77

BARD

FINAL REPORT

PROJECT NO. IS-2129-92

**Development and Application of a Molecular Genetic
Map for Melon (*Cucumis melo*)**

R. Perl-Treves, M. Kyle, E. Galun

1997

BARD Cover Page for Scientific Reports
To be sent to BARD Office in Israel:
BARD, P.O. Box 6, Bet Dagan 50250, Israel.

25/2/1997
Date: _____

IS 2129-92
BARD Project No. _____

Title

Development and Application of a Molecular Genetic Map for Melons

All Investigator's Names
(Principal listed first)

Rafael Perl-Treves

Molly Kyle

Esra Galun

Investigator's Institutions

Bar-Ilan University

Cornell University

Weizmann Institute of Science

1/10/1993
Project's starting date: _____

Type of Report: 1st Annual _____ 2nd Annual _____ Final x

Signature
Principal Investigator

Signature
Institution's Authorizing Official

Table of Contents

ABSTRACT

PROPOSED OBJECTIVES

RESEARCH ACCOMPLISHED RELATIVE TO PROPOSED OBJECTIVES

RESULTS AND DISCUSSION

DEVELOPMENT OF MOLECULAR MARKERS FOR MELON

Sources of RFLP clones

Optimization of RFLP protocols for *C. melo*

Extent and usefulness of RFLP polymorphism in *C. melo*

RAPD markers for mapping

DNA VARIATION IN MELON GERMPLASM

Survey of 13 accessions using RAPDs

Survey of 70 accessions using RAPDs and other techniques

Variation in fruit sugar metabolism

Variation in for genetic potential for regeneration in vitro

MAPPING of RAPD and RFLP markers

The F2 mapping population

Scoring morphological traits in the F2

Disease resistances scored at the F3

Scoring RAPD and RFLP markers at the F2

Targeting selected loci by Bulk Segregant Analysis

Generation of a permanent mapping population

DESCRIPTION OF COOPERATION

EVALUATION of RESEARCH ACHIEVEMENTS

CONCLUSIONS

PUBLICATIONS

ABSTRACT

This project has generated a systematic survey of DNA polymorphism in *Cucumis melo*. An RFLP and RAPD survey of the major cultivar groups and botanical varieties of this species has been conducted, with the purpose of assessing the degree of molecular variation and the phylogenetic relationships within the melon germplasm and, at the same time, develop sets of markers suitable for mapping the melon genome. Additional activities regarding variation in the melon germplasm in fruit traits and regeneration ability have been initiated as well.

The necessary populations required for the development of a molecular map of the *C. melo* genome have been prepared. An F2 that segregated for 4 viral resistances, powdery mildew resistance and sex type has been derived from a PI 414723 x Topmark cross, and a RILs population has been prepared from it. We have confirmed the resistances in the population and have analyzed the genetic relationships between these resistances. Progress toward the construction of a molecular map of *C. melo* and the development of markers linked to those traits is described. We have so far screened the first few tens of markers in the F2 population, and many additional ones were screened in DNA bulks prepared from such population.

OBJECTIVES

The general goal of this project was to develop and apply tools for genome analysis and for identification of markers for agronomic traits in melons. The three following objectives have been pursued:

1. Evaluation of DNA polymorphism within *Cucumis melo*. We proposed to survey a set of melon types and cultivars that were systematically selected to span the range of variability within the species, since preliminary results suggested that at least within cultivated varieties, there was quite a narrow germplasm. This type of survey allows the measurement of genetic distances between genotypes, helps to understand the genetic basis for diversity in the germplasm; and can be used to guide selection of the most diverged material for mapping purposes. We proposed to use different techniques, mainly RFLP (restriction fragment length polymorphism) with cDNA or single copy genomic DNA and RAPDs (random amplified polymorphic DNA) for this purpose.

2. Identification of markers linked to agronomically important traits. Parents were identified that differed in simply-inherited horticulturally significant traits and were sufficiently diverged at the DNA level. We proposed to construct segregating populations and identify markers linked to those traits. The pooling approach had been proposed for this purpose. An existing F2 population that segregated for resistance or tolerance to ZYMV (zucchini yellow mosaic virus), WMV (watermelon mosaic virus) and powdery mildew

(PM), as well as fruit characteristics, was selected as a mapping population.

3. Generation of a framework map of melon. We proposed that a moderate number (~100) of polymorphic markers were to be scored in a segregating population of at least 60 individuals derived from the most diverged parents. Linkage analysis was planned to obtain low-density map of the melon genome, and to provide a basis for more extensive mapping efforts in the future.

