IDENTIFICATION AND CONFIRMATION OF MOLECULAR MARKERS AND ORANGE FLESH COLOR ASSOCIATED WITH MAJOR QTL FOR HIGH BETA-CAROTENE CONTENT IN MUSKMELON

A Thesis

by

ALEXANDRA BAMBERGER NAPIER

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

MASTER OF SCIENCE

December 2006

Major Subject: Horticulture

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Approved by:

Co-Chairs of Committee,	Kevin M. Crosby
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ABSTRACT

Identification and Confirmation of Molecular Markers and Orange Flesh Color Associated with Major QTL for High Beta-Carotene Content in Muskmelon. (December 2006)

Alexandra Bamberger Napier, B.S., Washington State University

Co-Chairs of Advisory Committee: Dr. Kevin M. Crosby
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Beta-carotene presence or absence in muskmelon is controlled by two genes, green flesh *gf* and white flesh *wf*. In its dominant form the *wf* gene is responsible for orange flesh color; however, the epistatic interactions of *gf* and *wf* can create three flesh colors: orange, white and green. Two F_2 populations, consisting of 77 greenhouse grown and 117 field grown plants, from the cross of 'Sunrise' (white fleshed) by 'TAM Uvalde' (orange fleshed), were used to examine the relationships of beta-carotene content, flesh color, and flesh color intensity. Bulk segregent analysis was used with RAPD markers to identify molecular markers associated with high beta-carotene content. Flesh color and flesh color intensity both had significant relationships with beta-carotene content. A significant correlation between total soluble solids and beta-carotene content was also found. Molecular markers were identified in both F_2 populations and all significant, associated markers from 'TAM Uvalde' were linked with *WF*. A single QTL was also found to be linked with the *WF* locus. The identified QTL can be used to screen potential breeding lines for high beta-carotene. It was also confirmed that the visual ratings of flesh color intensity can be reliably used to select high beta-carotene content melons.

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INTRODUCTION

Muskmelons (*Cucumis melo* L.) are an economically important crop around the world and in Texas (Wang 1997). Annually there are 105,262 acres of melons harvested in the U.S., resulting in 1.9 billion pounds produced (Veneman 2004; USDA 2004). In Texas, in 2002, there were 706 melon producing farms and 10,084 acres harvested. The Texas harvest was surpassed only by Arizona (14,500+) and California (50,000+)(USDA 2004).

From a nutritional aspect melons are low in calories, fat and sodium and are good sources of potassium and vitamin C (Lester and Crosby 2002). Additionally muskmelons are considered excellent sources of beta-carotene (Lester 1997, 2006). Beta-carotene provides the orange coloring seen in the most popular variety of muskmelon, cantaloupe. Beta-carotene is an essential nutrient, required for human health and the production of vitamin A.

Muskmelons play a substantial role in the American diet and as a cultivated crop. The overwhelmingly positive nutritional characteristics of muskmelons, and their potential as a source for anti-oxidants, make muskmelons ideal candidates for crop improvement. Molecular markers associated with increased beta-carotene could be used to increase the levels of phytonutrients in muskmelon, while simultaneously increasing the overall breeding efficiency of the crop. The use of randomly amplified polymorphic DNA and bulk segregant analysis has successfully identified markers linked to traits in many crops.

This thesis has been prepared according to the style of the Journal of the American Society for Horticultural Science.

Traditional plant breeding is based around an increase in crop yield and quality, but with limited emphasis placed on the actual nutrient content of the fruit. Given the wide range of nutrient concentrations found between cultivars, Grusak and DellaPenna (1999) predict that increases in fruit nutritional quality can be made through plant selection. There is no doubt that the speed and efficiency of plant selection will greatly benefit from the advantage of marker assisted selection. The successful outcome to this project has laid the ground work for an improved melon breeding program in terms of the rate at which melons can be bred for elevated levels of beta-carotene.

Research Objectives

- To investigate inheritance of flesh color and its genetic relationship with β-carotene in F_2 populations from a muskmelon cross between orange and white fleshed parents in GH and F experiments.
- To identify molecular markers associated with major quantitative trait loci (QTL) for high beta-carotene content in the F_2 populations in the two environments.

LITERATURE REVIEW

Plant Description

Muskmelon (*Cucumis melo* L.), from the family Cucurbitaceae, originated in central Africa (Wien 1997). The Cucurbitaceae family is comprised over 90 genera and 750 species. The diverse species have been divided into six distinct, horticultural groups (not based on phylogeny). Muskmelons are found in the group cantalupensis, the sweet melons, which is the most economically important group worldwide (Robinson and Decker-Walters 1997).

Muskmelons are a warm season crop and as such are usually found in temperate and tropical regions. Melons exhibit a wide range of morphological, physiological, and biochemical diversity (Eduardo 2005). Their form is often prostrate and vining, they may also have curling tendrils that can facilitate climbing. The fruit produced is botanically known as a pepo.

Melons are an economically important crop around the world and in Texas (Wang 1997). When combined with watermelon, melons are second only to banana in terms of fresh fruit consumption per capita in the U.S (Lester and 1997). Annually there are 105,262 acres of melons harvested in the U.S., resulting in 1.9 billion pounds produced for a total gross value of 398.7 million dollars (Veneman 2004; USDA 2004). In Texas, in 2002, there were 706 melon producing farms and 10,084 acres harvested. The Texas harvest was surpassed only by Arizona (14,500+) and California (50,000+)(USDA 2004).

From a nutritional aspect melons are low in calories, fat and sodium and are good sources of potassium and vitamin C. Additionally muskmelons are considered excellent sources of pro-vitamin A, also called beta-carotene. A single 236 g (approximately 1 cup)

serving of muskmelon provides 160 percent of the recommended daily allowance of beta-carotene (Lester 1997).

Beta-Carotene

The positive health benefits associated with beta-carotene have been well documented (Lester 1997, 2006). Beta-carotene, also called pro-vitamin A, is a fat soluble vitamin and is an essential nutrient needed to maintain human health. Vertebrates are not able to synthesize carotenoids and therefore must rely on dietary intake to meet their carotenoid needs. Of the carotenoids, beta-carotene is necessary for retinol production, a compound necessary for sight and beta-carotene rich diets also reduce the risks of cataracts (Johnson et al 2003). In mammals, beta-carotene is also essential for high-quality bone-growth, reproduction, cell division, and cell differentiation (Abbo et al 2005). As an anti-oxidant beta-carotene protects against free radical damage (Johnson et al 2003), and is also known to aid in cancer prevention and guard against coronary artery disease (Abbo et al 2005; Lester and Eischen 1996; Grusak and DellaPenna 1999). Some controversy has arisen over the function of beta-carotene and its role in cancer prevention, but its necessity within the human diet remains unchallenged (Albanes et al 1996). The maximum adult recommended daily intact of beta-carotene is 1000 μgRE (expressed in retinal equivalents) (Grusak and DellaPenna 1999).

Beta-carotene is synthesized in melons during ripening when the chloroplasts change to chromoplasts. During the final stages of ripening the green colored chlorophyll is degraded and replaced by orange beta-carotene (Simandjuntak 1996). Many variables affect the final concentration of beta-carotene present in mature fruits. Fruit size, cultivar

and production soil type all influence final beta-carotene content. Larger fruit contain more beta-carotene than smaller fruit. Silty clay loam soil produces fruit with more beta-carotene than fine sandy loam. Beta-carotene in melon cultivars compared over a two year period revealed that different cultivars consistently produce different amounts of beta-carotene, indicating a genetic aspect to beta-carotene regulation (Lester and Eischen 1996).

Broad Sense Heritability

Physical, phenotypic traits are the results of a specific genotype that dictates trait expression. The transfer of traits from parents to offspring is called heredity. The extent by which the parental genotype influences the expression of the offspring's genotype is called broad sense heritability (H). Broad sense heritability is also called the degree of genetic determination and can be used to estimate the degree of heritability of a trait according to all genetic effects and influences. It can be described mathematically as the ratio of genotypic variance to phenotypic variance or V_G/V_P (Falconer and Mackay 1996; Poehlman and Sleper 1995).

Marker Development

The first genetic maps were made using loci controlling phenotypic traits. Genotype by environment interactions and a lack of marker loci made it difficult to develop highly saturated molecular maps in the early 20th century. However, modern technology and molecular markers now allow plant breeders extensive new methods for developing plant genome maps (Bradeen et al 2001). Molecular markers are tools that can provide insight into a plant's genetic composition. They can be used to identify cultivars,

determine genetic similarities, to calculate the rate of polymorphism occurrence, heterozygosity and self pollination, and they can also be used for marker assisted selection. Molecular markers can also be used to study relationships between phenotypic traits and their underlying genotypes. Genetic markers are perhaps most useful when applied to marker assisted breeding (Masojc 2002). Ideal markers include those that are located directly within a gene sequence or that can be recognized by a single nucleotide polymorphism (SNP). Such direct markers are often unattainable without previously sequenced genes. However, markers that are not part of an already known sequence can still be developed and used productively. These markers are said to be linked to the gene of interest and the distance from the actual marker to the gene can be measured in centimorgans, based on recombination frequency. Molecular markers are unique in that once they have been identified their presence can be determined at any stage of plant development and regardless of environmental factors (Masojc 2002).

The usefulness of molecular markers depends on the quality of the linkage between the target loci and the marker, and the reproducibility of the marker throughout related cultivars and species. The closer a marker is to an actual allele the tighter the linkage is said to be. A tight linkage is ideal because the chances of losing the marker or receiving false positive results decreases because the likelihood of a recombination event occurring between the allele and the marker is reduced (Masojc 2002).

Melon Genetics

The melon genome is diploid with a base number of chromosomes of n=12 (Robinson and Decker-Walters 1997). Over 160 loci have been described and include traits such as disease and pest resistance, and leaf, stem, flower, fruit and seed traits. Many melon genes have been cloned as either mRNA or as the complete gene, with the majority of the cloned genes relating to fruit maturity. The number of described genes may be somewhat inflated because of lack of allelism tests (Pitrat 2002). The apparent high levels of phenotypic variability in melon have been confirmed using molecular markers (Eduardo 2005). Many different methods of genome screening have been used to create melon genome maps (Baudracco-Arans and M. Pitrat 1996, Brotman et al 2000, Danin-Poleg et al 2000, Danin-Poleg et al 2002, Nakata et al 2005, Oliver et al 2001, Perin et al 2002, and Staub et al 2000).

Beta-carotene content is associated with melon flesh color. Carotenoids in muskmelons are responsible for the orange flesh color. Original flesh color studies done by Hughes (1948) found that beta-carotene content was controlled by two genes. The green flesh (*gf*) and white flesh (*wf*) genes have an epistatic relationship. The allelic combinations of *gf* and *wf* create orange flesh (*wf*+*-/gf*+- and *wf*+*-/gfgf*), white flesh (*wfwf/gf*+-), and green flesh (*wfwf/gfgf*), with segregation ratios of 12:3:1 and 3:1 (orange: white+green) (Clayberg, 1992).

Bulked Segregant Analysis

Segregating populations are the basis of bulk segregant analysis (BSA) as described by Michelmore et al (1991). In the BSA process two bulked DNA samples are generated

from a segregating population. The DNA bulks are made up of individuals that are identical for a particular trait, and presumed to be heterozygous at all other un-linked loci. The resulting bulks are then screened against each other to detect polymorphisms. Markers which segregate between the bulks are then screened against the entire population to determine the extent of the linkage (Michelmore et al 1991).

BSA works best when there is a tight linkage between the trait of interest and the molecular marker. For BSA to be successful, linkage disequilibrium must occur relative to the loci associated with the marker for the gene of interest. Linkage disequilibrium occurs when there is intermixing of populations with different gene frequencies or can occur by chance when small populations are used. Therefore larger populations are needed to reduce the risk of creating false linkage disequilibrium and false markers within the study population (Falconer and Mackay 1996).

When using a dominant marker system such as RAPDs, an F_2 population is commonly used in tandem with BSA (Mackay and Caligari 2000) (Uzun et al 2003) (Staub et al 2000). The use of RAPD markers and BSA has been successfully combined to develop molecular markers in melons for traits such as total soluble solids, sugars, and ascorbic acid (Sinclair et al 2006). Near isogenic lines (NIL) can be used for molecular marker development. However the development of near NIL can be costly and timing consuming, requiring up to six generations of backcrossing (Masojc 2002). The use of F_2 populations is quicker and the F_2 generation has also been found to be the best generation with which to conduct BSA (Mackay and Caligari 2000).

Random Amplified Polymorphic DNA Markers

Many different types of molecular markers exist and can be used to study different types of DNA. The use of randomly amplifed polymorphic DNA (RAPD) to identify molecular markers was developed by Williams et al (1990) as a low cost marker system. RAPD markers are dominant markers, meaning the PCR product will either be present or absent. This means that amplified polymorphisms can only distinguish between allele absence and presence, not the number of alleles (Falconer and Mackay 1996). The simplicity and ease of RAPDs is enabled by many factors including: a universal set of primers that can be used on any population, a short screening time, no requirement to isolate cloned DNA probes or to prepare hybridization filters, and the small quantities of DNA that are needed to perform screenings (Kelly 1995; Williams et al 1990).

The use of populations created from parents with opposite phenotypes, has been suggested as an ideal way to breed for increased nutrient content (Grusak and DellaPenna 1999). When using a dominant marker system such as RAPDs, an F_2 population is an ideal starting place to use the bulk segregant analysis technique (Mackay and Caligari 2000). RAPD markers have been effective in melon germplasm studies (Staub et al 2004; Park et al 2004) as well as other crop types (Haley et al 1994; Kelly 1995). Sinclair et al (2006) identified 45 potential RAPD markers using BSA, of which 15 were associated with fruit quality traits, as well as QTL in melon.

