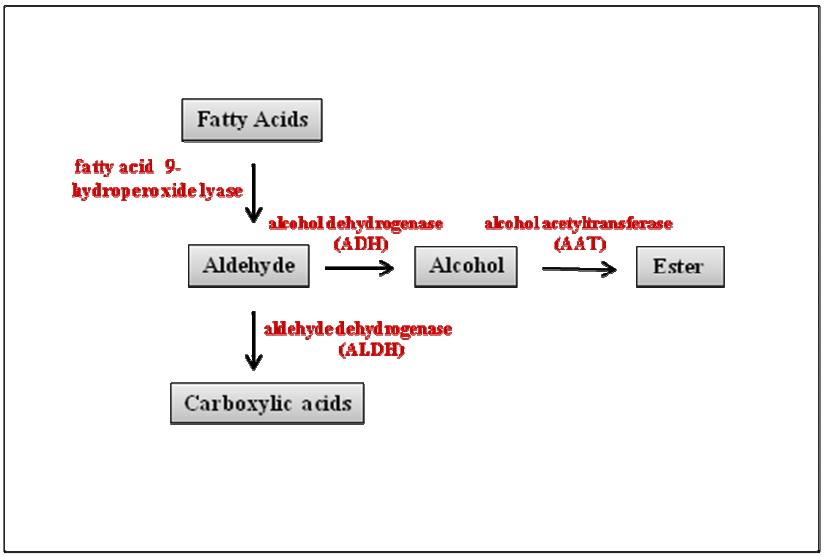


Figure 3.14 Aroma volatile biosynthesis pathway and related genes

Here showed the last few steps for aroma volatile biosynthesis in melon fruit and related genes that catalyzed the reactions of each step. All the four genes have been identified on melon chip and showed differential expression between Dulce and Rochet during ripening.

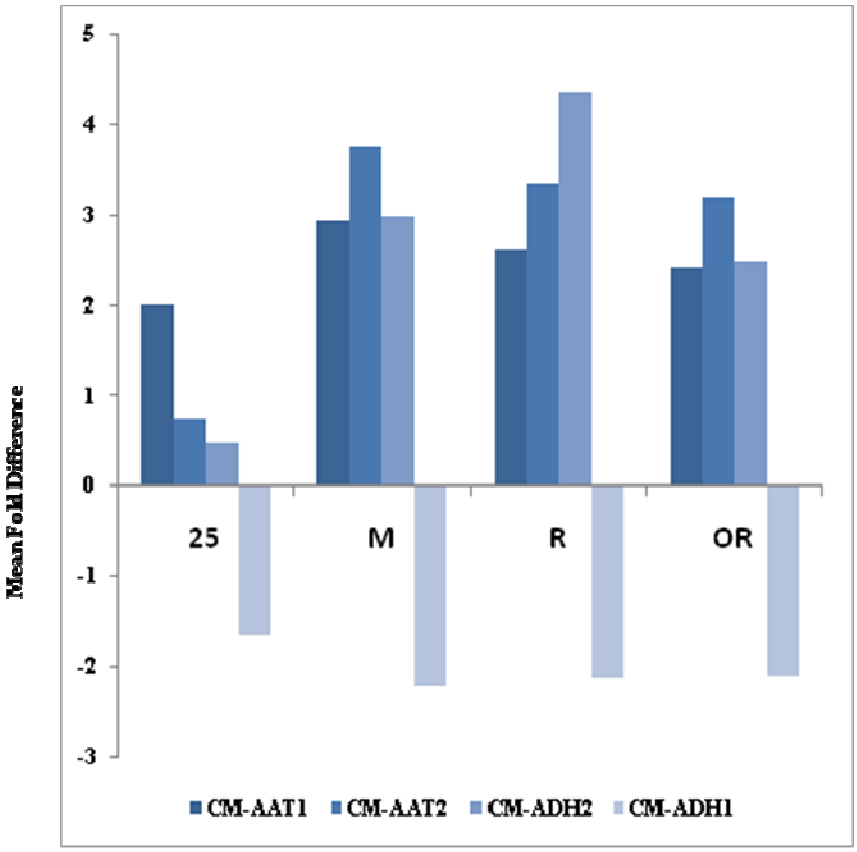


Figure 3.15 The expression pattern of AATs and ADHs in Dulce and Rochet

The differential expression of CM-AATs and CM-ADHs between Dulce and Rochet. The positive MFD value in the Y axis were obtained using Rochet as control and the negative MFD value in the Y axis were obtained using Dulce as control. Thus bars above the X axis showed the expression patterns of those genes in Dulce and the bar below the X axis represents the expression pattern of this gene in Rochet. Three genes, CM-AAT1, CM-AAT2 and CM-ADH2 were highly expressed in Dulce fruit during ripening. Only CM-ADH1 showed up regulation in Rochet at mature, ripe and over-ripe stage.