ACTUAL RESEARCH PROGRAM AS COMPARED TO ORIGINAL GOALS

In general, we adhered to the original objectives, and conducted a variability survey using RAPDs and RFLPs, and mapping in an F2 population. Progress in mapping has been slow, and additional work to accomplish this objective will continue as discussed below. Several activities that have not been finished in three years have been extended into 1997 using matching funds. The following activities, not included in the original objectives, have been added.

1. The F2 mapping population was also scored for resistance to papaya ringspot virus.
2. A set of recombinant inbred lines (RILs) have been derived from the F2 population by repeated selfing to create a permanent mapping population. This will greatly facilitate the coordination of maps between different groups, and the addition of further characters to the map.
3. A new marker technique, Inter-SSR PCR is being tried with the hope that this will accelerate determination of molecular genotypes and subsequent mapping.
4. The germplasm variability survey was expanded to include two important traits: regeneration in vitro, and metabolism relating to the production of fruit sugars.

RESULTS and DISCUSSION

A. DEVELOPMENT OF MOLECULAR MARKERS FOR MELON

A.1. Sources of RFLP markers

We have constructed a probe library of *C. melo* cv Topmark genomic DNA that was digested with PstI and cloned into the pBluescript vector. A collection of several hundred colonies was generated. A few tens were analyzed and coded PT-1 - PT-73. Average insert size was 1 kbp. In order to screen out repetitive DNA clones, we blotted PstI digests of our clones and hybridized them to radiolabeled total genomic DNA. Clones yielding strong signals were identified as repetitive. Out of 37 clones tested, 14 (38%) were scored repetitive. The others were retained as possible low/single copy; however this group still contained a significant proportion of repetitive clones. Partial characterization of a few of the repetitive DNA clones indicated the presence of a few classes of repeats in our library, and

that several of them included PstI sites. Inserts from different PT clones were radiolabelled with 32 -P and used as probes on Southern blots prepared from *C. melo* DNA to look for RFLPs between genotypes.

As a second source of RFLP probes we tried cucumber cDNAs from floral bud libraries constructed in our laboratory (Rosenman et al. 1995). These were applied to melon blots at lower stringency (550 °C, 5 x SSPE). Cucumber clones, both cDNA and genomic, were also obtained from Drs. Michael Havey and Jack Staub, University of Wisconsin at Madison, USA and tested as probes in the variability survey. Melon clones were also obtained from Dr. Michel Pitrat, INRA, France, in order to set up coordination between the French mapping effort and ours.

A.2. Optimization of RFLP protocols for *C. melo*

This phase of the project required substantially more time and effort than we had expected, but in view of the importance of reliability and reproducibility of signal in mapping, we persisted until the genomic Southern blots worked reproducibly and yielded clean, strong RFLP signals.

DNA preparation. Three protocols were initially evaluated. One was modified from Dellaporta et al. (1983), the second from McCouch et al. (1988), and the third from Baudracco-Arnas (1995). Both yield and quality of DNA extracted from fresh or frozen melon leaves were assessed. Average yields were not high, approximately 30 µg DNA/g fresh weight. The protocols did not differ in yield or digestability of DNA, but the latter one resulted in DNA that contained minimal amounts of polysaccharide-like materials that interfered with gel electrophoresis, thus improving the clarity of our results. For Southern blots, about 3 µg DNA samples were restricted and run on 0.8% agarose gels.

Hybridization membrane. We have tried supported nitrocellulose, nylon and charged nylon membranes from a few manufacturers and under our conditions, the best results were achieved with charged nylon (Gene Screen Plus, DuPont).

Blotting, fixation and stripping protocols. After trying a few protocols, we currently use capillary alkali blotting. DNA is fixed by the alkali treatment, followed by air drying. Resulting blots can be conveniently stripped for repeated use by boiling 0.1% SDS treatment.

Hybridization parameters were systematically evaluated in order to increase sensitivity and decrease "dirty" background. Currently our hybridization is performed in 6% PEG, 5% SDS, 5xSSPE and 50 µg/ml salmon DNA. Buffer (20 ml) is added to a 20 x 20 cm blot, with 25 ng labeled probe (labeled with 4 ml α^{32} P-dATP by the random hexamer method according to Bohringer Mannheim protocol), in a hybridization oven. Counts were checked and a typical autoradiogram exposure was 3 - 7 days with 2 intensifying screens.