Quantitative Trait Loci

Quantitative trait loci (QTL) in plants are often related to complex traits such as yield. The term QTL refers to individual genes that effect traits, but the effects are not

large enough to be apparent in a segregating population due to confounding effects of variation at other loci. This means that selection of QTL is more complex than selection for qualitative traits (Falconer and Mackay 1996). Use of markers to select for QTL aids traditional breeding methods (Masojc 2002). Segregating populations, like an F_2 generation and BSA are suggested for use in molecular marker identification in identifying major quantitative trait loci (QTL) and otherwise unmapped areas of plant genomes (Michelmore 1991).

To recognize markers related to QTL Falconer and Mackay (1996) have identified relevant guidelines for experimental design. First the marker loci should be highly polymorphic to guarantee that individuals will be carrying different alleles. The loci can not be linked to reproductive fitness, which could otherwise alter apparent allele frequencies. The use of co-dominant markers is also suggested as a way to identify all possible genotypes, but not required. The most efficient experimental designs related to QTL identification use crosses between parents that have fixed traits at opposing alleles. In the best case, the alleles associated with the trait of interest should increase the trait value in one parent while decreasing the value in the second parent. This type of experimental design will create the maximum amount of linkage disequilibrium within the F_2 population. Also, when alleles are in association it will help maximize the detection of QTL (Falconer and Mackay 1996).

Santos and Simon (2006) were able to successfully identify QTL in carrots related to the accumulation of beta-carotene and other carotenoids. Their analysis was based around two populations of F_2 plants, formed from the initial cross of a dark-orange carrot by a white carrot. In the study a total of eight loci associated with beta-carotene were

identified that explained 26.4% of the phenotypic variation in population 1 and 40.4% of the variation in population 2. Three QTL related to beta-carotene were also identified.

Marker-Assisted Selection

Traditional plant breeding is based on the transfer of alleles from a donor plant into a recipient cultivar. Following the initial cross creating an F_1 generation backcrossing is often required to eliminate linkage drag. Linkage drag occurs when undesirable traits from the donor plant are also present with the genes of interest. Six or more generations of backcrossing are often needed to fully restore the recipient cultivar with the desirable genes. Not only is this process time consuming, but it can also be further hindered if selection is impinged by environmental affects or low heritability, quantitative control of the trait, and possible costly or difficult methods of trait evaluation (Haley et al 1994; Masojc 2002).

The aim of MAS is to bypass problems such as low heritability, problematic field conditions, and expenses by tagging genes of interest and thereby allowing for indirect, selection based on linkage of markers to the target genes. When compared to traditional phenotypic screening, marker assisted selection has been found to be cost affective, once the tools are in place to carry out MAS. The time saving benefits of MAS are also not disputed when compared to traditional breeding methods (Dreher et al 2002; Morris et al 2002).

MATERIALS AND METHODS

Plant Material – Source and Selection

Two phenotypically opposite seed sources were selected for this experiment. The original parents consisted of 'TAM Uvalde' and 'Sunrise.' Both are commercially available melon cultivars from the horticultural grouping cantalupensis. The key difference between the two cultivars is flesh color. 'TAM Uvalde' is orange fleshed while 'Sunrise' is white fleshed. Other fruit quality traits and characteristics that differ between the cultivars are listed in Table 1.

Table 1. A summary of fruit characteristics that differ between the cultivars, 'TAM Uvalde' and 'Sunrise', used in the marker development experiment.

Fruit and quality trait	'TAM Uvalde'	'Sunrise'
Total Soluble Solids	Moderate	High
Flesh Color	Orange	White
Beta-Carotene	High	Low
Fruit Weight	Moderate	High
Fruit Length	Moderate	High
Fruit Flesh Weight	Moderate	High
Fruit Cavity Closure	High	Low

An initial cross was made between 'Sunrise' and 'TAM Uvalde' to create an F_1 population in 2004. The F_1 population was manually self-pollinated to produce an F_2 population. All crossing was completed in greenhouses to avoid outside pollination sources and cross pollination caused by flying insects and wind.

Plant Material – Treatment and Care

The produced seed was propagated in a greenhouse in Weslaco, Texas at the Texas A&M Experiment Station in the summer of 2005. Ninety F_2 seeds were planted along with eight seeds of 'Sunrise', eights seeds of 'TAM Uvalde', and eight seeds off 'Sunrise' x 'TAM Uvalde' F_1. Each seed was placed in a three-gallon, black plastic, self-draining pot with a growing medium of Sunshine mix #4 (Sun Gro Horiculture Inc., Bellevue, WA). Greenhouse care consisted of daily or twice daily watering as needed and weekly fertilizing consisting of an application of 1 liter of a 5% solution of Peters 20-20-20 water soluble fertilizer to each pot (Smurfit-Stone, Wellsburg, WV).

Plants were grown vertically, and growth was limited to a central climbing vine by the weekly removal of lateral branches. Flowers were hand pollinated and tagged to produce desired fruit and fruit set was limited to a single fruit per plant to ensure the highest possible fruit quality. Spray applications of pesticide were used to keep the plants and greenhouse free from whiteflies and powdery mildew.

Fruit was harvested at maturity, as indicated by the slipping of the fruit from the vine. At time of harvest fruit weight, fruit length, fruit diameter, flesh weight, total soluble solids, cavity space, and flesh color were recorded. Next the seeds were cleaned and packaged, and flesh samples were placed in plastic bags and frozen at -20°C for future use.

A second population of 120 plants from the same F_2 seed was also planted in Weslaco, Texas at the Texas A&M Experiment Station in the summer of 2005. The second population was field grown, and the seeds were sown three weeks after the greenhouse plants.

Beta-Carotene Extraction

The beta-carotene in each fruit sample was measured using the spectrophotometric assay procedure described by Fish et al (2002) with some slight modifications.

Before beginning the assay fruit samples were removed from the freezer and allowed to thaw overnight in a refrigerator. To avoid beta-carotene degradation, samples were stored on ice and kept in the dark or in amber colored glass for the duration of the procedure.

Approximately 15 g of cold tissue was placed in a 25 mL plastic test tube and homogenized for 30 seconds using a homogenizer set at speed level 5 (Brinkman Instruments, Inc., Westbury, NY). From the homogenized mixture 1 gram was weighed out into an amber colored jar and placed on ice. To each jar 10 mL of hexane, 5 mL of acetone, and 5 mL of ethanol were added. The jars were securely capped and mixed, by placing them in an ice bath on a shaker table for 15 minutes. Three mL of pure water was added to each sample and the samples were mixed for an additional five minutes.

Jars were removed from the ice bath and allowed to rest at room temperature for 30 minutes. The hexane phase was removed from each sample and placed into a clean cuvette. Absorbance values were recorded at 450 nm using a spectrophotometer (Unicam 8625 UV/Vis Spectrometer, ATI Unicam, Cambridge, UK). Pure hexane was used to blank the spectrophotometer and then the absorbance of the samples were read.

The amount of beta-carotene in each sample was calculated using the molecular weight of beta-carotene (536.88 g/Mol) and its extinction coefficient at 450 nm (138,730 cm/M).

DNA Extraction

Total genomic DNA was extracted from young leaf tissue following the method described by Skroch and Nienhuis (1995) with slight modification. Young leaf tissue was collected from each greenhouse plant during their fifth week of growth. As it was harvested tissue was placed on ice until it could be moved to a -80°C. Just prior to DNA extraction samples were moved to a -20°C freezer.

One to 2 grams of leaf tissue were ground with a sterilized mortar and pestle to break cells and homogenize tissue. Approximately 1 mL of ground tissue was placed in a 1.5 mL micro centrifuge tube, and 500 μL of potassium ethyl xanthagenate (PEX) extraction buffer containing 1 M Tris (pH=7.5), NaCl, PEX, and 0.5M EDTA (pH=8.0) were added to the tube and the mixture vortexed. Mixed tubes were placed in a heating block for a minimum of 60 minutes at 65°C. Tubes were vortexed after 10 minutes and again at 30 minutes to increase the success of the extraction.

Tubes were then centrifuged at >10,000 RPM for 10 minutes in a micro centrifuge (Spectrafuge 16M; National Labnet Co., Edison, NJ). The resulting supernatant liquid was transferred to a clean 1.5 mL micro centrifuge tube. DNA was precipitated by adding 1000 μL of a 6:1 mixture of ethanol and 7.5 M ammonium acetate to each tube. Tubes were carefully mixed by slowly inverting ten times and allowing to rest for 30 minutes. Samples were stored overnight at -20°C.

Tubes were removed from the freezer and mixed again by inverting slowly five times. The samples were centrifuged at 6,000 RPM for 5 minutes using a micro centrifuge. The supernatant liquid was poured off, leaving the DNA pellet; remaining liquid was removed from the microfuge tube by blotting it gently against a paper towel.

Next, 300 µL of TE buffer (1mM Tris (ph=7.5), 0.1 mM EDTA (pH=8.0)) was added to each tube along with 10 µL of RNAse A (10mg/mL solution). After the additions each tube was vortexed to mix, and placed in a heated block at 37°C for 60 minutes. After which they were vortexed again to dissolve any precipitate and then returned to the heated block for an additional 30 minutes at 37°C. Tubes were centrifuged for 14,000 RPM for 30 seconds. As they were removed from the micro centrifuge the supernatant was poured into a clean microfuge tube. A 20:1 mixture of ethanol and 3 M sodium acetate was very slowly added to each tube. Tubes were mixed by very slowly inverting 10 times. The samples were stored at -20°C overnight.

Samples were removed from the freezer and allowed to rest for at least 60 minutes. They were then centrifuged at 6,000 RPM for 5 minutes. The ethanol was poured from each tube, leaving pelleted DNA; tubes were dried by inverting and blotting on a paper towel. DNA pellets were washed by adding 1 mL of 70% ethanol to each tube and vortexing to rinse tube. DNA pellets were collected by vortexing at 14,000 RPM for 30 seconds. The ethanol was poured off and tubes were dried by inverting and blotting on a paper towel. Tubes were left open and inverted for 30 minutes to dry completely.

The DNA pellets were rehydrated using 400 µL of TE buffer and allowed to rest for 60 minutes. Tubes were vortexed to resuspend pellet and then allowed to rest again. The suspended DNA was boxed and labeled as ‘Original DNA’ and stored at -20°C.

DNA from the field F_2 population was extracted in the same manner as described above, but the initial tissue used for extraction was different. At the time of harvest rind samples were taken from each fruit during the initial assessment and frozen at -80°C. The rind samples were used in place of young leaf tissue.

DNA Concentration

The concentration of each DNA sample was measured using a spectrophotometer (DU 530 Lifescience; Beckman, Fullerton, CA). Diluted DNA samples were created to use during the RAPD marker screening. Known amounts of DNA from each sample were added to a clean microfuge tube containing 400 μL of TE buffer to arrive at the final concentration of 10 ng/μL.

Development of DNA Bulks

DNA bulks were created from six low and six high beta-carotene content F_2 individuals. Separate bulks of each parent DNA were also made and consisted of four plants per bulk. Phenotypic characteristics such as days to maturity, weight, size, flesh color and flesh color intensity were also considered; plants exhibiting signs of vine decline were omitted whenever possible. Specific plants used in the DNA bulks are listed in Appendices A, B and C along with additional phenotypic data.

Screening RAPD Markers

Random 10-mer primers (Operon Technologies, Almedia, CA) were used to screen between the high and low DNA bulks and between the two parents. A total of 510 10-mer primers were screened. Markers that segregated between bulks during the initial primer screening were screened with the entire greenhouse population of 77 F_2 individuals. After the initial goodness-of-fit test, positive markers were screened again using the field population of 117 F_2 individuals. The RAPD marker screening techniques, linkage analysis and QTL detection were performed the same way on both F_2 populations.

Polymerase Chain Reaction

A thermalcycler machine (PTC-100 Programmable Thermal Controller; MJ Research, Waltham, MA) and 96-well PCR plates (96 well polypropylene microplates MJ Research, Waltham, MA) were used to perform polymerase chain reaction (PCR). Ten primers could be tested on each plate. Each test consisted of one row on the PCR plate containing the following DNA samples combined with a primer in prepared solution (1 = 'Sunrise' bulk, 2 = 'TAM Uvalde' bulk, 3 = low white GH bulk, 4 = low orange GH bulk, 5 = high orange GH bulk, 6 = low white FLD bulk, 7 = low orange FLD bulk, and 8 = high orange FLD bulk). The primer solution consisted of pure water (34.5 µL), 5x buffer (19.8 µL), individual primer (19.8 µL), dNTP (4 µL), and Taq polymerase (1.2 µL). The buffer, primer, and dNTP were each vortexed before adding to the solution to ensure adequate mixing (Scroch and Neinhuis, 1995).

Each solution was thoroughly mixed by stirring with a pipette tip. Eight µL of primer solution was added to each PCR plate well. The DNA samples were vortexed and then 2.1 µL of each was added to the appropriate well. The final volume of each well was 10 µL, including 8 µL of primer mixture and 2 µL of DNA. Microseal 'A' Film (MJ Research, Waltham, MA) was used to seal the top of the PCR plates. The film was secured by using a pipette handle to firmly and evenly adhere the film to the plate and seal the wells.

Sealed PCR plates were placed in the PCR machine with the lid fastened tightly. The PCR run consisted of two cycles at 92°C for 60 seconds, 42°C for 7 seconds, and 72°C for 70 seconds. Denaturation, annealing and elongation followed by using 38 cycles

of: 1 second at 92°C, 7 seconds at 42°C, and 70 seconds at 72°C. The final step consisted of 4 minutes at 72°C before cooling and holding at 4°C (Scroch and Neinhuis 1995). Once finished, samples were removed from the thermalcycler, placed in a plastic bag and stored at 4°C for up to 10 days.