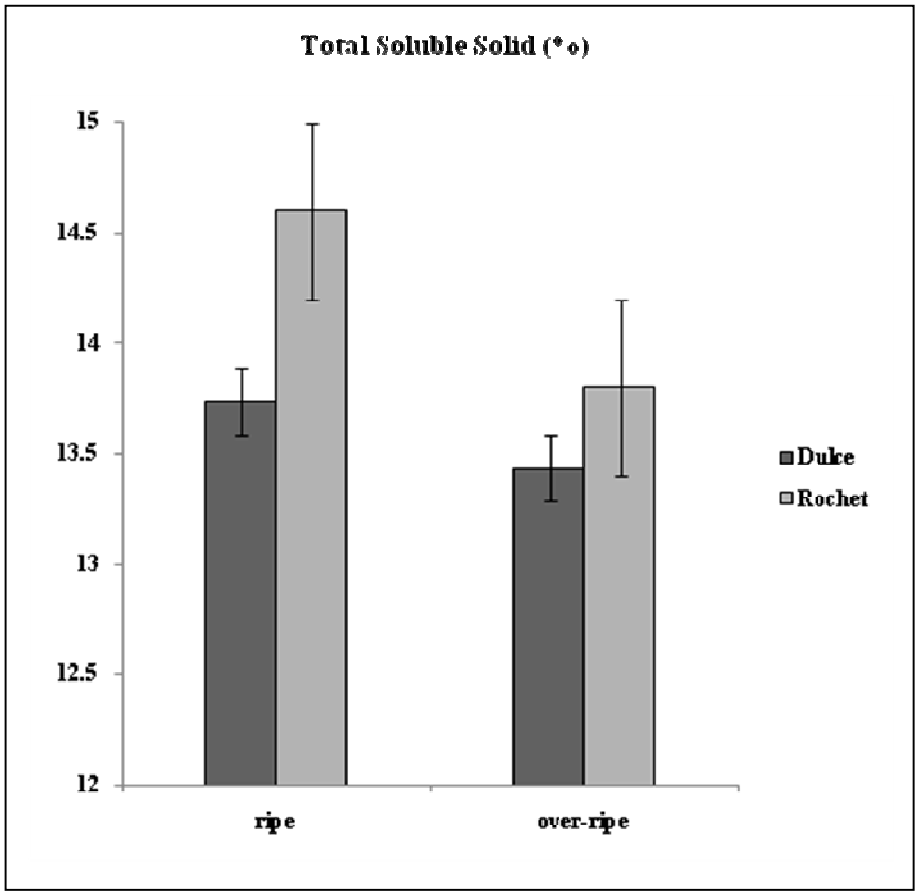


Figure 3.16 Total soluble solid contents in Dulce and Rochet at mature and over-ripe stage

The Total Soluble Solid (TSS) were measured from mature and over-ripe stage fruits harvested . The result from T- test showed that the TSS values between these two varieties had no statistical difference ($p > 0.05$) for both stages.

overripe stages the total soluble solid (TSS) levels in Dulce and Rochet reached approximately the same level (T test $p \leq 0.05$) (Figure 3.16, data kindly provided by N. Katzir for the same fruit used in our microarray analysis). Sucrose concentration in melon fruit is determined by both acid invertase and sucrose phosphate synthase (SPS) activity (Hubbard et al., 1989).

In this transcriptome comparison between Dulce and Rochet fruit, most genes involved in sucrose biosynthesis and the TCA cycle were similarly expressed in all stages compared. Included are genes putatively encoding the main enzymes involved in sugar metabolism such as acid invertase, neutral invertase, sucrose synthase, sucrose-phosphatase, sucrose-phosphate synthase 1, sucrose transporter, sugar transporter, UDP-sugar pyrophosphorylase, UDP-glucose pyrophosphorylase, in addition to genes catalyzing the principle reactions in organic acid (malic and citric) metabolism. The latter include malate dehydrogenase, citrate synthase, succinate dehydrogenase, succinyl-CoA ligase beta subunit and aconitase C-terminal domain-containing protein. This result is consistent with the TSS data of Dulce and Rochet provided by our collaborators in Israel.

Genes up-regulated in Dulce or Rochet through all four fruit stages

Four unigenes were up regulated in Dulce through 25 dpa, mature, ripe and overripe stages (MFD $\geq$2.0, FDR$\leq$0.05). They are alcohol acetyltransferase (*Cm-AAT1*), anthocyanin acyltransferas-like protein, putative major latex protein and a hypothetical protein of unknown function (Table 3.7). Six unigenes were up regulated in Rochet throughout all four stages. One has no sequence similarity with any known sequence in GenBank. The other five include a low molecular weight heat-shock

Table 3.7 Genes upregulated in Dulce through all four stages compared

Clone ID	GB #	Best hit in GenBank	e value
FR13M11	DV633416	hypothetical protein [Vitis vinifera]	8.00E-43
FR10B2	DV632367	putative major latex protein [Momordica charantia]	4.00E-48
FR11E20	DV632676	anthocyanin acyltransferase-like protein [Arabidopsis thaliana]	3.00E-57
SSH6E12	DV631963	alcohol acetyltransferase [Cucumis melo]	4.00E-295

Table 3.8 Genes upregulated in Rochet through all four stages compared

Clone ID	GB #	Best hit in GenBank	e value
SSH6A2	DV631884	low molecular weight heat-shock protein [Nicotiana tabacum]	3.00E-79
FR18P23	DV635128	wound-responsive protein, putative [Arabidopsis thaliana]	1.00E-27
SSH6C13	DV631918	No hit fund	
SSH1A13	DV631408	NADP-dependent malic protein [Ricinus communis]	2.00E-31
FR12L18	DV633100	NADP-dependent malic enzyme (NADP-ME)	0.00E+00
SSH6J17	DV632080	putative glutamate decarboxylase [Glycine max]	3.00E-33

protein, wound-responsive protein, DANP-dependent malic enzyme (two different unigenes) and glutamate decarboxylase (Table 3.8).

Microarray data validation

Microarray data were validated in two ways - result from published studies and real-time quantitative PCR analysis. *Cm-ACO1* (FR13N15, FR15J17) and *ACS* (SSH1N10) were up regulated in Dulce during ripening which is consistent with previous reports (Lasserre et al., 1996) (Katzir N. et al. personal communication). In addition, an E8 gene (SSH4B19) was also highly expressed in Dulce during ripening. In tomato, the expression of E8 is induced by ethylene and showed ripening related expression (Deikman and Fischer, 1988; Lincoln et al., 1987; Lincoln and Fischer, 1988). These results are all consistent with our microarray data and additional validation of the array through comparison to published data is described in Chapter 2.