A.3. Extent and usefulness of RFLP polymorphism in *C. melo*

RFLP probes were assayed on Southern blots containing DNA from 8 *C. melo* accessions, digested with 6 restriction enzymes: EcoRI, EcoRV, HindIII, DraI, XbaI and MvaI (an isoschyzomer of BstNI). Blots showing RFLP patterns with cucumber cDNA probes are shown in Fig. 1. Table 1 summarizes the results obtained with 25 genomic clones and 34 cDNA clones. Of the 25 genomic clones surveyed, 3 were copies of a repetitive element. Out of 23 different clones, 19 (83%) were polymorphic among 8 varieties (i.e. at least one variety could be differentiated from the rest). About 48% of the probes detected polymorphism between the two accessions used in our laboratory as mapping parents, namely TM (Topmark) and MOM (PI 414723). Out of 33 different cDNA probes scored with 6 enzymes, 27 (82%) were polymorphic among at least 2 of the 8 varieties, and 21 (64%) were polymorphic between the mapping two parents. We observed that most of the polymorphic probes detected polymorphism with a single restriction enzyme, suggesting that most DNA polymorphism in melon results from nucleotide substitutions.

Only a fraction of the polymorphic probe x enzyme combinations were graded "very good" for mapping (Table 1). Some of the polymorphic patterns considered less useful for mapping consisted of presence / absence of a band (e.g. the XbaI marker with probe AC-10, Fig. 1B), exhibiting dominant inheritance at the F2. Other patterns could be distinguished by small differences in the migration of allelic bands (e.g., the slight difference between the heaviest DraI bands of MOM and TM in Fig. 1B), rendering their accurate scoring in a segregating population difficult. The MvaI differences between MOM and TM in Fig. 1A and 1B are examples of fully-useful polymorphism. Clones of melon genomic DNA from the PstI probe library revealed a smaller proportion of mappable polymorphism than the cucumber floral bud cDNAs. In addition, repetitive clones were quite frequent among the PstI genomic clones. Even when polymorphic, they were hard to score, and they also reduced the efficiency of sampling new markers from the probe library. Although cDNAs that represent abundant species may also be sampled repetitively, only 1 out of 34 cDNA clones sampled turned out to be redundant. When used at moderate stringency, all the cucumber clones produced clear and strong patterns on melon genomic blots.

A.4. RAPD markers for mapping

RAPD reactions were performed at Bar Ilan using random decamers (Operon Technologies, Alameda - A, B, C, D, L and R primer-sets), according to standard protocols. At Cornell, RAPD primers from British Columbia University were used.

We first analyzed RAPD patterns in the two mapping parents of this study, cultivar Topmark (TM) and the Indian var. momordica (MOM). One hundred and fifteen decamer

primers were tried with these two samples. Almost all yielded clear banding patterns that were replicated at least twice; 61% of the primers detected polymorphism (Table 2). The proportion of polymorphic bands computed for all primers was 24% when only strong bands were considered. This result indicates that RAPDs are an abundant source of polymorphic markers in melon.

The reproducibility and consequently, the credibility of RAPD patterns obtained in different experiments have often been questioned. Some researchers have observed that even minor changes in the protocol or chemical batches may result in different patterns. Since our project required integration of data from two laboratories, we systematically examined this question by replicating some RAPD data in both labs. Eight primers used in the US were sent to Israel and RAPD reactions were run using a common subset of 8 varieties. Photographs of gels run at both labs were compared. The major bands were the same, but many minor bands were scored differently. Most of the discrepancies resulted from minor bands present in the IS pattern and missing in the US patterns. The two labs used different DNA batches, enzyme sources and Thermal cyclers.

Our conclusion is that when attempting to reproduce RAPDs, the chance of introducing significant errors can be reduced by scoring only major bands that are consistently present. Both labs should standardize RAPD yield with respect to the number of bands obtained per primer. In order to make sure that negative scores do not result from poor amplification, bands missing from a weak amplification profile should not be considered absent. If minor bands are of interest, our results indicate that they should be probably converted to single-locus PCR products defined by sequence characterized amplified regions (SCARs) before using them in other genetic backgrounds, other labs etc.