Gel Electrophoresis

1.5% W/V agarose gels for electrophoresis were made from a solution consisting of 4.5 g agarose (Invitrogen corporation, Carlsbad, CA) and 300 mL of 0.5x TBE buffer. The solution was mixed by swirling in a 1000 mL flask and then placed in a microwave on high power for 3 minutes. The solution was mixed again and returned to the microwave for an additional 30 seconds on high power. Upon removal from the microwave the flask was placed in a water bath and stirred using a stir bar until the agarose solution had cooled to 60°C. Once cooled the solution was poured into a prepared electrophoresis gel tray. Four 30 well combs were placed into the gel at even distances from each other. The gel was allowed to solidify for 30 minutes, then the combs were carefully removed and the resulting gel and plate were placed into the electrophoresis box (Submarine/Horizontal Gel Unit; C.B.S. Scientific Co., Del Mar, CA). 0.5x TBE buffer was used to fill the electrophoresis box, additional buffer was added as needed to just cover the top of the agarose gel.

Gels were filled by pipeting the primer samples directly into the wells. Using two electrophoresis boxes, 240 wells were available, enough to run 3 PCR plates or a total of 30 primer samples. Once the gels were loaded the covers were replaced and connected to

the power source. The voltage was set at 200 and the current was allowed to run for 1.5 hours.

After the current was turned off, the gel was removed from the electrophoresis box and cut in half along the third row of wells using a razor blade. The two gel halves were placed in a staining solution consisting of 20 μL of ethidium bromide and 600 mL of water. After 1 hour in the staining solution the gels were destained in pure water for 10 minutes. Once destained each gel half was placed onto an ultraviolet illuminator (T1202; Sigma, St. Louis, MO.) with light box and digital camera setup attached (EDAS 290; Eastman Kodak Company, Rochester, NY) and photographed. The gels and destaining solution were disposed of appropriately after photographing.

Names of RAPD Markers

Marker fragments that segregated between parents were given identification names. The names consisted of an 'O' referring to the Operon kit, the Operon label specific to that primer, and the estimated length of the marker, in base pairs. A molecular weight base pair ladder was used to measure the size of the RAPD marker bands on the agarose gel.

Linkage Analysis

Band presence and absence was scored within the F_2 population for marker analysis. Band presence was scored with a 'D' while band absence was scored with a 'B'; missing data was notated with a hyphen. Each marker was tested for goodness-of-fit to a 3:1 ratio to detect possible segregation distortion (Tables 2 and 3). The linkage analysis for markers obtained from 'Sunrise' was done separately from markers obtained from

'TAM Uvalde', because of the dominant aspects of RAPD markers. The flesh color recorded for each fruit was also treated as a marker. Orange was scored with a 'D' while white and green flesh colors were both scored with a 'B'. During linkage analysis the orange flesh color marker was included with the other 'TAM Uvalde' markers.

MAPMAKER version 3.0 (Lander et al., 1987) was used for linkage analysis. The parameters used included a minimum logarithm of odds (LOD) score of 3 and a maximum map distance of 37.2 centimorgans (cM). The Kosambi mapping function was used to determine map distance between ordered marker loci.

Detection of QTL

The Statistical Analysis System (SAS, 2003) was used to conduct all analyses. Simpler linear regression was used to determine associations between markers and beta-carotene content. A marker trait association was considered significant if the P-value was less than 0.05. An R^2 value was calculated for all positive markers to determine their contribution to observed phenotypic variation. Stepwise multiple regression was used to determine the overall variation accounted for by the significant markers. Significant markers were ranked according to their R^2 value and then added in a stepwise regression to select the best set of markers to use for trait selection.

RESULTS

Inheritance of Flesh Color

Of the 90 greenhouse F_2 plants, 77 were used in the analysis. The remaining 13 plants were omitted due to lack of phenotypic data. Phenotypic data was also successfully collected for 117 of the original 120 field grown F_2 plants.

The genotypes for the parent plants were assumed to be *wf+wf+/gf-gf* for 'TAM Uvalde' and *wf-wf-/gf+gf+* for 'Sunrise'. Given the epistatic interactions of the *wf* and *gf* genes, the segregation ratio for the F_2 populations was expected to be 12:3:1, (orange:white:green). A 3:1 (orange:white+green) segregation ratio for the *wf* locus is also expected to be apparent in the F_2 populations. Both of the segregation ratios are illustrated in Figure 1.

wf+wf+/gf+gf+	*wf+wf+/gf+gf-*	*wf+wf-/gf+gf+*	*wf+wf-/gf+gf-*
wf+wf+/gf+gf-	*wf+wf+/gf-gf-*	*wf+wf-/gf+gf-*	*wf+wf-/gf-gf-*
wf+wf-/gf+gf+	*wf+wf-/gf+gf-*	*wf-wf-/gf+gf+*	*wf-wf-/gf+gf-*
wf+wf-/gf+gf-	*wf+wf-/gf-gf-*	*wf-wf-/gf-gf+*	*wf-wf-/gf-gf-*

Figure 1. Expected genotypes and flesh colors of F_2 plants from the cross of 'Sunrise' x 'TAM Uvalde'.

The actual values from each F_2 population were tested against the proposed models (Tables 2 and 3). Chi-square goodness-of-fit tests confirm the expected 12:3:1 segregation ratio in the greenhouse F_2 population (Ott and Longnecker 2001). The field and combined F_2 populations do not fit the expected 12:3:1 ratio. However, the greenhouse, field and combined F_2 populations do fit to the 3:1 ratio.

Table 2. The Chi-square tests for segregation of F_2 populations to a 12:3:1 ratio.

	Number of Plants					
Location	**Orange**	**White**	**Green**	**Expected Ratio**	**χ2**	***P***
Greenhouse	57	12	8	12:3:1	2.524	0.283
Field	92	4	21	12:3:1	40.504	0.000
Combined	150	16	29	12:3:1	35.039	0.000

Table 3. The Chi-square tests for segregation of F_2 populations to a 3:1 ratio.

	Number of Plants				
Genotype	***wf*++ & *wf*+-**	***wf*- -**			
Location	**Orange**	**White+green**	**Expected Ratio**	**χ2**	***P***
Greenhouse	58	20	3:1	0.004	0.948
Field	92	25	3:1	0.641	0.423
Combined	150	45	3:1	0.296	0.587

Beta-Carotene Inheritance

Beta-carotene content was measured for each fruit sample. 'Sunrise' contained the lowest mean beta-carotene while 'TAM Uvalde' had the highest. The F2 populations exhibited mean values between 'Sunrise' and 'TAM Uvalde'; however the range of values in the F_2 populations is quite large, as can be seen from the calculated variances (Table 4).

Table 4. Mean beta-carotene content with standard deviations and variances of all sampled melons.

Generation & Description	Mean Beta-Carotene (µg/g)	Standard Deviation	Variance
'Sunrise'	1.41	0.41	0.16
'TAM Uvalde'	25.91	8.20	67.24
F_1	14.45	6.48	41.99
F_2, Greenhouse	12.81	9.32	86.86
F_2, Field	15.32	9.36	87.60
F_2, Combined	14.37	9.39	88.17

The frequency distributions for beta-carotene were skewed towards low values in both populations (Figures 2 and 3). When the populations were combined the data showed the same skewed distribution (Figure 4).

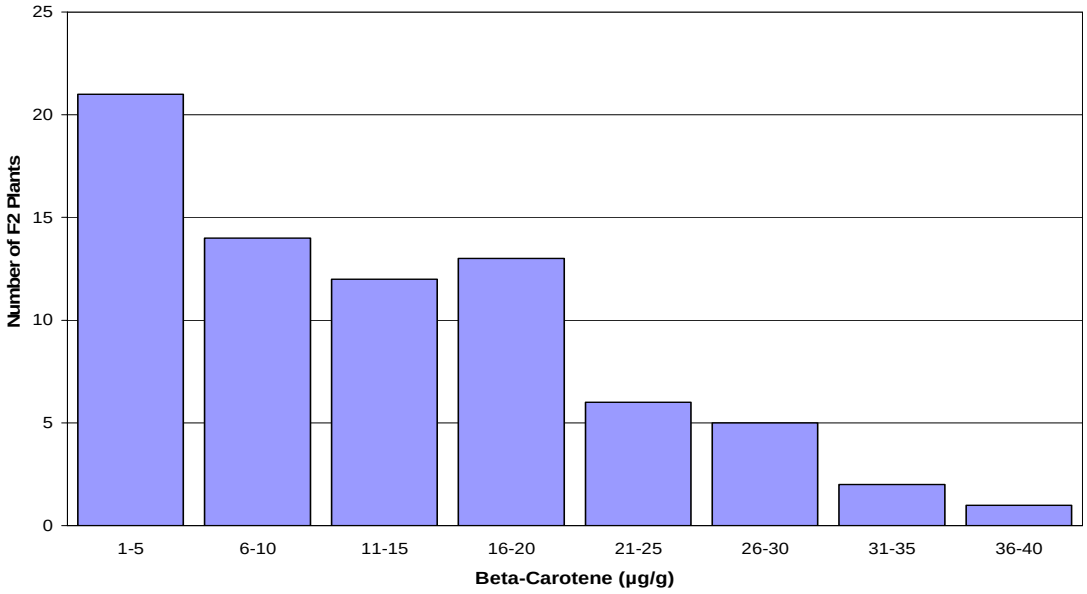

Figure 2. Beta-carotene content frequency distribution for greenhouse grown F_2 plants derived from the cross of 'Sunrise' x 'TAM Uvalde'.

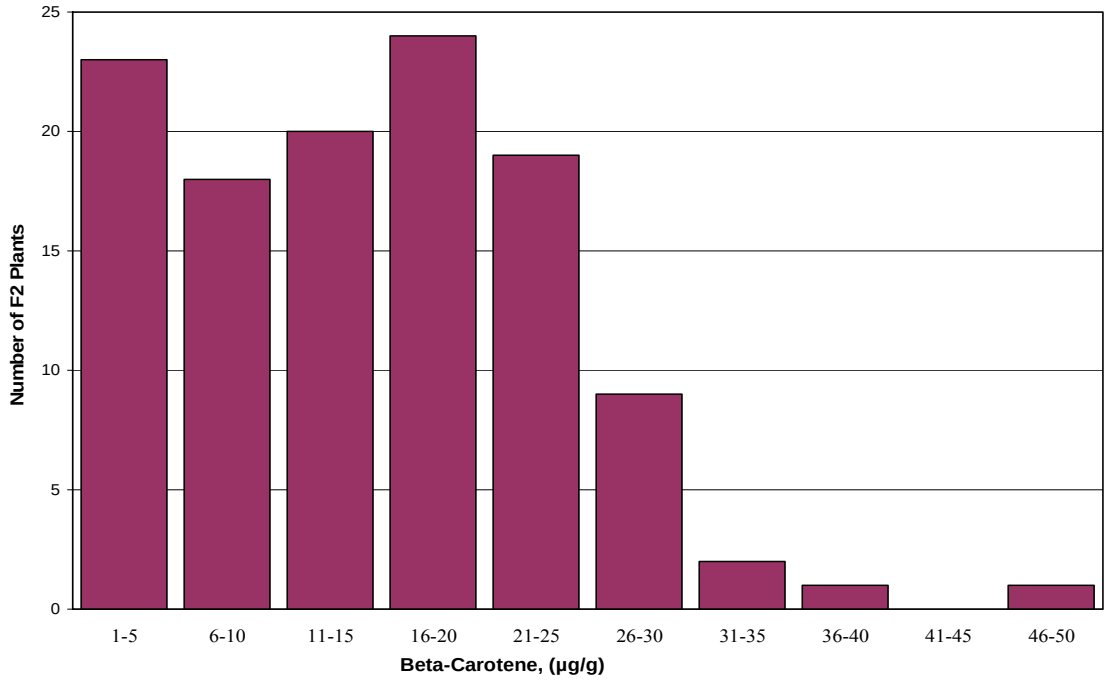

Figure 3. Beta-carotene content frequency distribution for field grown F_2 plants derived from the cross of 'Sunrise' x 'TAM Uvalde'.

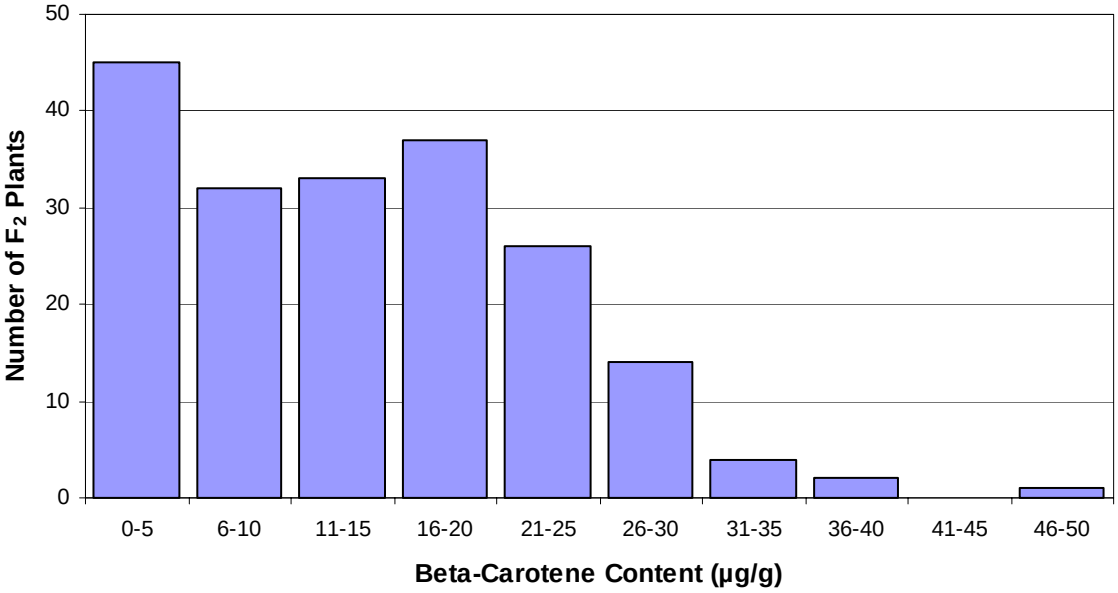

Figure 4. Beta-carotene content frequency distribution for combined F_2 plants derived from the cross of 'Sunrise' x 'TAM Uvalde'.