Quantitative Real-time PCR analysis was also performed not only to validate the differential expression of genes identified by microarray but also to support our hypothesis of possible regulatory mechanism between Dulce and Rochet in certain pathways. To reach this aim, nine genes involved in carotenoid metabolism were chose and there expression patterns were re-evaluated by qRT-PCR (*DXR, GGPS, GGHY, PSY, PDS, ZDS, CHY-b, b-LCY* and *ZED*). Primer Express software (Applied Biosystems) was used to design primers for each gene (Table 3.9). Two-step quantitative RT-PCR was performed using SYBR Green PCR master kits (Applied Biosystems) with the Standard Curve method and run on an ABI 7900 (Applied Biosciences) quantitative real-time PCR unit and employing three technical replicates for each gene. A melon 18S sequence (GenBank: AY030241) was used as an internal

control to normalize gene expression levels. Comparison of the results from real-time RT-PCR with those from microarray analysis revealed similar patterns of expression for eight of the nine genes thus lending support to our interpretation of microarray data in this study (Fig 3.17).

Discussion

The molecular biology of melon fruit ripening has been primarily studied in climacteric type melons. Little is known regarding the molecular biology of climacteric versus non-climacteric melon ripening. As far as we are aware, this is the first documented transcriptome comparison for a range of fruit developmental stages between a climacteric and non-climacteric fruit of the same species. In this study, we focused on the transcriptome difference between two melon varieties at four distinct developmental stages and with an emphasis on pathways related to fruit quality and whose end products are known to differ between climacteric and non-climacteric melons.

Cell wall metabolism

The softening process of fruit is the result of degradation of cell wall polysaccharides which is a network comprised of the cellulose matrix, cross-linked hemicellulose and highly hydrated pectins. Solubilization of pectin rich middle lamella, de-esterfication of pectins and depolymerization of the polysaccharide matrix are the major events associated with softening during fruit ripening (Rose et al., 1998). It is believed that the softening of fruit is the result of co-ordination of several enzymes as knock-outs of single genes, such as PG

Table 3.9 Primers for qRT-PCR analysis

	Forward Primer	Reverse Primer
XDR	GACCTATGGGCTAGGGATTATGCT	TTATGCGGCTACGGGACTAAA
GGPS	CCAATCGACTTCGCCAACTC	TGTATCGACGATCGGCGTATC
PSY	CCTCCCTTGGAAAAGCTCTTC	CTTCACACATAACACCATCAGCATT
PDS	TTCCACCATTGTCGCATCAAA	AGTCTTCGCACCTGCAGAAGA
ZDS	GTCACCGCCAACTTCGGTATA	GCCTTACTGCCTCGAACGTT
b-LCY	CCGGTCGAGCTACCAAAGAA	GCAATGCCCTTTTCATCAAATC
bCHY	CAGGAACAAGCCCTTTATGGAA	GCTGTGCCGGCCATAGC
ZEP	TCCCGTACGCTTCCATCAG	CTTCACACGAAATATTGCCTTCTTAT
GGHY	ATTTCTTTATATCGGCTTTGTGAGTCA	TGTTCCCAAAATGCGACCAT
18S	TGCATGGCCGTTCTTAGTTG	GGCTGAGGTCTCGTTCGTTAA

Figure 3.17 Quantitative RT-PCR analysis of microarray data

Nine genes that involved in carotenoid metabolism were chose for qRT-PCR analysis. Eight of them showed matched or basic matched expression patterns with that to qRT-PCR data.