B. DNA VARIATION IN THE MELON GERMPLASM

B.1. Survey of 13 accessions using RAPDs

The intra-specific botanical classification of *Cucumis melo* dates back to 1859 but to date remains rather unclear and controversial. One of our objectives was to clarify this situation by providing molecular fingerprinting data to augment previous classification schemes based strictly on the dramatic morphological variation seen within this species. This study was also intended to provide information that could identify cultivar groups, or geographical sites, where such variation is particularly abundant. As a first attempt to evaluate the extent of polymorphism in melon germplasm, we selected a panel of 13 melon accessions and one *Cucumis metuliferus* accession as an outgroup (see Fig. 2 legend). We included representatives of all the main groups that some consider to correspond to botanical varieties, following the classification of Munger and Robinson (1991).

A total of 176 RAPD bands, produced with 18 primers, were scored. Fig. 2 shows an example of such a gel. Only 5 bands were monomorphic. Seventy five bands were

polymorphic between the outgroup *C. metuliferus* and *C. melo*. Within *C. melo*, 96 bands (54.5%) were polymorphic (Table 2). Using parsimony analysis and distance-matrix-based analysis (Swofford 1993; Felsenstein 1993), we derived phylogenetic trees such as the one shown in Fig. 3. As expected, the longest branch separates *C. metuliferus* (the outgroup species) from all melon accessions. The next node separates MOM (var. *momordica*) from the rest. Other well diverged taxa are AGR (var. *agrestis*, wild small fruited melons of Asia and Africa) and the cluster of non-sweet varieties from the Far East: CHI (var. *chito*), DUD (var. *dudaim*) and CON (var. *conomon*). The relationships among the rest of the taxa were not consistent, although the distance values indicate that var. *flexuosus* (FLX; non-sweet snake melons) and the mildly sweet Indian PI 124111 derivative (YC) are more distant from the Western dessert cultivars. The two major groups of dessert melon are the crisp fleshed types var. *inodorus*, including Cassaba and Honeydew melon that lack aroma and exhibit slow, non-climacteric ripening (HON, CAS), and var. *cantalupensis*, represented by netted soft orange fleshed types, (OGN, END, TM) and Charentais (CHA). These two groups could not be separated by our present data suggesting that some of the most important morphological distinctions between fruit characters may not be reflected by large changes at the DNA level. Adding more RAPD bands to the data set did not resolve these groups. The significance of this phylogeny is being further evaluated by a more extensive geographical sampling of the germplasm (see below).

B.2. Survey of 70 accessions using RAPDs and other techniques

In order to examine the genetic variation in melons and compare the results to the geographic distribution, morphological characteristics and traditional botanical classifications, a larger survey was required (its size was limited by our capacity to process fingerprinting reactions).

We grew a set of about 70 accessions from 25 countries in a field plot near Rehovot, Israel in May-August 1995. Seeds came mainly from the IPK, Gatersleben (Courtesy of Dr. Klaus Hammer) and the PI Station at Ames Iowa (Courtesy of Ms. K. Reitsma). Samples were also provided by cucurbit breeders in Israel, US and Spain. Representatives of the typical Casaba and cantaloupe varieties and the main subspecific groups were included, as well as a number of accessions from Africa, South and Central Asia, and the Far East (Fig. 4). Morphological records and photographs were taken, and fruit characteristics were examined (see below). Leaf material for DNA extraction was collected.

The laboratory at Bar-Ilan started the fingerprinting work in summer, 1996 and we plan to gather and analyze the data by fall 1997. RFLP is inappropriate for varietal fingerprinting. RAPDs worked, but were too slow and seemed to introduce "noise" in the form of ambiguous scores. We will use RAPDs but also will compare them to more novel techniques including a method based on using simple sequence repeats to anchor

polymerase chain reaction amplification of polymorphic regions (Inter-SSR PCR). This method detects variation within and between microsatellite repeats using PCR primers that correspond to the repeat itself (Zietkiewitch et al. 1994, Gupta et al. 1994). A second method that should be explored is the use of amplified fragment length polymorphism (AFLP) (Vos et al. 1995). Each of these will be tried also on a segregating F2 population to assess the relative efficiency of the methods for our second objective, namely genetic mapping. We have already adapted for our use the Inter-SSR method with rather promising preliminary results in terms of ease and amounts of variation. Inter-SSR primers from British Columbia University were used. Two variants of the technique were tried: radiolabeled PCR run on sequencing gels that yielded numerous bands per reaction, and non-labeled agarose gel detection with fewer bands. In the latter case, patterns resemble RAPDs with respect to complexity and amount of variation, but because they are produced at more stringent temperatures, they may be more reliable for phylogenetic or linkage analysis (Fig. 5).