Broad Sense Heritability Estimate

To determine a broad sense heritability estimate for beta-carotene content, the F_1 and F_2 populations from the greenhouse were used. The F_1 population consisted of genetically uniform plants. The variance of beta-carotene content between the plants was calculated and assumed to equal the environmental variance (V_E). The variation of the F_2 population was assumed to be the phenotypic variance of the population. By subtracting V_E from V_P, the V_G was determined (Falconer and Mackay 1996; Poehlman and Sleper 1995). The variances used in the calculation are shown in Table 5. The broad sense heritability estimate that was calculated was 51%. This indicates that 51% of the observed beta-carotene content variability in greenhouse grown F_2 plants can be attributed to genetic differences between the plants.

Table 5. Observed variance and corresponding broad sense heritability estimate for beta-carotene content in greenhouse grown plants from the melon cross of 'Sunrise' by 'TAM Uvalde'.

V_P	86.86
V_G	44.87
V_E	41.99
H	51%

Genetic Relationship of Beta-Carotene and Flesh Color

Beta-carotene frequency distributions were repeated, using only orange flesh color plant data. The resulting distributions were still skewed toward low values, but only three orange fleshed plants had less than 5 μg/g of beta-carotene in the greenhouse F_2 population (Figure 5), and there were no orange fleshed plants with less than 5 μg/g of beta-carotene in the field F_2 population (Figure 6). The data was combined and the resulting graph is still skewed towards low values (Figure 7). The majority (75%) of the plants have between 6

and 20 µg/g of beta-carotene, while the remaining approximately 25% have between 20-50 µg/g of beta-carotene. These distributions indicate an apparent relationship between white and green flesh color and low beta-carotene content.

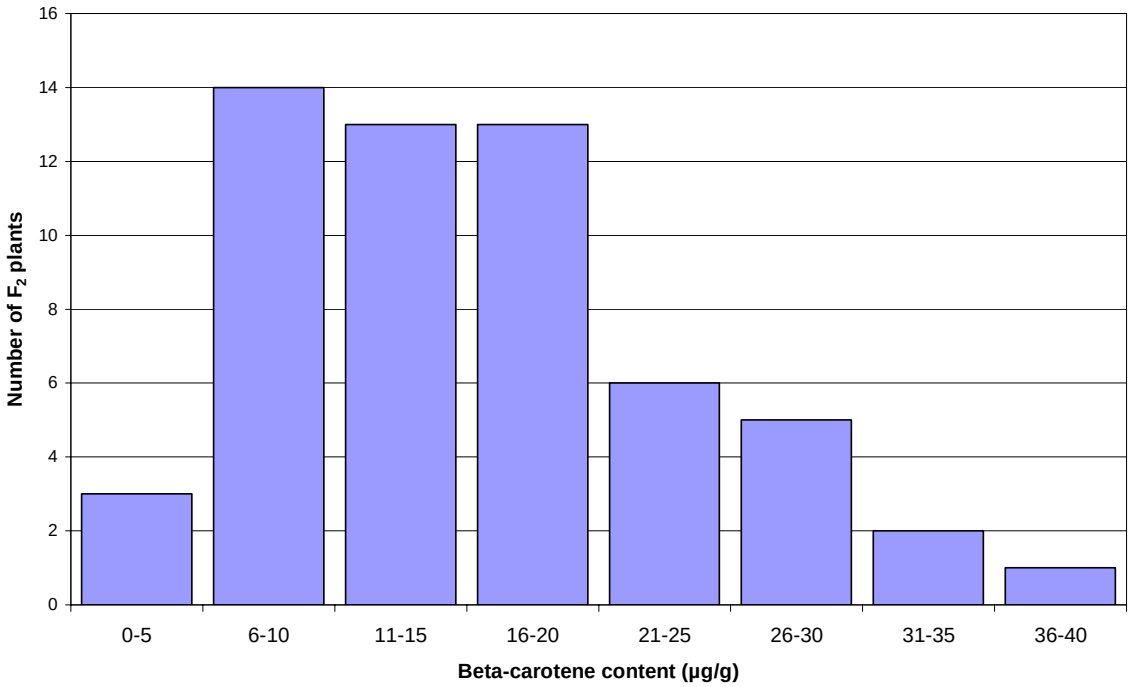

Figure 5. Beta-carotene frequency distribution for orange flesh colored F_2 plants from a greenhouse population.

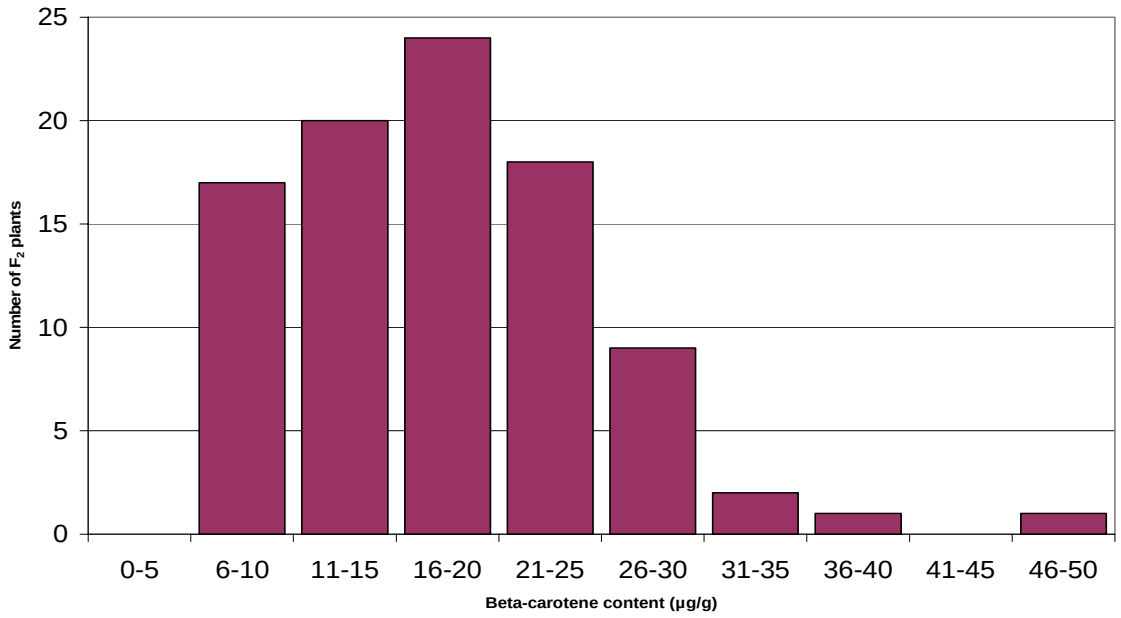

Figure 6. Beta-carotene frequency distribution for orange flesh colored F_2 plants from a field population.

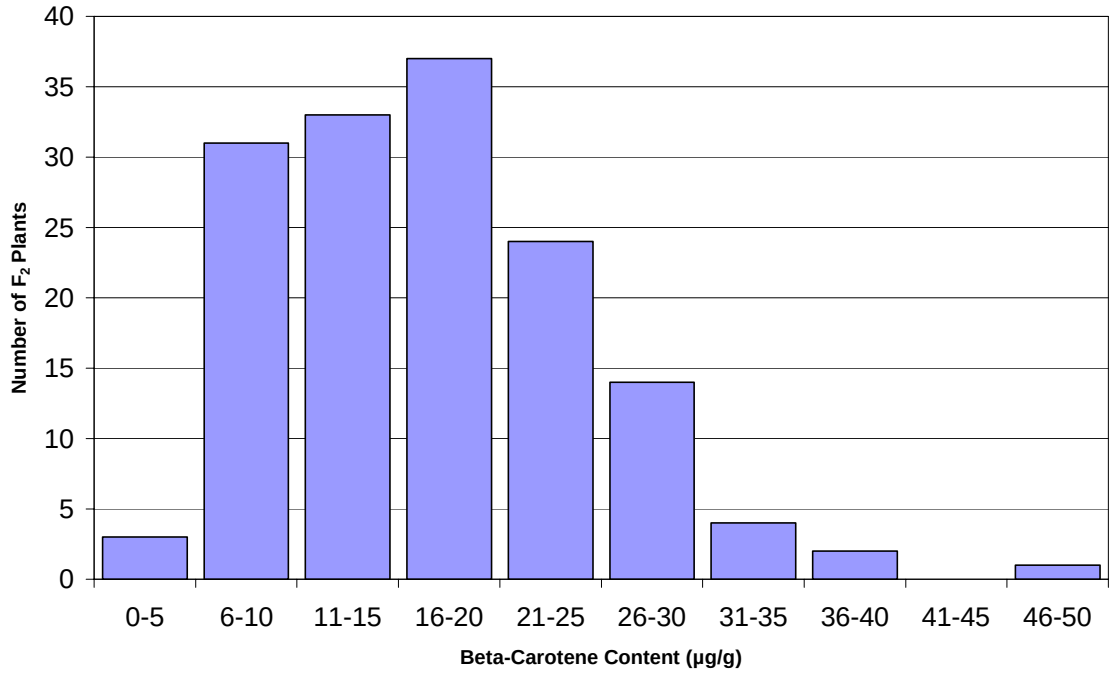

Figure 7. Beta-carotene frequency distribution for orange flesh colored F_2 plants in a combined population.

Each F_2 population was divided according to flesh color (orange, green and white) and white and green flesh colors were also combined to form a fourth sample (white+green). The mean beta-carotene values were determined for each flesh color group (Figure 8). All flesh color groups were found to be significantly different within each population (P=0.05) with the exception of the white and green fleshed groups in the field population. The combined white and green color groups were not significantly different from the white and green fleshed groups, but were significantly different from orange flesh color (P=0.05). Within color groups (i.e. white fleshed melons from greenhouse, field and combined data) beta-carotene content was not significantly different (P=0.05).

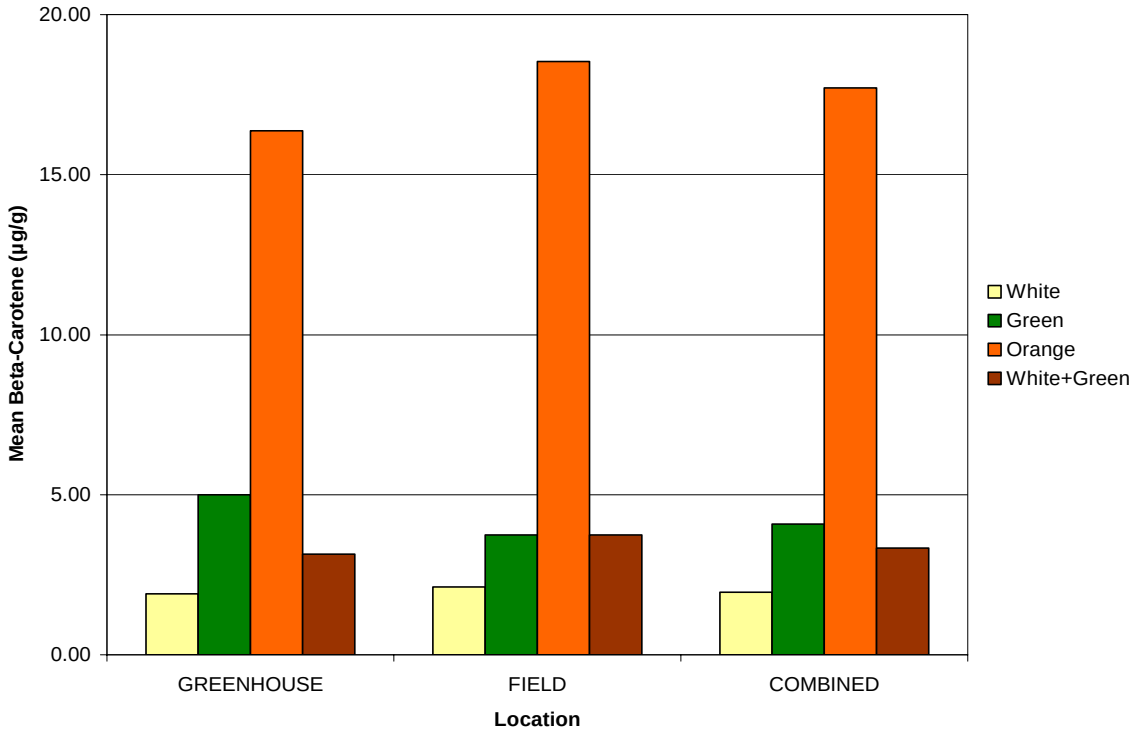

Figure 8. Mean beta-carotene content for melon flesh color for two F_2 populations and combined data.

At the time of harvest the orange fleshed fruit were given a visual rating based on the intensity of their orange flesh color. The lightest orange fleshed fruits were ranked '1', intermediate orange fleshed fruits were ranked '2' and the darkest orange fleshed fruits were ranked '3'. These ratings were used to examine the relationship between orange flesh color intensity and beta-carotene content. Using Pearson correlations a significant relationship was found between orange flesh color intensity and beta-carotene content in the greenhouse (r=0.423), field (r=0.698) and combined (r=0.545) F_2 populations (Table 6).

Table 6. Results of Pearson correlations between flesh color intensity and beta-carotene content in greenhouse, field and combined F_2 populations.