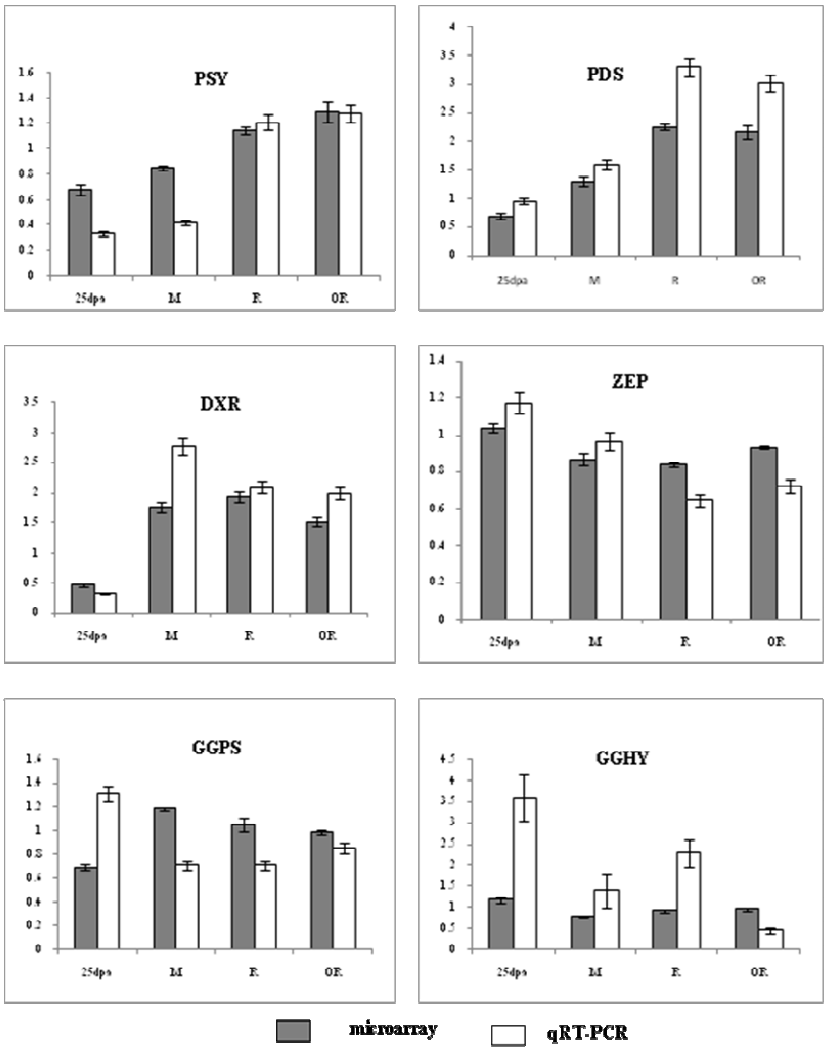
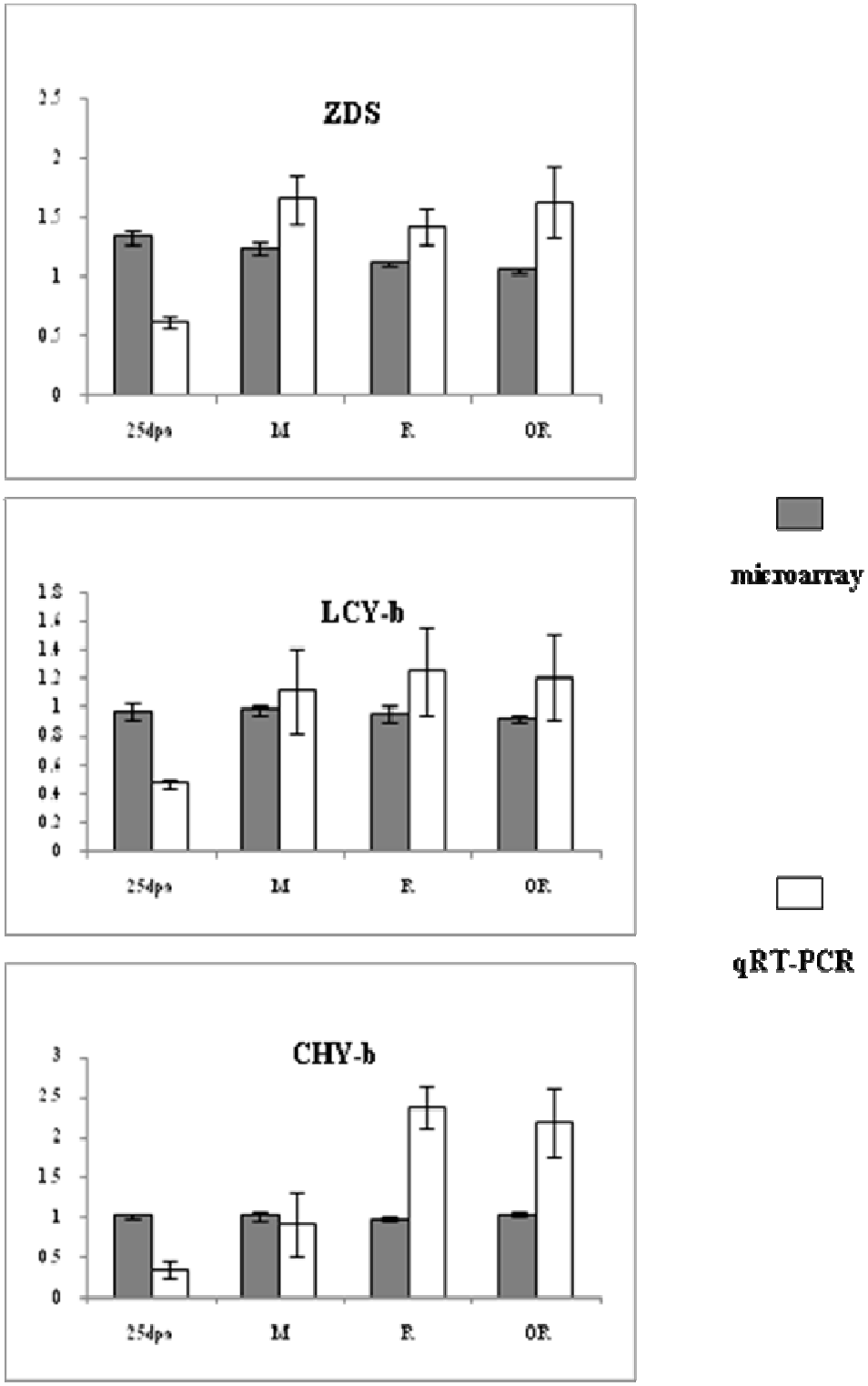

PSY
PDS
DXR
ZEP
GGPS
GGHY
25dpa
M
R
OR
microarray
qRT-PCR

Figure 3.17 (Continued)



(Sheehy et al., 1987; Smith et al., 1988), GALs (Carey et al., 2001), XTH (Asada et al., 1999; de Silva et al., 1994) and ripening related Expansin (Brummell et al., 1999) had little effect on fruit firmness, while co-suppression of PG and EXP genes together in tomato increased firmness by 20% (Powell et al., 2003).