B.3. Variation in fruit sugar metabolism

Melon breeders classify cultivars mainly with respect to fruit morphology and physiological criteria. Do these traits reflect true genetic distances between varieties? What are the most reliable traits to be used as the basis for phylogenetic relationships within *C. melo*? What traits represent milestones in the domestication of this species? Since the fruit of the plant is the agricultural commodity and a physiological and economically important aspect of the fruit is its sugar content, we examined the variation in fruit sugar metabolism in a sample of over 60 varieties in collaboration with Dr. A. Schaffer from ARO (Volcani Center). Examining genetically determined variability in melon fruit sugars is clearly of central importance for breeding programs and may also provide physiological insight for a most important trait, fruit taste and quality. Towards this end, approximately 3 mature fruits per accession were sampled, tasted, and the following measurements and assays were performed: total soluble solids (TSS) and pH of flesh juice; HPLC quantification of extracted flesh sugars; acid- and basic-invertase activities. As expected, a very large variation in sucrose content was observed between accessions that ranged from very nearly 0 mg/gfw in some *agrestis* and *flexuosus* accessions to ~70 mg/gfw. We also detected significant variation in reducing sugars, glucose and fructose. Usually sucrose accounts for about a half of the total sugars in sweet varieties, but a few accessions with higher hexose sugars were also encountered. This variation should be further evaluated because it undoubtedly reflects significant physiological and biochemical differences that relate to commodity quality. It was interesting to note that sweet varieties are found also in exotic germplasm, and not only in the *cantalupensis* and *inodorus* groups. Fig. 6 is a scatter plot of mean total sugar content per fruit versus pH. Accessions with pH<5 usually had sugar

levels <30 mg/gfw. Interestingly, there are a few mildly acidic types with high sugars. The varieties with intermediate pH (5-6) are heterogeneous with respect to sugar levels, but those with high pH (>6) include mostly sugary ones. The reasons for the apparent correlation between pH and sugar (and the possibility to by-pass it) are unknown and may be worth pursuing.

Another physiological correlation that had been studied in a few selected varieties was between acid invertase and sucrose content of fruit (Schaffer et al. 1987). From this study, it appeared that sucrose accumulation is a function of a decrease in acidic invertase, and the final sweetness correlated to the time lapse between invertase decrease and fruit dehiscence/ harvest. The distribution of sucrose versus invertase in our varietal survey (Fig. 7) generally supports the hypothesis that sucrose can accumulate only in low-invertase fruits. However, many fruits with decreased invertase do not accumulate sucrose. The decrease in invertase may be therefore necessary but not sufficient for the process.

Prospects for future experiments. We grew a partial replicate of the varieties for the sugar survey in the field in 1996 to assess the reproducibility of the observed variation in a second season. We included all the exceptional accessions, and representatives of the more typical ones. We also examined a few F1 hybrids between sweet and non-sweet varieties. Results from this work suggest that a more extensive test of F1 populations may suggest whether distinct sugar profiles could be combined and whether sweetness genes from diverse genetic backgrounds may be different. Based on our preliminary results, it appears that may be distinct "sweetness genes" with additive genetic relationships between them. The implications for breeding and commodity quality may be very significant. Also, as has already occurred in species for which molecular maps are well-developed, e.g., tomato, these kinds of characters have proven extremely amenable to genetic analysis (analysis of the quantitative trait loci, QTL). As molecular mapping work progresses, this phenotype may represent one of the most interesting to pursue once a denser map is available. For this purpose, we have produced 58 different F1s including 25 sweet x sweet, 3 nonsweet x nonsweet and 30 sweet x nonsweet combinations during summer 1996, including also diverse sucrose:hexose ratios from various genetic backgrounds.

B.4. Variation in Genetic Potential for Regeneration in vitro

The regeneration response of melons in tissue culture is important for biotechnological improvement of this crop, and extreme variability with respect to this capacity exists in the species. We asked whether the capacity to regenerate in vitro correlates with the phylogenetic groups of melons, i.e., are there groups or types that are clearly better or worse than others. For obvious reasons, most of the existing work has focused on a few commercial cultivars so we checked also the more exotic groups. In collaboration with Dr.