Statistic	Greenhouse	Field	Combined
r	0.423	0.698	0.545
P	<0.01	<0.01	<0.01

To investigate the extent of this correlation, the orange fleshed colored melons were divided according to their flesh color intensity rating. A mean beta-carotene value was found for each group (Figure 9) and a t-test was used to test for significant differences (Table 7). Significant differences were found between each intensity rating in the field population, between the light and intermediate intensities in the greenhouse population and between the light and intermediate, and light and dark intensities in the combined data (P=0.05).

A significant positive correlation was also noted between beta-carotene and total soluble solids in the field and combined F_2 populations (r=0.236). No correlations were

found between beta-carotene and fruit weight, fresh weight, cavity diameter, and cavity weight (Table 8).

Table 7. P-values used to determine the significant differences between mean beta-carotene content of orange flesh color intensity groups.

	F_2, Greenhouse		F_2, Field		F_2, Combined	
	Flesh Color Intensity group					
	Intermediate	Dark	Intermediate	Dark	Intermediate	Dark
Light	0.0008	NS	0.0000	0.0005	0.0000	0.0004
Intermediate	-	NS	-	0.05	-	NS

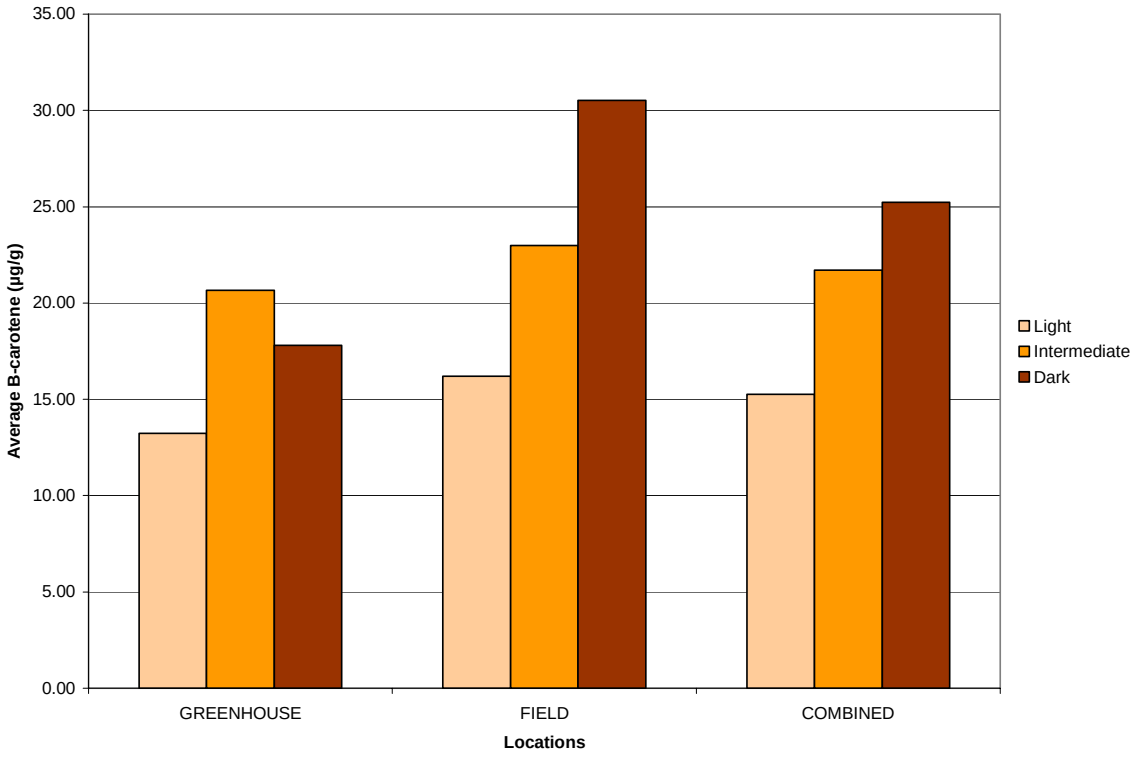

Figure 9. Mean beta-carotene content of orange fleshed melons based on a visual flesh color intensity rating for greenhouse, field and combined F_2 populations.

Table 8. Pearson correlations of beta-carotene and fruit weight, total soluble solids, cavity closure, fresh weight and cavity weight.

		Fruit quality and size traits				
	Location	**Weight**	**Total Soluble Solids**	**Cavity Closure**	**Fresh weight**	**Cavity weight**
β-carotene	**Greenhouse**	NS	0.182 / (0.1122)	NS	NS	NS
	Field	NS	0.236 / (0.01)	NS	NS	NS
	Combined	NS	0.236 / (0.0009)	NS	NS	NS

Screening RAPD Primers for Beta-Carotene Content Using BSA

Greenhouse F_2 Population

A Total of 510 random 10-mer primers were used for the RAPD analysis of the single bulk pairs developed from the low and high beta-carotene content F2 plants and their parents 'Sunrise' and 'TAM Uvalde'. 47 primers exhibited polymorphisms that differentiated between parents and the corresponding bulks (Figure 10). The identified segregating primers were screened in the greenhouse F2 population. Of those screened, seven were identified to be associated with beta-carotene on the basis of simple linear regression. The remaining 40 markers were false positives.

Four of the resulting markers were associated with the parent 'TAM Uvalde' and 3 were associated with 'Sunrise'. An example of the marker OAC09.900 obtained from 'TAM Uvalde' is shown in Figure 11. An example of the marker OAI17.2000 obtained from 'Sunrise' is shown in Figure 12.

The presence of segregation distortion within each identified marker was tested for using a Chi-square goodness-of-fit test to a 3:1 ratio. Five of the markers fit the expected ratio, while markers OAI09.800 and OAC14.700 did not. However, their P values are still

quite close to the 5% acceptable limit and it is thought that the small population size may be the result of the noted distortion (Table 9).

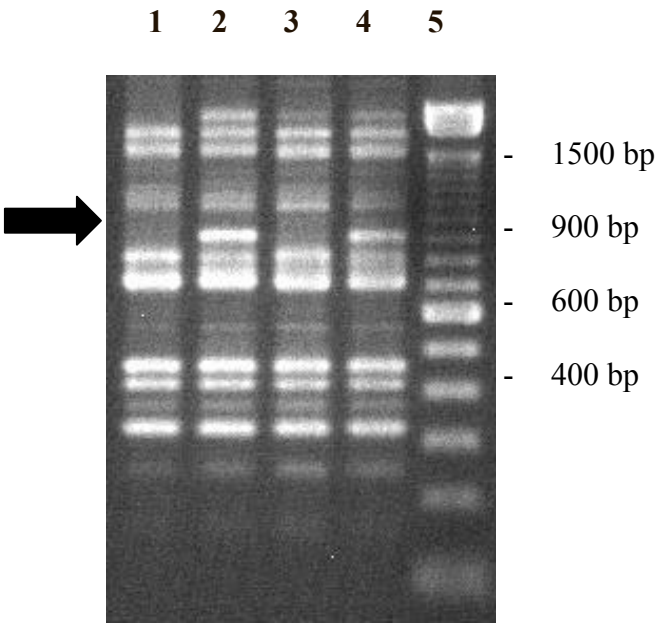

Figure 10. RAPD marker OAC09.900 expressing polymorphism between two DNA bulks from high and low beta-carotene F_2 plants. 1 = Sunrise (low parent), 2 = TAM Uvalde (high parent), 3 = DNA bulk from low beta-carotene F_2 plants, 4 = DNA bulk from high beta-carotene F_2 plants, and 5 = molecular size marker.

Table 9. The chi-square tests for segregation of RAPD markers for four markers from 'TAM Uvalde' and three markers from 'Sunrise' associated with high beta-carotene content in a greenhouse F_2 population derived from the melon cross of 'TAM Uvalde' (high beta-carotene) and 'Sunrise' (low beta-carotene).

		plants (no.)				
Marker	**Source**	**Present**	**Absent**	**Expected Ratio**	**$\chi 2$**	**P**
OAC09.900	TU	57	20	3:1	0.004	0.948
OAK03.1000	TU	54	22	3:1	0.446	0.504
OAI09.800	TU	67	10	3:1	5.303	0.021
OAC14.700	TU	47	29	3:1	6.264	0.012
OAI17.2000	SR	50	27	3:1	3.641	0.056
OAO18.600	SR	50	27	3:1	3.641	0.056
OAO18.1500	SR	62	15	3:1	0.974	0.324

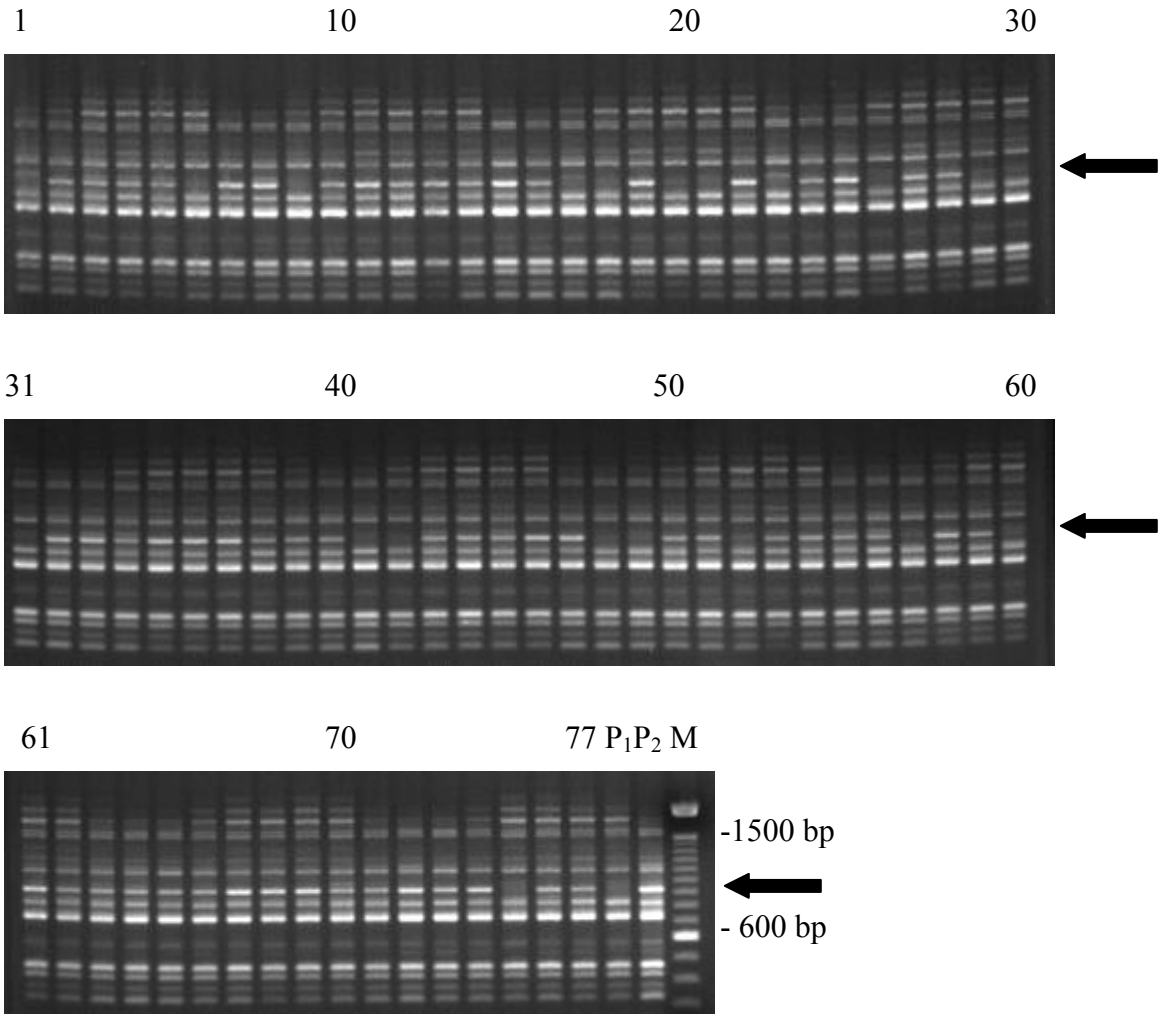

Figure 11. Segregation of RAPD marker OAC09.900 from 'TAM Uvalde' in an F_2 population derived from the cross of 'Sunrise' x 'TAM Uvalde' in a greenhouse population. First image = F_2 plants from 1-30, second image = F_2 plants from 31-60, third image = F_2 plants 61-77, P_1= 'Sunrise', P_2= 'TAM Uvalde' and M=100 bp molecular marker ladder. Arrows indicate band of interest.