Here we observed several major cell-wall modifying enzymes that were up regulated in Dulce fruit during ripening (*PG, GAL, XTH, PME* and Expansin). It is reasonable to hypothesize that the coordinated increased activity of these enzymes is a contributing factor in the rapid softening process of ripening Dulce fruit. Beta-Galactosidase (GAL) was the first enzyme involved in cell wall degradation that has been identified in climacteric melon. GAL's activity increased notably during ripening (File-Lycaon B., and Buret M. 1991; Ranwala AP., 1992). Pectin depolymerizarion and solubilization has been associated with ripening and in correlation with the accumulation of PG mRNA (Rose et al. 1998; Hadfield et al.1998; Nishiyama K. et al., 2007). Fruit ripening related expansins (*Cm-EXP1*) and XTH (*Cm-XTH1* and *Cm-XTH3*) have also been isolated from melon and showed up-regulation during Dulce climacteric melon ripening

Our microarray data suggest that regulation of melon fruit cell wall metabolism may be more complicated than even the available literature suggests and provide new insights into the genes that may be most important for cell wall metabolism and associated textural changes. For example, of the four *GAL*s detected on our melon chip, three of them were highly induced and one was constitutively expressed in Dulce during ripening. Of the three *Cm-PG*s (*Cm-PG1*, *Cm-PG2* and *Cm-PG3*), *Cm-PG1* and *Cm-PG2* were up regulated in Dulce and *Cm-PG3* was equally expressed between Dulce and Rochet during ripening suggesting that *Cm-PG1*

and *Cm-PG2* likely are two of the main *PG*s causing the degradation of cell wall pectins during Dulce ripening. So far six *PG* genes have been cloned from melon. Only *Cm-PG1*, *Cm-PG2* and *Cm-PG3* were expressed in fruit. The regulation of their expression has been studied in wild type, ACO antisense and 1-MCP treated melon fruit (Rose .et al. 1998). Their results also suggested that *Cm-PG1*, *Cm-PG2* and *Cm-PG3* may contribute differently to the softening process of climacteric melon fruit. *Cm-PG1* expression is totally ethylene-dependent and thus may represent the major PG activity difference between climacteric and non-climacteric fruit. *Cm-PG1* has been functionally validated (Hadfield et al.1998). This proposal is basically consistent with our microarray data where we observe high levels of *Cm-PG1* transcripts in Dulce and similar expression of *Cm-PG3* during ripening in both Dulce and the firm fruit Rochet cultivar. mRNA abundance of *Cm-PG2* in Dulce was much higher than that in Rochet during ripening (MFD=3.2 at mature, 5.6 at ripe and 14.8 at overripe stage) suggesting that the expression of *Cm-PG2* might also be under the control of ethylene though the expression of this gene also increases during maturation of the relatively firm Rochet fruit (indicating response to a non-ethylene signal but not supporting a strong role in softening).

In Rochet, the non-climacteric melon, the firmness of fruit decreases slowly during ripening but remain firm relative to Dulce. Two enzymes associated with ripening related cell wall degradation were found to be up regulated in Rochet and may be associated with the textural changes observed: Xyloglucan endotransglycosylase (*XTH-38)* and pectinmethylesterase (PME). The mRNA of *XTH-38* was high in Rochet at the mature stage while that of pectinmethylesterase accumulated at the ripe and over-ripe stages. Assessment of the roles of these genes in non-climacteric melon softening will require *in planta* functional analysis (e.g.

antisense, RNAi or over-expression). As we mentioned above, the main function of PME is to remove the methyl groups from galacturonic acid residues of pectins which makes the polyuronides more susceptible to degradation by other enzymes such as PG. In antisense PME transgenic tomato plants, the firmness of fruits was not affected indicating that PME alone contributes little to fruit softening during the tomato ripening process (Tieman.et al. 1994). Similarly, fruit softening behavior was not reduced by suppression of the tomato ripening related XTH gene (Brummell et al. 2001). These results might suggest that the slow softening process of Rochet fruit during ripening is due to the activity of other major cell wall modification enzymes such as PG and GAL or it may indicate the need to test the effect of suppressing both genes simultaneously.

Carotenoids biosynthesis

Carotenoid composition analysis showed that the orange Dulce fruit accumulated a high level of b-carotene indicating that the orange color of Dulce flesh is due to the accumulation of beta-carotene. While the major steps and key enzymes involved in the carotene biosynthesis pathway have been identified, little is known regarding the mechanisms of how this pathway is regulated during ripening. Here we showed that two of the eight carotenoid synthesis genes on the melon chip showed differential expression between Dulce and Rochet (*DXR* and *PDS*).

Up regulation of mRNA levels of several early genes in the carotenoid biosynthesis pathway have been shown to correlate with the accumulation of certain downstream carotenoids in plant. In Citrus fruit Satsuma mandarin (*Citrus unshiu*

Marc.) which accumulate beta-cryptoxanthin (beta-ring hydroxylase of beta-carotene) during ripening expression of several early pathway genes such as *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, and *CitZEP* increased simultaneously (Kato et al. 2004). In tomato, a lycopene and beta-carotene accumulator, high expression of DXS, PSY and PDS was observed at the breaker stage when lycopene started to accumulate (Lois et al. 2000; Giuliano et al. 1993; Fraser et al.1994; Bramley et al.; Peck I. et al. 1996; Ronen G. et al.1999).