V. Gaba at ARO (Volcani Center) we have set up a regeneration experiment from cotyledon explants. The first experiment surveyed 8 varieties that represented the major sub-specific groups within the species. We examined regeneration initiation from explant edge, callus formation and plantlet formation on 3 different media. Differences between cultivars were quite large, and all the variables checked (cultivar, medium, seedling stage and explant position) appeared to contribute significantly to the variance in the response. A simpler experiment with only one medium and one seedling-stage was devised based on results from the first experiment, and more cultivars were included to assess whether the variation correlates with some phylogenetic pattern. This experiment has not been completed yet, and we plan to finish and analyze its results during early 1997.

C. MAPPING of RAPD and RFLP markers

C.1. The F2 mapping population

PI 414723 (var. *momordica*, MOM) and cv. Topmark (var. *cantalupensis*) were selected as the mapping parents for this project. The former has been used by breeders as donor of several resistances, while Topmark is a popular recurrent parent in breeding cantalopes for both North America and increasingly, for European markets. Based on previous work they were known to be genetically distant. The parental line of PI 414723 had been selfed 4 generations with selection for resistance to powdery mildew, ZYMV and WMV. The F1 between this parent and Topmark was obtained at Cornell. F1 plants were grown and self-pollinated at the Weizmann Institute. In 1994, we grew 150 F2 individuals (seed sample #2003) derived from a single selfed F1 fruit. Leaves (10-20 g) were harvested and stored from 125 plants, and F3 seeds were obtained from 98 plants. F3 seed samples (40 seeds each) were sent to Cornell for the virus resistance tests.

The PI 414723 accession had been reported to harbor a variety of resistance genes (McCreight et al. 1992). With the help of Prof. Yigal Cohen at Bar-Ilan we checked whether our specific parental line expressed additional resistance genes. Resistance of PI 414723 to 4 races of *Fusarium* and 3 races of downy mildew was checked at seedling stage, but no resistance was observed, while resistance to powdery mildew races 1 and 2 was confirmed. Topmark was susceptible to all the pests mentioned above.

It has also been reported that the same parent carries aphid resistance, so attempts were made in the U.S. to develop a method of evaluating levels of aphid resistance that would be suitable for use in a mapping study. In Israel, natural infestation with *Aphis gossypii* occurred in the growth room, and it was observed that PI 414723 became infested somewhat later than other material. In the U.S., we tried various methods to grow plants in cages, infest them with a small defined population of aphids, and observe statistically significant differences in levels of either colonization or survival. While we observed that PI 414723 generally supported many fewer insects consistent with the field observations upon

which the initial report was based, we were unable to work out a screen that we felt would reliably reflect individual plant phenotypes for this character.

C.2 Scoring morphological traits at the F2

The parental lines differed with respect to many aspects of plant morphology which were segregating and scored in the F2. These phenotypes may be mapped as simple or quantitative traits once the map based on this population includes sufficient markers so that we are likely to see linkages emerge. Plant morphology was scored following the descriptor lists found in the Genetic Resources of the Cucurbitaceae, IBPGR report (1983), edited by Esquinas-Alcazar and Gulick. The following traits showed segregation in the F2 and were scored: Hairiness (at leaf petiole), sex of flowers (andromonoecious versus monoecious - inherited as expected as a single gene with monoecy fully dominant), shape of ovary, immature fruit color, mature fruit color (external, flesh and jelly); fruit size, shape (these traits are problematic in greenhouse, hand-pollinated plants); design on fruit skin; appearance of net on fruit skin; fruit bursting at maturity. In general, segregation appears skewed in favor of the MOM morphology in many traits and we did not attempt to study the mode of inheritance of any of the traits (except for verifying monoecy gene inheritance).

C.3. Disease resistances scored at the F3

All available F3 families were scored for resistance to three viruses and powdery mildew. Sets of at least 16 seedlings per family were grown in individual pots and inoculated at the seedling stage with one of the three viruses, papaya ringspot virus, watermelon mosaic virus or zucchini yellow mosaic virus. Mildew occurs in our greenhouse if plants are not protected, and once the plants were established and viral phenotypes had been determined, we allowed mildew infestation to proceed, and made a determination as to whether a population was segregating or fully susceptible or resistant. Our results indicated linkage between the WMV and ZYMV resistance loci (see Fig.8), with a recombination fraction of about 5%. The resistances to PRSV and PM-1 segregated independently from the other traits.