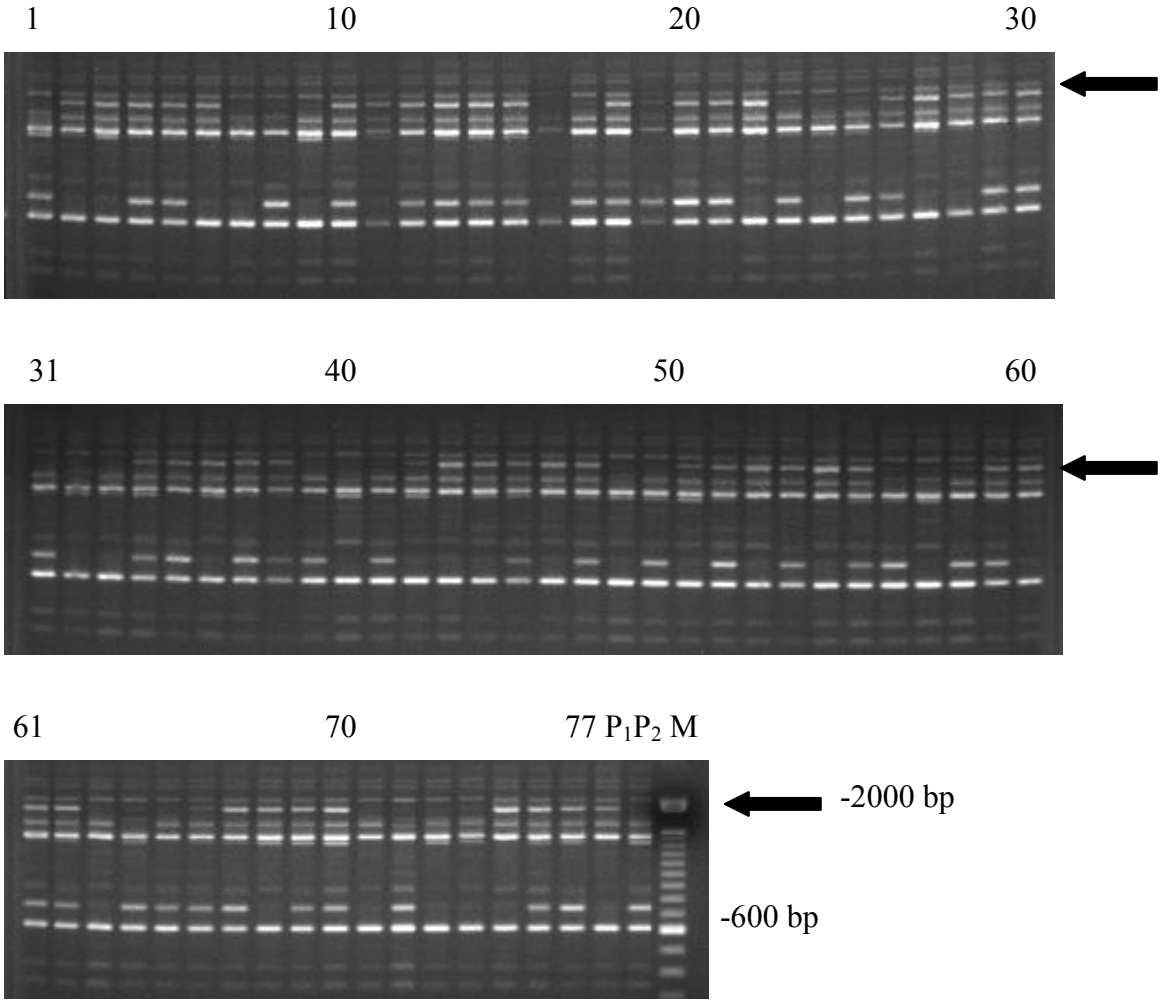

Figure 12. Segregation of RAPD marker OAI17.2000 from 'Sunrise' in an F_2 population derived from the cross of 'Sunrise' x 'TAM Uvalde' in a greenhouse population. First image = F_2 plants from 1-30, second image = F_2 plants from 31-60, third image = F_2 plants 61-77, P_1= 'Sunrise', P_2= 'TAM Uvalde' and M=100 bp molecular marker ladder. Arrows indicate band of interest.

Identification of Major QTL for Beta-Carotene

Of the seven markers associated with beta-carotene in the greenhouse population the four markers from the 'TAM Uvalde' parent were all included in a single linkage group. The *WF* locus was also included in the linkage group. The linkage group included 5 loci and covered a distance of 62.7 cM (Figure 13). Stepwise multiple regression found

that the linkage group could account for 39% of the observed phenotypic variation (Table 10). The three remaining RAPD markers from 'Sunrise' were unlinked.

Table 10. Simple linear regression and stepwise multiple regression analyses of greenhouse RAPD markers and data for detection of major QTL associated with high beta-carotene content in a F_2 population derived from the melon cross of 'Sunrise' (low beta-carotene) x 'TAM Uvalde' (high beta-carotene).

		SLR		β-carotene mean		SMR	
Marker	**Source**	***P***	***R^2* (%)**	**Present**	**Absent**	***P***	***R^2* (%)**
WF	TU	0.0000	39	16	3	**0.0001**	**39**
OAK03.1000	TU	0.0000	24	16	6		
OAC14.700	TU	0.0000	20	16	8		
OAC09.900	TU	0.0001	19	15	6		
OAI09.800	TU	0.0302	6	14	6		
					Cumulative R^2 =		**39**
OAV10.600	SR	0.0003	17	9	17		
OAO18.600	SR	0.0003	16	11	19	**0.0003**	**16**
OAG18.1500	SR	0.0057	10	11	16	**0.0154**	**7**
					Cumulative R^2 =		**23**

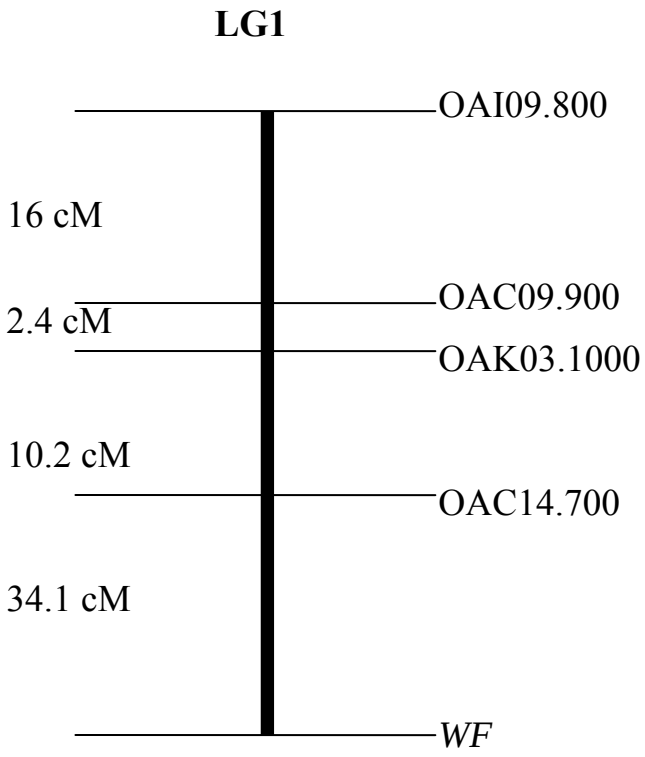

Figure 13. Linkage group 1 including four RAPD markers and *WF* from 'TAM Uvalde' associated with high beta-carotene content in the greenhouse F_2 population of the melon cross 'Sunrise' x 'TAM Uvalde'. Marker names are given on the right and the distance in centiMorgans between the markers is indicated on the left.

Field F_2 Population

The seven RAPD primers that segregated and were positively correlated to high beta-carotene content in the greenhouse F_2 population were selected for screening in the field F_2 population. An additional six primers that appeared to show a level of segregation, even if unlinked in the greenhouse F_2 population, were also screened. A total of 13 RAPD primers were screened in the field F_2 population, from those primers three markers were found to be linked to beta-carotene content. Two of the resulting markers were associated with the parent 'TAM Uvalde' and one was associated with 'Sunrise'.

Segregation distortion within each identified marker was tested using a Chi-square goodness-of-fit test to a 3:1 ratio. All three of the markers fit the expected ratio (Table 11). Linkage analysis failed to detect a linkage group between the two markers from 'TAM Uvalde'. The *WF* data again accounted for the majority of the phenotypic variation as determined by simple linear regression and stepwise multiple regression (Table 12).

Table 11. The chi-square tests for segregation of RAPD markers for two markers from 'TAM Uvalde' and one marker from 'Sunrise' associated with high beta-carotene content in a field F_2 population derived from the melon cross of 'TAM Uvalde' (high beta-carotene) and 'Sunrise' (low beta-carotene).

		plants (no.)				
Marker	**Source**	**Present**	**Absent**	**Expected Ratio**	**χ2**	***P***
OAC09.900	TU	79	36	3:1	2.397	0.119
OAK03.1000	TU	76	34	3:1	2.315	0.126
OAI17.2000	SR	76	33	3:1	2.028	0.152

Table 12. Simple linear regression and stepwise multiple regression analyses of field RAPD markers and data for detection of major QTL associated with high beta-carotene content in a F_2 population derived from the melon cross of 'Sunrise' (low beta-carotene) x 'TAM Uvalde' (high beta-carotene).

		SLR		β-carotene mean		SMR	
Marker	**Source**	***P***	***R^2* (%)**	**Present**	**Absent**	***P***	***R^2* (%)**
WF	TU	0.0000	44	19	3	0.0000	44
OAC09.900	TU	0.0362	4	17	13		
OAK03.1000	TU	0.0015	9	17	11		
					Cumulative R^2 =		44
OAI17.2000	SR	0.0468	4	17	13	0.0468	4
					Cumulative R^2 =		4

Combined F_2 Population

After marker analyses were completed for the greenhouse and field F_2 populations the data from both populations were combined into a single study population. Linkage analysis was conducted on thirteen combined markers. Of those markers, seven were false positives and six showed a positive trait association. The six linked markers were tested for goodness-of-fit to a 3:1 ratio using a Chi-square test (Table 13). Four of the markers segregated as expected, while two from 'Sunrise' did not.

Table 13. The chi-square tests for segregation of RAPD markers for three markers from 'TAM Uvalde' and three markers from 'Sunrise' associated with high beta-carotene content in a combined F_2 population derived from the melon cross of 'TAM Uvalde' (high beta-carotene) and 'Sunrise' (low beta-carotene).

		plants (no.)				
Marker	**Source**	**Present**	**Absent**	**Expected Ratio**	**$\chi 2$**	***P***
OAC09.900	TU	136	56	3:1	1.758	0.182
OAK03.1000	TU	130	56	3:1	2.785	0.094
OAC14.700	TU	130	52	3:1	1.886	0.168
OAI17.2000	SR	126	60	3:1	5.303	0.021
OAS08.550	SR	128	65	3:1	7.672	0.005
OAV10.600	SR	130	59	3:1	3.892	0.048

Identification of Major QTL for Beta-Carotene

From the six markers associated with beta-carotene in the combined population the three markers from the 'TAM Uvalde' parent were all included in a single linkage group. The *WF* data was also included in the linkage group. The linkage group included 4 loci and covered a distance of 81.5 cM. Stepwise multiple regression found that the linkage group could account for 42% of the observed phenotypic variation (Table 14). The three remaining RAPD markers from 'Sunrise' were unlinked.

Table 14. Simple linear regression and stepwise multiple regression analyses of combined RAPD markers and data for detection of major QTL associated with high beta-carotene content in a F_2 population derived from the melon cross of 'Sunrise' (low beta-carotene) x 'TAM Uvalde' (high beta-carotene).

		SLR		β-carotene mean		SMR	
Marker	**Source**	***P***	***R^2* (%)**	**Present**	**Absent**	***P***	***R^2* (%)**
WF	TU	0.0000	42	18	3	0.0000	42
OAK03.1000	TU	0.0000	14	17	9		
OAC09.900	TU	0.0001	8	16	10		
OAC14.700	TU	0.0008	6	16	12		
					Cumulative R^2 =		42
OAI17.2000	SR	0.0213	3	16	12	0.0137	5
OAS08.550	SR	0.0375	2	13	16	0.0481	3
OAV10.600	SR	0.0332	2	13	17		
					Cumulative R^2 =		8

DISCUSSION

Our findings concur with already well-known flesh-color genes. While only the greenhouse population fit the 12:3:1 segregation model, all three F_2 data sets fit the 3:1 model. The lack of goodness-of-fit in the field population to the 12:3:1 model was primarily caused by an overabundance in green fleshed fruit and a lack of white fleshed fruit. The variation found between the field and greenhouse populations could be caused by uncontrollable environmental factors or, as mentioned by Clayberg (1992), the inherent difficulties associated with differentiating white and green flesh color. However, the fact that the greenhouse, field and combined F_2 populations all fit the 3:1 ratio suggests that the parental genotypes in relationship to *wf* were still as proposed. To achieve the 3:1 ratio the genotypes of TAM Uvalde and Sunrise would be *wf+wf+/gf-gf-* and *wf-wf-/gf+gf+*, respectively.

The beta-carotene values for 'Sunrise' and 'TAM Uvalde' were low and high, as expected. The association of orange flesh color and high beta-carotene in 'TAM Uvalde' and white flesh color and low beta-carotene in 'Sunrise' made them ideal parents for our QTL study (Falconer and Mackay 1996). Beta-carotene in the F_2 populations appeared to be skewed towards low values. This skew could be caused by the epistatic interaction of *gf* and *wf*. However in some instances single F_2 plants had beta-carotene levels higher than 'TAM Uvalde'. This might have occurred through heterosis, the increased vigor often seen in hybrid plants that allows them to outperform their parents. Or it could be the result of specific environmental interactions that favored beta-carotene production in a few individuals. Similarly it was noted that flesh color plays a major role in beta-carotene accumulation. When white and green flesh colored fruits are removed from the

population, the lowest values of beta-carotene are removed. Orange, white and green fleshed melons all produce significantly different amounts of beta-carotene, regardless of environment.

A relationship was found between orange flesh color intensity and beta-carotene content. It is possible to select melons high in beta-carotene based on a visual flesh color intensity rating system. However in some instances the removal of lower beta-carotene fruit appears to be more feasible than direct selection for only high beta-carotene fruit. Progeny tests are needed to further determine the flesh color interaction of *wf* and *gf* and their relationship to beta-carotene content.

Our calculation of the broad sense heritability of beta-carotene content in the greenhouse plants resulted with 51% of the observed variation being attributed to genetic differences between the plants. This indicates that beta-carotene content has a relatively low heritability; 49% of the variation that occurred was caused by environmental factors.

The combination of BSA and RAPD primers is an ideal way to screen large amounts of primers in a relatively short time. Of course, the results of BSA are limited to the traits of interest associated with the study. In the greenhouse population seven markers were found to be associated with high beta-carotene content. Four of those markers and *WF* came from 'TAM Uvalde' and were linked to a major QTL associated with high beta-carotene (R^2= 39%). The same QTL was not duplicated in the field population, but a similar QTL linked to three of the same markers and *WF* was detected in the combined F_2 populations and also contributed to a large portion of the genetic variation for the trait (R^2= 42%). Progeny testing could be used to help determine the exact map distance and placement of markers within this genomic region around *WF* (Falconer and Mackay 1996).