Among the genes involved in carotenoid biosynthesis, the regulatory roles of phytoene synthase and phytoene desaturase have been most studied and shown to be critical in carotenoid accumulation. For example, over-expression of bacterial phytoene synthesis *CrtB* with *CrtI* (bacterial phytoene desaturase) and *CrtY*(bacterial lycopene beta cyclase) was sufficient to lead to accumulation of beta-carotene in canola seed (Schewmaker, et al. 1999; Ravanello, et al. 2003) and potato tuber (Ducreux, et al. 2005; Diretto, G. et al. 2006; Giuliano et al. 2008), both of which normally accumulate very little carotenoid. Over-expression of *CrtB* alone in tomato delivered enhanced levels of lycopene while over-expression of *CrtI* lead to accumulation of beta-carotene in tomato (Fraser et al 2002; Romer et al 2000). Similarly, over-expression of a plant PSY in tomato increased lycopene in fruit while co-overexpression with *CrtI* lead to increased beta-carotene in rice (Fray .et al. 1995; Ye, et al. 2000; Paine et al. 2005). These results indicated that the step controlled by *Crt I* is essential for beta-carotene accumulation. Normally in plants the four sequential desaturation reactions that convert phytoene to lycopene are catalyze by PDS, ZDS (zeta-carotene desaturase), CrtISO (carotenoid isomerase) and ZISO (zeta-carotene isomerase). In bacteria, all four reactions are performed by a single bacterial phytoene desaturase Crt I. Thus in plants, the accumulation of beta-carotene can

potentially be regulated at multiple steps in addition to *PSY* and *PDS.* In this study using microarrays, even though Dulce contains about 30 times higher beta-carotene than Rochet, *PSY* and *LCY-b* were equally expressed between theses two varieties and only PDS was up-regulated in Dulce. qRT-PCR analysis confirmed expression patterns derived from microarray data indicating that PDS is likely a main control for b-carotene biosynthesis in orange flesh Dulce.

The second carotenoid synthesis gene upregulated in Dulce in association with ripening and as compared to Rochet was *DXR*. Blocking DXR activity in tomato fruit affected the accumulation of carotenoids suggesting that DXR also plays a role in regulation of fruit carotenoid biosynthesis (Rodriguet-Concepcion. et al. 2001; Yao 2008).

A transcriptome comparison between wild type and *Nr* (ethylene receptor) mutant tomato using tomato cDNA microarray performed by Alba et al (2005) showed that the transcription of *PDS* and *PSY* both increased during fruit ripening in wild type fruit and that only the expression of *PDS* is ethylene regulated. The relatively high expression of *PDS* in Dulce and the uniform expression of *PSY* between these two cultivars may reflect similar regulatory characteristics of *PDS* and *PSY* in melon fruit.

Post-transcriptional mechanisms may also influence *PSY* and/or *LCY-b* regulation. Such regulation has been observed in *Narcissus pseudonarcissus* chromoplasts. Although carotenoid accumulation was strongly induced during flower development of *Narcissus pseudonarcissus*, the transcripts and corresponding protein of PDS were very low. The activation of the inactive soluble form of PDS required FAD and galactolipids for PSY as membrane bound cofactors, indicating the

contribution of post-transcriptional mechanisms (Al-Babili et al.1996; Schledz 1996). Measurement of PSY activity in melon *in situ* and *ex situ* will help answer whether or not this is also the case for the corresponding melon peptides.

The carotenoid biosynthesis pathway may also be regulated by end-products. A carotenoid cleavage dioxygenase (CCD), which is able to cleave beta-carotene to produce beta-ionone was up regulated on the microarry in Dulce from the mature through overripe stages. It has been reported that the orange flesh Dulce accumulates high amounts of beta-ionone (12-33ng/gFW), an important component for the unique aroma of orange fleshed melon fruit and that pale green and white fleshed varieties such as Rochet produce very little (0-5 ng/gFW) (Ibdah 2006). CCD can generate additional apocarotenoids from phytoene, lycopene and delta-carotene. The conversion of carotenoids to apocarotenoid compounds serves as a driving force to influence GGPP flux and the accumulation of downstream carotenoid products. In agreement with this hypothesis, a chromoplast-specific carotenoid-associated protein (FR15B13) also showed up regulation in Dulce during ripening. FR15B13 encodes a protein that is a major component of carotenoid-lipoprotein complexes in *Cucumis sativus* chromoplasts and may participate in carotenoid sequestration within chromoplasts (Vishnevetsky et al. 1996; Micheal.1997).

In summary, our results in concert with published data from others suggests that the accumulation of beta-carotene in Dulce is likely regulated by multiple mechanisms, including transcriptional regulation, post-transcriptional control and end-product regulatory pathways. Genes associated with these possible regulatory mechanisms show expression patterns that when combined with the carotenoid

properties of Dulce and Rochet are consistent with each playing some role in carotenoid regulation of maturing melon fruit.

Melon Aroma Volatiles Biosynthesis

The aroma volatiles of melon are composed of a mixture of ester, alcohols and volatile aldehyde compounds. Among them, volatile esters are the main contributors to the unique aromatic fragrance of melon. In climacteric melon, esters are synthesized by esterification of alcohols in reactions catalyzed by alcohol acyltransferases (AAT). Alcohols are derived from the reduction of aldehydes through the action of alcohol dehydrogenases (ADH). Aldehydes are generated by degradation of unsaturated fatty acids catalyzed mainly by lipoxygenases (LOX). The intermediates from this oxidation, unsaturated fatty acid hydroperoxides (HPOs) can be further metabolized to produce volatile compounds by hydroperoxide lyases, such as fatty acid 9-hydroperoxide lyase (Alexander and Grierson, 2002). In addition to reduction to alcohols, aldehydes can also be oxidized to carboxylic acids by aldehyde dehydrogenases (ALDH).

In the ripe fruit of Rochet, 60% of the volatiles were alcohols, 20% was aldehydes and about 7% were esters. In ripe Arava fruit (which is related to Dulce and also contains orange flesh and high aroma), the volatile compound composition was reported to be predominantly esters (83.3%), alcohols (4.3%) and aldehydes (0.4%) (Shalit et al., 2001). Of the four genes (*Cm-AAT1, Cm-AAT2, Cm-ADH1* and *Cm-ADH2*) that are involved in the last two steps of ester biosynthesis, *Cm-AAT1, Cm-AAT2* and *Cm-ADH2* were highly expressed in Dulce during ripening while only *Cm-ADH1* was up-regulated in Rochet. This expression profile is consistent with the lack

of esters and accumulation of alcohols in Rochet fruit. In addition, the AAT activity in Rochet was shown to be lower than that in Arava which further supports the relative volatile profiles previously reported for these fruit (Shalit et al., 2001).