C.4. Scoring RAPD and RFLP markers at the F2

During the third year of the project, several RAPDs and RFLPs that produced clear differences between the parental lines were scored for each of the F2 individuals in the mapping population. The rate of analyzing markers on the F2 was rather modest for the following reasons: 1. it took us a long time to optimize the RFLP technique; 2. many markers, both RAPDs and RFLPs have shown extremely skewed segregation ratios, and they are not contributing to the linkage analysis; 3. a serious health problem occurred beginning in Sept., 1995 for the Ph. D. student who worked at Cornell on the project and

who was supposed to carry out much of this work. This problem has resulted in a permanent and complete disability that has affected even his ability to manage the work he had already accomplished. Table 3 indicates the markers scored and the ratios between parental classes for each marker. Chi-square tests for the segregation of each marker with respect to the expected 3:1 or 1:2:1 model were run using the SAS program, and are given in Table 3. Analysis of the data using MAPMAKER is under way. An initial assignment of some of the markers to linkage groups is shown in Fig. 8 and Table 3. Many of the markers appear linked to Group 1 that includes the sex gene A. While this group suffered from severe skewing that prevented accurate analysis of most of them, a few of the RAPD markers were shown to be indeed sex-linked using another population (see below).

C.5. Targeting selected loci by bulked segregant analysis

As a complementary strategy to the random scoring of markers in the F₂, we have also performed bulked segregant analysis (Michelmore et al. 1991). The segregation data for resistance to WMV, ZYMV and PM race1 were used to generate pools of DNA samples from resistant versus susceptible individuals, and such pools were screened for diagnostic RAPDs that are likely to be linked to the trait that differentiates the pools. We also compiled segregation data at the F₂ (with partial F₃ data) for the sex gene A (A- monoecious, aa andromonoecious). Because very few individuals of the mapping population displayed the aa phenotype, we used pools from a separate F₂ population (from the same parents) where the sex genotypes did not deviate from the expected 3:1 ratio. We have thus assembled 3 pairs of pools: one for virus resistance with 5-8 individuals scored as homozygous resistant (i.e. uniformly resistant in the F₃) to both ZYMV and WMV which are linked traits, and 5-8 F₂ individuals whose progeny was uniformly susceptible to both viruses. The other 2 pairs were for resistance / susceptibility to powdery mildew race 1 and for the sex determination locus A. Sets of 8 RAPD reactions (two parents and six pools) were run with 70 decamer primers that were polymorphic between parents. In about 12 cases, a band appeared to correlate with a pool. The next step was to run individual PCR reactions for all the samples included in the pools that exhibited the difference. Here for reasons that we could not explain, many of the differences were not confirmed by the individual tests. In case there was correlation between the individual reactions and the pools, we ran additional individuals from the F₂ and a few of the putative-linked markers could be discarded at this stage. The yield of the pooling screen was lower than one would expect from theoretical consideration on the probability of hitting a single genomic target by scoring 70 primers (~100 polymorphic bands). We ended up with no virus or PM-linked RAPDs but with 3 sex linked bands that mark the a allele and 1 band that marks the A allele. We are presently cloning these bands and converting them to SCARs to test their usefulness in breeding. We will also try to map them more precisely with respect to the A locus.

C.6. Generation of a RILs population for future mapping

The present mapping project was based on F2 DNA and seed samples, which cannot be permanently maintained and therefore are limited with respect to the number of characters that can be mapped and the amount of DNA per mapping individual that will be available. The technical problem of storing large samples of leaf material, the low yields of DNA, as well as the limited number of F3 seeds obtained, convinced us that it was necessary to develop a population of RILs (Recombinant Inbred Lines). This consists of a set of homozygous lines derived by 6-7 selfed generations from F2 individuals. Such a collection can then be propagated and distributed, and experiment requiring replicated trials (e.g. QTL mapping) or additional seed samples, can be performed. We have thus carried out and nearly finished - the series of self-crosses starting from the mapping individuals. Additional individuals from the same F2 seed sample (# 2003) and individuals from samples # 2014, #2002 and #2022 were used to obtain a larger set of lines, and compensate for segregation distortions observed in sample 2003. We have right now 56 lines at the F4 seed stage, 82 lines at the F5 seed stage and 32 lines from which we obtained F6 seeds. We may project therefore to have by fall 1997 100 -120 lines at the required F6 or F7 stages.