In each of the populations *WF* was responsible for the highest amount of phenotypic variation. This is somewhat discouraging, as *WF* is a trait that can be easily selected for at harvest time. Nonetheless, the fact that four markers were all placed in the same linkage group suggests that a major QTL for beta-carotene content could be present in melons, on the same chromosome as *WF*.

From this point SCAR markers can be developed to increase the ease of marker assisted selection. The markers can also be used to survey muskmelon germplasm for identification of high beta-carotene genotypes for introduction into breeding programs. These markers can also be used to locate the major beta-carotene QTL on the melon linkage map.

Marker OAC14.700

When the linkage analysis data from the three populations were compared the presence and absence of marker OAC14.700 becomes apparent. In the greenhouse population OAC14.700 is the marker with the tightest linkage with *WF*. However, in the field population, OAC14.700 and *WF* are not linked. When the greenhouse and field population data are combined, OAC14.700 is again linked to *WF*, though not as closely. To further examine the relationship between OAC14.700 and *WF* in the field population the field population data was reevaluated.

When only the markers that segregate according to the dominant marker model are examined, the 'TAM Uvalde' markers that compose linkage group one in the greenhouse and combined data are missing in the field population. However, linkage analysis can still be done using the distorted markers that did not segregate according to the 3:1 model. In

the field population there were eight markers, including orange flesh color, identified as coming from 'TAM Uvalde'. Six of those eight segregated to the expected ratio while two did not. Mapmaker was used to determine the relationships between all eight markers and found one linkage group of two markers; the remaining six markers were unlinked. All eight markers with their expected segregation ratios are shown in Table 15.

Table 15. The chi-square tests for segregation of RAPD markers for eight markers from 'TAM Uvalde' associated with high beta-carotene content in a field F2 population derived from the melon cross of 'TAM Uvalde' (high beta-carotene) and 'Sunrise' (low beta-carotene).

		plants (no.)				
Marker	**Source**	**Present**	**Absent**	**Expected Ratio**	χ^2	**P**
Orange	TU	92	25	3:1	0.804	0.364
OAC09.900	TU	79	36	3:1	2.397	0.119
OAI09.800	TU	79	36	3:1	2.397	0.119
OAK03.1000	TU	76	34	3:1	2.315	0.126
OAU09.700	TU	75	31	3:1	1.937	0.162
OAX01.2000	TU	36	63	3:1	69.287	0.000
OAC14.700	TU	64	47	3:1	17.112	0.000
OAX19.800	TU	73	33	3:1	2.931	0.085

The fact that marker OAC14.700 appears to be unassociated with beta-carotene in the field population, but has the closest association with *WF* in the greenhouse and combined populations creates a discrepancy. It could be that the small population in the greenhouse created a false positive marker through chance. However, it seems doubtful that chance is responsible for the presence of the marker's association in the combined data. It seems more likely that the data from the field population holds the discrepancy. There were some experimental factors that could indicate that this is true. For instance, the DNA that was extracted from the field plants had on average a concentration 10-fold less than that of the greenhouse plants. The DNA for the two populations was extracted at

different times, under different circumstances. This might have led to slight variations in the procedure that reduced the final concentration. The DNA from the greenhouse plants was also extracted from the young leaf tissue, while the DNA from the field plants was extracted from the rind. The different tissues used to harvest the DNA could have also affected the results. The DNA concentration is important because if it is not accounted for in the RAPD procedure the gel pictures produced will be low quality. The initial RAPD gels that were run using the field DNA had a noticeably poor quality. It was only after doubling the DNA concentration used in the PCR reaction and doubling the number of cycles performed by the thermalcycler, that clearer, visible bands could be produced using the field DNA. Even after the mentioned modifications to the RAPD process, the DNA fragments from the field population were still somewhat smeared and difficult to score. Impurities left in the DNA samples from the extraction process could have caused the unclear appearance of the DNA fragments.

Besides the DNA concentration and quality making the field RAPD images difficult to score, the phenotypic data might have also influenced the outcome of linkage analysis. Vine decline was present in the field population. Of the 117 plants in the field, 49 had visible symptoms of vine decline. Vine decline creates a significant problem in melon growing areas of south Texas, like the Rio Grande Valley. It is caused by the soil borne pathogen *Monosporascus cannonballus*. The symptoms of the disease include vine wilting just prior to fruit harvest. Ironically, while the pathogen infects the root system of the plant early on, the symptoms do not become apparent until the fruit are reaching maturity (Bertelsen et al 1994). The vine decline fungi could have influenced the fruit quality of the infected plants. The expressed phenotypes of the infected plants could have

been changed by the vine decline infection. If this did occur, it seems that the likelihood of finding a relationship between the RAPD DNA fragments and the beta-carotene data would be reduced.

A few ideas have been presented regarding the appearance of OAC14.700 in the greenhouse population and combined population, and its disappearance in the field population. Scoring the RAPD gels produced from the field DNA was more difficult, which could have led to increased experimental error, while the presence of vine decline could have affected the phenotypes of enough plants in the field population to change the outcome of linkage analysis. This seems like a probable explanation, because the linkage was recovered when the data was combined.

SUMMARY

Two F_2 populations, one greenhouse grown and one field grown were used to study the inheritance of flesh color and beta-carotene and their genetic relationship with regard to beta-carotene content.

A positive correlation was found between beta-carotene content and total soluble solids, this suggests that breeding for melons high in beta-carotene as well as sugars should be possible. No correlations were found between fruit size traits and beta-carotene. The relationship between flesh color intensity and beta-carotene content is a positive breeding tool that can be used to easily select melons high in beta-carotene at harvest time.

The heritability of beta-carotene content was determined to be 51% in the greenhouse population. This is a relatively low heritability, leaving 49% of the beta-carotene variation seen between plants related to environmental effects. To increase selection for high beta-carotene more field trials would be necessary to determine what types of environments can positively influence beta-carotene content.

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APPENDIX A

F_2 plants from field population used in Bulk Segregate Analysis.

Table 16. BSA bulk, low beta-carotene, orange flesh color, field.

Sample Name	Beta-Carotene, µg/g	Weight, g	Size	Flesh Color	Flesh Color Intensity	Vine Decline
FLD -51	6.06	1312	15	orange	1	No
FLD -8	7.34	1422	12	orange	1	No
FLD -3	7.49	1103	15	orange	1	No
FLD -82	7.66	2420	6	orange	1	No
FLD -65	7.77	1736	12	orange	1	No
FLD -110	9.36	1232	189	orange	1	No
MEAN:	7.61			MEAN:	1	

Table 17. BSA bulk, high beta-carotene, orange flesh color, field.

Sample Name	Beta-Carotene, µg/g	Weight, g	Size	Flesh Color	Flesh Color Intensity	Vine Decline
FLD -60	30.51	1768	9	orange	2	No
FLD -47	30.99	1083	18	orange	2	No
FLD -26	34.36	1025	18	orange	3.5	No
FLD -74	34.86	1680	12	orange	2	Yes
FLD -31	36.52	1165	18	orange	3.5	No
FLD -66	50.26	1233	15	orange	4	Yes
MEAN:	36.25			MEAN:	2.83	

Table 18. BSA bulk, low beta-carotene, white/green flesh color, field.

Sample Name	Beta-Carotene, µg/g	Weight, g	Flesh Color	Vine Decline
FLD -40	1.41	1858	white	No
FLD -58	1.61	1625	green	No
FLD -7	1.61	1159	green	No
FLD -107	1.66	2913	green	No
FLD -97	1.72	3785	green	No
FLD -90	1.85	2869	green	No
MEAN:	1.64			

APPENDIX B

F_2 plants from greenhouse population used in Bulk Segregate Analysis.

Table 19. BSA bulk, low beta-carotene, orange flesh color, greenhouse.

Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh Color	Flesh Color Intensity
F2-9	6.02	32	1479.9	12	orange	1
F2-88	7.95	33	1473.1	12	orange	1
F2-44	8.35	32	1130.7	18	orange	1
F2-89	8.75	33	1036.1	18	orange	2
F2-14	8.75	33	871	23	orange	1
F2-52	8.93	34	732.4	23	orange	2
MEAN:	8.12				MEAN:	1

Table 20. BSA bulk, high beta-carotene, orange flesh color, greenhouse.

Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh Color	Flesh Color Intensity
F2-25	26.74	32	1130.3	18	orange	4
F2-72	27.79	34	903.4	18	orange	2.5
F2-38	27.84	36	1155.4	15	orange	2.5
F2-81	32.28	37	1498.3	12	orange	2.5
F2-69	34.35	41	1212.8	15	orange	2.5
F2-43	40.17	33	1111.1	18	orange	3
MEAN:	31.53				MEAN:	2.83

Table 21. BSA bulk, low beta-carotene, white/green flesh color, greenhouse.

Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh color
F2-17	1.13	38	1090.7	18	white
F2-71	1.25	30	1197.8	15	white
F2-35	1.33	38	1171.1	15	white
F2-74	1.57	32	1136	18	white
F2-34	1.57	33	1108	15	white
F2-36	1.59	38	1183.3	15	white
MEAN:	1.41				

APPENDIX C

Table 22. BSA bulk, sunrise parent, low beta-carotene, greenhouse.

Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh Color	Flesh Color Intensity
SR3	1.50	34	1715.1	12	white	1
SR4	1.54	34	1249.2	15	white	1
SR5	1.51	35	1360	15	white	1
SR9	1.41	35	925.9	18	white	1
MEAN:	1.49				MEAN:	1

Table 23. BSA bulk, TAM Uvalde parent, high beta-carotene, greenhouse.

Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh Color	Flesh Color Intensity
TU2	25.48	32	842.6	23	orange	4.5
TU3	22.12	34	913.9	23	orange	4
TU4	37.32	35	826.1	18	orange	3.5
TU7	29.30	33	1091.5	18	orange	4
MEAN:	28.56				MEAN:	4

APPENDIX D

Initially, the Amplified Fragment Length Polymorphism (AFLP) marker screening technique, as described by Vos et al (1995), was used to detect possible markers in the greenhouse population. During the BSA screening process 128 primer combinations were used, but no polymorphic markers were positively identified. Since markers were not identified during the BSA screening process, AFLP primers were not further screened in either the greenhouse or field populations.

DNA Quantification and Purification

The DNA used to carry out the AFLP procedure came from the same DNA samples that were used in the RAPD procedure. An explanation of the DNA extraction procedure can be found in the Materials and Methods section.

Before beginning, each DNA sample was washed with an ethanol solution to remove excess sodium acetate or other residues left from the original extraction procedure. To wash the DNA, the samples were centrifuged for 5 minutes at 10,000 RPM in a microfuge. After spinning down, 100 μL of each sample were transferred to a clean, labeled microfuge tube. During the transfer care was taken to remove the supernatant from the top of the original tube, avoiding any debris that may have settled out while centrifuging. The DNA was then precipitated from the new sample by the addition of 500 μL of a 70% ethanol solution. The precipitate was gathered into a pellet by centrifuging again for five minutes at 10,000 RPM. Next the supernatant liquid was poured off leaving only the pellet. The pellet was washed again; the second time using 1 mL of 70% ethanol. The cleaned DNA samples were then allowed to dry overnight in a dry, draft free location.

The following day the samples were rehydrated using 20 µL of ½ X TE buffer. After the addition, each tube was gently mixed for 30 seconds and then centrifuged for 1 minute at 4,000 RPM. Rehydrated samples were stored on ice or frozen until needed.

The DNA quantity of each cleaned sample was measured using a fluorometer. Prior to use, the fluorometer was blanked and calibrated using a solution of 100 mL of 1 X TNE and 10 µL of Hoecht dye. Two mL of the solution were placed in the sampling cuvette to blank the fluorometer. Next, 2 µL of calf Thymus DNA were added to the blanked sample to calibrate the fluorometer. To read a sample in the calibrated fluorometer two mL of the TNE and Hoecht dye solution were added to a cleaned cuvette, along with 2 µL of the DNA sample. The solution was then mixed well, after sealing the cuvette with parafilm. Then the cuvette was placed in the fluorometer and measured, the displayed value was recorded and the process was repeated with the next sample. Between samples the cuvette was washed with sterilized water and dried using a vacuum tube. Throughout the calibration process and while samples were being quantified, the light sensitive solution of TNE and Hoecht dye was covered with aluminum foil. The desired DNA concentration for each sample was 100 ng/µL; however when the concentration was too low or high, the quantity of each DNA sample was adjusted accordingly as described in the first phase of the AFLP process.

Amplified Fragment Length Polymorphism Procedure

The AFLP process consists of four phases. They are: restriction digestion, adapter ligation, preamplification, and selective amplification. The first three phases are necessary to prepare the DNA sample for selective amplification. Once a DNA sample has

completed those three phases the created DNA template can be used repeatedly for the fourth phase, selective amplification. The following AFLP technique is that of Vos et al (1995).

To begin restriction digestion a master mix was created. The mix consisted of the following ingredients: 4μL of restriction enzyme buffer (New England BioLabs), 5 units of EcoRI (New England BioLabs), 5 units of MseI (New England BioLabs), and enough water to create a final volume of 30 μL. The amount of each ingredient was adjusted according to the number of DNA samples being prepared. The master mix solution was mixed and 30 μL were pipetted into labeled, .5 mL microfuge tubes. Next the DNA samples were added to each microfuge tube and the final volumes were adjusted to 40 μL using water.

The amount of DNA added was based on the concentration of each sample. If the concentration of the DNA sample had been measured at 100 ng/μL then 5 μL of the sample and 5 μL of additional water were added to the sample tube. However, if the DNA sample was not at 100 ng/μL, then the amounts of DNA and water added to each microfuge tube were adjusted accordingly. For instance, if a DNA sample had a concentration of 250 ng/μL, then only 2 μL of that sample were added to the microfuge tube. In this example the amount of water would be increased to 8 μL to maintain the final volume at 40 μL.