The genes encoding AAT and ADH have been isolated from Charentais melon. The AAT gene family contains four members (*CmAAT1- CmAAT4*). All are expressed in melon fruit during ripening and all are capable of converting a wide range of substrates to their respective esters except for *CmAAT2*. Both enzyme activity and the expression of *Cm-AAT*s during ripening were shown to be ethylene dependent in climacteric melon which is consistent with our microarray data showing that both *Cm-AAT1* and *Cm-AAT2* were up regulated in Dulce fruit relative to Rochet. Several lines of evidence indicate that AAT1 is the main enzyme for generating diverse volatile esters in melon (El-Sharkawy et al., 2005; Lucchetta et al., 2007; Yahyaoui et al., 2002). Though both *Cm-AAT1* and *Cm-AAT2* were up regulated in Dulce, only *Cm-AAT1* is likely responsible for the synthesis of esters in Dulce fruit as *Cm-AAT2* is not functional. The role of *Cm-AAT3* and *Cm-AAT4* in volatile biosynthesis in Dulce could not be addressed in our analysis as they are not represented on the melon microarray. The function of *Cm-AAT2* remains a mystery though its homology to *Cm-AAT1* and its ripening-related expression make it an interesting candidate for functional analysis.

ADH is encoded by a gene family consisting at least two members (*ADH1* and *ADH2*). They are able to interconvert aldehyde to alcohol but are more active in converting aldehyde to alcohol. *Cm-ADH1* uses NAPDH as a co-factor and is able to reduce branched aldehydes, aromatic aldehydes and aliphatic aldehydes. The substrate for *Cm-ADH2* is restricted to aliphatic aldehydes using NADH as a co-factor. The

enzyme activities and the expression of both ADH genes are fruit specific and are up regulated during fruit ripening in Charentais melon. Ethylene has been identified to be the main regulator for gene expression and enzyme activities of both *Cm-ADH1* and *Cm-ADH2* in melon while tomato ADH2 has not been found to be ethylene inducible (Speirs et al., 1998). In addition, ADH activity was also found to be increased in non-climacteric grapes suggesting the existence of ethylene-independent regulation for this gene (Sarno-Manchado et al., 1997). The expression patterns observed when comparing *Cm-ADH1* and *Cm-ADH2* between Dulce and Rochet also indicates that regulatory factors other than ethylene may be involved. ADH1 is likely the major enzyme catalyzing the biosynthesis of alcohol from aldehydes in non-climacteric melon fruits. While in climacteric melons, this step is probably accomplished by ADH2 as evidenced by relative gene expression patterns.

Comparing expression patterns of genes involved in volatile compound biosynthesis pathways between Dulce and Rochet, we find that genes encoding the main steps from fatty acid oxidation to ester formation are more highly expressed in Dulce than in Rochet as reflected by the relative up-regulation of three groups of genes in Dulce during ripening: fatty acid 9-hydroperoxide lyase, ADH (*Cm-ADH2*) and AAT (*Cm-AAT1* and *Cm-AAT2*). The coordinate activity of these enzymes likely drives volatile biosynthesis toward the direction of esters in Dulce, while in the non-climacteric Rochet fruit the most active gene expression steps result in the net catabolism of aldehydes to alcohols by ADH1 or oxidation to carboxylic acid by ALDH.

In summary, our results indicate that volatile biosynthesis is differentially regulated between climacteric and non-climacteric melon fruit in ways consistent with

documented volatile profiles in Rochet and a climacteric cultivar related to Dulce. Based on our expression results, the key regulatory step is likely to be the last step of ester biosynthesis catalyzed by AAT. It has been suggested that this step is controlled by both ethylene-dependent and –independent regulatory mechanisms (Flores et al., 2002) as only 50% of ATT activity was inhibited in ACO antisense melon fruit. The low but significant activity of ATT remaining in Rochet fruit also suggests that this final step in volatile determination may be regulated by signals other than ethylene.

Conclusions

Using the melon microarray developed and described in chapter 2 a transcriptome comparison was performed between climacteric and non-climacteric melon varieties. By focusing on transcript abundance of genes involved in four biological pathways related to important melon fruit qualities (cell wall metabolism, carotenoid composition, aroma volatile biosynthesis and sugar and organic acids accumulation), we found that genes involved in three pathways (cell wall metabolism, carotenoid composition, aroma volatile biosynthesis) were differentially regulated between Dulce and Rochet in correlation with physiology and metabolite data generated by our collaborators and available in the literature. The rapid softening phenotype of Dulce is consistent with the up- regulation of several groups of cell wall metabolizing genes including *PG, GAL, EXP, XTH* and *PME,* during ripening. The mRNA levels of most of these genes were correspondingly low in Rochet through fruit development. The regulation of beta-carotene accumulation in Dulce during ripening likely involves targeted regulation of several steps in the carotenoid biosynthesis pathway and candidate transcription factors correlated with differential carotenoid gene expression were identified and serve as candidates for future functional analyses.

The enzyme activities of DXR and PDS in particular are likely regulated at the transcriptional level though post-transcriptional modifications may well control the activity of these and possibly other genes. In addition, the conversion of carotenoids to apocarotenoid compounds is supported by our expression data and may also serve as an element of the regulatory mechanism controlling the accumulation of beta-carotene in maturing Dulce fruit. Finally, the low aroma volatile production in Rochet is consistent with the low expression and activity of AATs in this genotype as compared to Dulce. These hypotheses are based on the analysis of mRNA levels via microarray analysis in conjunction with phenotypic data generated by ourselves, collaborators and available through the literature. The result is a small set of genes that would be interesting to pursue via functional analyses in transgenic or otherwise genetically altered plants.

Methods and Materials

Plant material

Plants were grown in the Newe Ya'ar Research Center in Israel under standard irrigation and fertilization during the summer seasons of 2005-2007. Flowers were open pollinated and tagged. Fruit were harvested at the following stages:(1) young green fruits (25 days after anthesis); (2) mature fruits (after change of color and development of abscission zone). For this experiment, Dulce (*C .melo* var. *reticulates*) fruits were picked at 42 DPA for mature stage and Roshet (*C. melo*. var. *inodorus*) fruits were pick at 45 DPA for mature stage. (3) Ripe stage. Mature fruits were picked and kept for 2-4 days at 20^0C. 46 DPA for Dulce and 47DPA for

Rochet. (4). Over-ripe stage. Mature fruits were picked and kept for 10-12 days at 20^0C, 54 DPA for Dulce and 55 DPA for Rochet.

RNA Extraction

Total RNA was extracted from fruit flesh using a modification to the method of LaClaire and Herrin (1997). The RNA concentration was determined by spectrophotometry, and its integrity was assessed by electrophoresis in 1% (w/v) formaldehyde-agrose gels.

Microarray Hybridization and Scanning

Total RNAs were isolated using the protocol mentioned above. The first-strand cDNA synthesis, cDNA labeling and purification were performed using SuperScript™Indirect cDNA Labeling System (Invitrogen™ Crop., Carlsbad, CA,USA). 15 ug of total RNA was used as templates for first strand cDNA synthesis. The efficiency of the labeling procedure was evaluated as described in "5.5 Analysis of labeling reaction" in TIGR's Standard Operating Proceedure #M004 (http://pga.tigr.org/sop/M004_1a.pdf).

A total of 200 pmol of Cy3™ coupled and an equivalent amount of Cy5™ coupled cDNA were combined and used to hybridize the slides. First the combined dye-coupled cDNA was dried using VacumFuge™ evaporator and re-suspended in 50 ul of hybridization solution [33 µl Milli-Q® H_2O, 30 µl de-ionized formamide (SIGMA, catalog no.9037), 25 µl 20x SSC, 10 µl 50x Denhardt's Soln (Invitrogen; catalog no.50-018), 1 µl 10 µg/ µl PolyA RNA (Amersham Biosciences; catalog no.

27-4110) and 1 µl 10% SDS]. After heating at 95℃ for 5 min and spinning at 14,000rpm for 1 min, the cDNA probes were then applied to a slide that has been pre-hybridized at 43°C for 45 min in buffer containing 5 x SSC, 0.1% SDS and 1% BSA and covered with LifterSlip glass (50mm, catalog no. 22x501-2-4711; Erie Scientific, Portsmouth, NH, USA). The hybridization was performed at 43℃ for 12-16h under dark. Following hybridization, 3 washes were applied to the slides in Coplin jars: Wash 1: 1 x SSC with 0.2% SDS, 10 min at 43°C; Wash 2: 0.1 x SSC with 0.2% SDS, 10 min at room temperature (RT); wash 3: 0.1 x SSC only, 10min at RT. Then slides were dried by spinning at 1,500 rpm for 30 seconds under dark. Slides were scanned by ScanArray scanner (ScanArray 5000; GSI Lumonics, Billerica, MA,USA) at 10 um resolution. The 90 % laser power and different PMT value were used for each slide in order to balance Cy3/Cy5 intensity.

Data Acquisition, Filtering and Processing

ImaGene software (v5.6, BioDiscovery Inc., EI Segundo, CA. USA) was chose to convert obtained raw fluorescence image to numerical signal data. Spots were flagged by setting ImaGene's ignored pixels threshold to 69%, the area-to-perimeter threshold to 0.6 and the offset value threshold to 17.5; Only spots that showed fluorescence intensity at least two times the mean background value for both channels were retained. Log transformation, normalization and further statistic analysis were all carried out within the R statistical package (R 2.4.0, Bioconductor package). Differential expressed genes were identified by PaGE program.

Quantitive RT-PCR

The total RNA from the same resource for microarray experiments were reverse transcribed into cDNAs using polyA primers under the condition suggested by SuperScript^{TM}Indirect cDNA Labeling System (Invitrogen^{TM} Crop., Carlsbad, CA,USA).Gene specific primers were designed using Primer Express software (Applied Biosystems). The (two-step) quantitative RT-PCR were performed using SYBR Green PCR master kits (Applied Biosystems) under Standard Curve method on ABI 7900 (Applied Biosciences). The reaction volume was 10 ul (cDNA template 2.5 ul, 2x SYBR 5 ul, ddH_2O 1.3 ul, forward primer (5 nmol/ul) 0.6 ul, reverse primer (5 nmol/ul) 0.6 ul). The quantitative PCR condition was: 50^{o}C 2 minutes, 95 ^{o}C 10 minutes followed by 40 cycles of 95 ^{o}C 15 seconds and 60 ^{o}C 1 minute. Three technical replicates were performed for each samples and 18s rRNA was designed as endogenous control (Forward primer: TGCATGGCCGTTCTTAGTTG; Reverse primer: TGCATGGCCGTTCTTAGTTG).

Measurement of Ethylene production

Ethylene was measured from fruits of different developmental stages by sealing whole fruits in airtight jars for 2 h at 22°C after which a 1-mL sample of the headspace was taken and injected on to a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector. Samples were compared to a standard of known concentration and normalized for fruit mass. For each stage measured, four biological repeats were performed.

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