After preparing all the microfuge tubes with the proper amounts of DNA for restriction digestion they were placed in a hot water bath at 37°C for 2 hours. Next the samples were spun down in a centrifuge for 5 seconds at 4,000 RPM and placed on ice.

To begin adapter ligation, the second phase of the AFLP procedure, another master mix was created. 10 μL of the following solution were added to each microfuge tube: 1 μL

of 5 pmol EcoRI adapters (MWG-Biotech), 1 µL of 50 pmol MseI adapters (MWG-Biotech), 1 µL of 10 mM ATP (Promega), 0.3 µL of 1 Unit T4 DNA ligase (Promega), 1 µL of 10 X RE buffer (Promega), and 5.7 µL of water. The quantity of the master mix was adjusted according to the number of samples being prepared. After adding the solution to each sample the samples were mixed well and centrifuged at 4,000 RPM for 5 seconds. Next they were placed in a hot water bath at 37°C overnight, but not longer than 16 hours.

The following day, the tubes were removed from the water bath and spun in a centrifuge at 4,000 RPM for 5 seconds. The samples were then diluted using 450 µL of 1 X TE buffer. The samples were then relabeled as 'Dilute Template DNA' and stored at -20°C until needed.

The next step of the AFLP process is preamplification of the dilute DNA templates. This step requires the use of sealable, 96-well PCR plates and a thermalcycler. To begin, 5 µL of each dilute template DNA sample was placed in a separate well of a PCR plate. A master mix was prepared using the following items: 2 µL of 10 X PCR buffer (Promega), 2 µL of 10 mM $MgCl_2$ (Promega), 1.6 µL of 2.5 mM dNTPs (Promgea), 1 µL of 30ng/µL EcoRI preamplification primer (MWG-Biotech), 1 µL of 30 ng/µL MseI preamplification primer (MWG-Biotech), 0.08 µL of Taq DNA polymerase (Promega), and 7.32 µL of water. The volumes listed are the initial quantities needed to prepare enough master mix for one 15 µL sample. Increase the volume of the master mix by multiplying the quantities of each solution by the final number of samples needed. 15 µL of the master mix were added to each PCR plate well. A clean pipette tip was used to transfer the solution to each well. The dilute template DNA was thoroughly mixed with the master mix by drawing it up into the pipette tip 8-10 times. Once the master mix had been distributed to all samples

the PCR plate was sealed and placed in the thermalcycler. The PCR preamplification consisted of : 20 cycles of 94°C for 30 seconds, 56°C for 60 seconds, and 72°C for 60 seconds, after the cycles were completed the cycle was held at 4°C.

Following the PCR run the samples were diluted again using ½ X TE buffer. 180 µL of the TE buffer were added to each well. The samples were then labeled as dilute preamplified template DNA and have a final DNA concentration of 25 pg/µL. At this point the samples were stored at -20°C until needed.

The fourth and final stage of AFLP PCR, selective amplification, utilizes the dilute preamplified template DNA. During selective amplification unique primer combinations are used to amplify specific areas of the DNA that have been prepared during the first three stages of the AFLP process. Different primers combinations, each consisting of an MseI selective primer and an EcoRI selective primer are used to create different DNA fragments than can be visualized using the Licor automated sequencer. Each EcoRI selective primer is labeled with a light sensitive infrared dye. The dye allows the automated sequencer to read the DNA fragments. The Licor system that was available for our use was capable of reading two different infrared frequencies simultaneously, the 700 nm and 800 nm ranges. This capacity allowed two samples to be loaded into the same gel and visualized at the same time.

To prepare the PCR plate for the Licor system the samples were loaded so that each sample corresponded with an available well in the gel. Only 64 samples were prepared on each PCR plate as only 64 wells were in each gel. To begin, 2 µL of each dilute preamplified template DNA were added to their corresponding well on the PCR plate. A master mix was then prepared consisting of: 1 µL of 10X PCR buffer (Promega), 1 µL of

10mM $MgCl_2$ (Promega), 0.8 µL of 2.5mM dNTPs (Promega), 2 µL of 7.5 ng/µL MseI selective primer (MWG Biotech), 0.3 µL of IRD labeled EcoRI selective primer (MWG Biotech), 0.04 µL Taq DNA polymerase (Promega), and 2.86 µL water for a final volume of 8 µL per sample. The volumes shown are the initial quantities needed to prepare enough master mix for one 8 µL sample. To increase the volume of the master mix, multiply the quantities of each solution by the final number of samples being prepared. Add 8 µL of the master mix to each well, mixing each one by pipetting up and down into the pipette tip 8-10 times. The PCR plate was sealed and selective amplification was run on the thermalcycler as follows: 1 cycle of 94°C for 2 minutes, 13 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 60 seconds. During this phase the annealing temperature is lowered by 0.7°C after each cycle. The cycle continues with 23 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72° for 60 seconds. It ends with 5 minutes at 72°C and the final hold is at 4°C. The finished reactions were covered in aluminum foil and stored at -20°C.

An acrylamide gel was prepared using 25 mL of Gene Page Plus 6%, 15 µL of 7 M urea, and 150 µL of 10% ammonium persulfate solution. A solution of 1X TBE buffer was used to cover the gel during the run. Before loading the samples, the 700 nm labeled samples were combined with the 800 nm labeled samples. 4 µL of Licor loading dye was added to the combined samples. The samples were then denatured for 5 minutes at 95°C in a thermalcycler. From there they were transported on ice to the Licor automated sequencer. Using a micro-multichannel pipetter eight samples were loaded into the gel concurrently. 1 µL of each sample was loaded, the unused portions of the samples were returned to the -20°C freezer.

The gels were allowed to run for at least 2.5 hours, which allowed the majority of the fragments to be visualized. Afterwards the gel plates were removed from the Licor system and the computerized visualizations were condensed to a single page and printed. The images were read and scored by hand, as described below.

AFLP Primers

The sequences of the EcoRI and MseI enzymes and adapters that were used in the techniques described above are shown in Tables 22 and 23. In total 128 AFLP primer combinations were screened in a subset of the greenhouse F_2 population. The primers used to perform the screening are listed in Tables 24 and 25. The term primer combination refers to a single EcoRI primer paired with any single MseI primer. There were 16 available MseI primers and 8 EcoRI primers, resulting in 128 combinations between them. The first unique base of each MseI primer was 'C' to match the sequence of the MseI adapters used during the adapter ligation phase. No additional bases were used with the EcoRI adapters, so each EcoRI primer was completely unique.

Table 24. EcoRI and MseI enzymes and their sequences used in the restriction digestion phase of the AFLP process. Each set of enzymes were annealed prior to use by mixing equal volumes of each in a microfuge tube and then placing in a boiling water bath for 5 minutes. After which the heat was turned off and the tubes were allowed to remain in the water until it returned to room temperature.

AFLP Phase	Adapters	Sequence
Restriction Digestion	EcoRI-1	5'-CTCGTAGACTGCGTACC-3'
Restriction Digestion	EcoRI-2	5'-AATTGGTACGCAGTCTAC-3'
Restriction Digestion	MseI-1	5'-GACGATGAGTCCTGAG-3'
Restriction Digestion	MseI-2	5'-TACTCAGGACTCAT-3'

Table 25. Adapters and sequences used in the adapter ligation phase of the AFLP procedure.

AFLP Phase	Preamplification Primers	Sequence
Adapter Ligation	EcoRI+0	5'-GTAGACTGCGTACCAATTC-3'
Adapter Ligation	MseI+C	5'-ACGATGAGTCCTGAGTAAC-3'

Table 26. The MseI primers used during the selective amplification phase of the AFLP process. Their sequences and unique endings are also shown.

AFLP Phase	Primer Name	Base Sequence	Ending Sequence
Selective Amplification	M+CCC	5'-GATGAGTCCTGAGTAA-3'	CCC
Selective Amplification	M+CCA	5'-GATGAGTCCTGAGTAA-3'	CCA
Selective Amplification	M+CCT	5'-GATGAGTCCTGAGTAA-3'	CCT
Selective Amplification	M+CCG	5'-GATGAGTCCTGAGTAA-3'	CCG
Selective Amplification	M+CAC	5'-GATGAGTCCTGAGTAA-3'	CAC
Selective Amplification	M+CGC	5'-GATGAGTCCTGAGTAA-3'	CGC
Selective Amplification	M+CTC	5'-GATGAGTCCTGAGTAA-3'	CTC
Selective Amplification	M+CAT	5'-GATGAGTCCTGAGTAA-3'	CAT
Selective Amplification	M+CAG	5'-GATGAGTCCTGAGTAA-3'	CAG
Selective Amplification	M+CAA	5'-GATGAGTCCTGAGTAA-3'	CAA
Selective Amplification	M+CTA	5'-GATGAGTCCTGAGTAA-3'	CTA
Selective Amplification	M+CTG	5'-GATGAGTCCTGAGTAA-3'	CTG
Selective Amplification	M+CTT	5'-GATGAGTCCTGAGTAA-3'	CTT
Selective Amplification	M+CGG	5'-GATGAGTCCTGAGTAA-3'	CGG
Selective Amplification	M+CGA	5'-GATGAGTCCTGAGTAA-3'	CGA
Selective Amplification	M+CGT	5'-GATGAGTCCTGAGTAA-3'	CGT

Table 27. The EcoRI primers used during the selective amplification phase of the AFLP process. Their sequences and unique endings are shown along with the IRD label attached to each primer.

AFLP Phase	Primer Name	Base Sequence	Ending Sequence	IRD Label
Selective Amplification	E+ACT	5'-AGACTGCGTACCAATTC-3'	ACT	700
Selective Amplification	E+TAC	5'-AGACTGCGTACCAATTC-3'	TAC	700
Selective Amplification	E+TAT	5'-AGACTGCGTACCAATTC-3'	TAT	700
Selective Amplification	E+TAG	5'-AGACTGCGTACCAATTC-3'	TAG	700
Selective Amplification	E+GAG	5'-AGACTGCGTACCAATTC-3'	GAG	800
Selective Amplification	E+CAT	5'-AGACTGCGTACCAATTC-3'	CAT	800
Selective Amplification	E+AAG	5'-AGACTGCGTACCAATTC-3'	AAG	800
Selective Amplification	E+CAG	5'-AGACTGCGTACCAATTC-3'	CAG	800

Bulk Segregant Analysis

The bulk segregant analysis technique described by Michelmore et al (1991) was used to screen the greenhouse population for potential markers. However, some modifications were used. Instead of creating bulks of the DNA from the high and low beta-carotene content F_2 plants, the DNA was left unbulked. Parent DNA bulks were not employed either; however two parents from ‘Sunrise’ and ‘TAM Uvalde’ were always tested with each primer combination. The parent plants and F_2 plants used are shown in Table 26.

Table 28. Parent and F2 plants from the greenhouse population used to test 128 AFLP primer combinations.

Origin	Sample Name	Beta-Carotene, µg/g	Growing days	Weight, g	Size	Flesh Color	Flesh Color Intensity
'Sunrise' Parent	SR-4	1.54	34	1249.2	15	white	1
'Sunrise' Parent	SR-9	1.41	35	925.9	18	white	1
'TAM Uvalde' Parent	TU-4	37.32	35	826.1	18	orange	3.5
'TAM Uvalde' Parent	TU-7	29.30	33	1091.5	18	orange	4
High F2	F2-11	24.37	32	1069.3	18	orange	2.5
High F2	F2-12	30.19	34	971.8	18	orange	1.5
High F2	F2-25	26.74	32	1130.3	18	orange	4
High F2	F2-38	27.84	36	1155.4	15	orange	2.5
High F2	F2-42	11.72	34	709.4	23	orange	2.5
Low F2	F2-17	1.13	38	1090.7	18	white	1
Low F2	F2-18	1.99	34	1185.7	15	white	2
Low F2	F2-26	1.69	35	1001.9	18	white	1
Low F2	F2-34	1.57	33	1108	15	white	1
Low F2	F2-35	1.33	38	1171.1	15	white	1

Results

A total of 128 different primer combinations were screened following the techniques described for the AFLP PCR process combined with BSA. No marker polymorphisms could be detected between the parents and the high and low F2 groups.

Discussion

Our inability to find markers after screening a large number of AFLP primer combinations is confusing. AFLP primers have been used to successfully identified markers in *Cucumis* varieties by many researchers (Oliver et al 2001; Perin et al 2002;

Bradeen et al 2001; Perin et al 2000; Wang 1997). A comparison between the efficiency of RAPD, RFLP, and AFLP marker analysis in their ability to distinguish genetic diversity between six melon cultivars found that AFLPs are the most efficient and reproducible (Gracia-Mas et. al. 2000). Perin et al (2000) also found the use of AFLPs in melon breeding to be the most reliable and repeatable.

There are a number of factors which might have interfered with our own successful marker identification. The AFLP technique requires a great deal more precision than when working with RAPD markers. Smaller volumes are used in every aspect of the procedure and loading the gels can be quite difficult without properly working pipetters and a good deal of experience. The set up of our BSA was also somewhat unusual, however the decision to not bulk the DNA should have only increased the amount of time needed to test primer combinations as opposed to the outcome of each test. Even without identifying polymorphisms between the F_2 plants, polymorphisms between 'Sunrise' and 'TAM Uvalde' would have been expected.

The gel pictures that were produced were for the most part uniform looking. This suggests that the initial phases of the procedure were carried out successfully to the dilute template DNA stage. The uniformity seen between parents and the selected F_2 population plants with each selective amplification primer combination also suggests that the primers were amplifying the same DNA fragments in every sample. It could be that the random selections of primers used in the screening were ill matched with our DNA samples, making it quite possible that other primers would have been effective.

VITA

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