DESCRIPTION of COOPERATION

This project was conceived while Dr. Perl-Treves was at Cornell and population development and accession selection for the surveys were done collaboratively during the time the Pis were together. When Dr. Perl-Treves returned to Israel, we planned that the field components and quality characters would be done in Israel while disease screening and optimization of DNA isolations were to be done at Cornell. Both labs were to be involved in generating the data involved in the variability studies and in the mapping. The collaboration progressed as planned, with a visit in November, 1994 from Dr. Perl-Treves to Ithaca and very regular email exchange with one major difficulty. The graduate student who was the person supported by this grant and who was responsible for carrying out all of the experiments in the lab and greenhouse in the US became acutely mentally ill in Sept., 1995 and was involuntarily hospitalized in January, 1996. With support of the professionals involved in treating him, we tried to salvage as much as possible from his work, but he departed the country in November, 1996 and we have been told this will be a permanent condition. Despite this very unfortunate development and the technical problems in working out protocols for melon and with the skewing in the mapping population, we have prepared two manuscripts that will be submitted for publication very soon.

EVALUATION of RESEARCH ACHIEVEMENTS

This project was meant to set up the basis for using molecular markers in an important crop that had not been subjected previously to such approach. At the time we

started we did not have any markers, maps or hands-on experience with melon genome analysis. As a result of this specific project, both laboratories have gained the necessary experience and tools to continue and use markers for diverse applications, both for research and for breeding purposes. From this perspective this has been a very successful project. Sets of RFLPs and RAPDs have been developed and tried and protocols for reliable DNA extraction, RFLP and RAPD analysis have been optimized. We have measured the amount of DNA variation that may be detected in the melon germplasm; and we can undertake further mapping projects with a realistic assessment of the amount of labor, cost and time that will be required, and the kind of genetic material that we may need, all based on insight from this work. The objective of attaining a linkage map has not been reached so far but work towards this goal is progressing steadily. It is hoped that Dr. Perl-Treves will continue in melons, while Dr. Kyle's commodity responsibilities have been shifted by staff changes due to retirements. This project has contributed to the global effort in *C. melo* and if Dr. Perl-Treves and his colleagues receive funding to continue, it will be an excellent example of international coordination and cooperation in the genome analysis of a very valuable crop that is often somewhat marginalized for research.

Following is a summary of the on-going and planned activities that are projected from the present program:

1. Score more RAPD and RFLP markers on the F2 during 1997.
2. Analyze an expanded variability survey of the melon germplasm, with emphasis on botanical classification and trial of novel fingerprinting techniques.
3. Complete the preparation of a mapping population of recombinant inbred lines.
4. Tag the sex-locus A with SCAR markers.
5. Complete the studies on variation in sugar composition and regeneration responses in the melon germplasm.
6. Set up a framework for future collaboration in mapping of melons between our project and the following laboratories involved in mapping of Cucumis: N. Katzir at Newe-Yaar (Katzir et al. 1996); J. Staub from Wisconsin (Kennard et al. 1994); and M. Pitrat at Monfavet (Baudracco-Arnas and Pitrat 1996). The latter has published recently the first map of melon using a pair of mapping parents from different backgrounds than ours, with different agronomic traits. It would be very productive to relate the mapping data we develop with his, and with data developed in the two other labs, in order to join efforts and gain mutual access to the markers mapped by each group.

CONCLUSIONS

1. The methods for RFLP and RAPD mapping in melon have been worked out. Sets of polymorphic markers among melon accessions were developed. Both genomic and

cucumber cDNA probes worked well. Amounts of variation are workable, although faster techniques should be sought. Pairs of mapping parents may be selected among accessions that belong to different groups or types of melons, but not from the same group. A major technical difficulty with RFLPs was the low DNA yield from leaves while progress with RAPDs was slowed down because it was often necessary to replicate results for verification.

2. Mapping progress was rather slow due to technical problems and to frequent segregation distortion. The first set of about 40 markers has been scored on the F2 progeny. The DNA pooling approach did not work very well in our hands but sex-linked markers have been developed.

3. Resistances to WMV and ZYMV were genetically linked. Monogenic resistances to PM race 1 and to PRSV were scored in the population and were unlinked to the other traits.

4. The melon germplasm harbors extensive variation in morphology, fruit sugar metabolism and other characteristics and it is interesting to try and relate it to a molecular phylogeny based on DNA fingerprints.

Publications related to this project

Proceedings Papers

Kovalski I, Silberstein L, Huang R, Perl-Treves R. (1995) Development of molecular markers for genome analysis in *Cucumis melo*. In: Cucurbitaceae'94, Proceedings of the Cucurbitaceae Conference, South Padre, Texas, 1-4 November 1994, Lester G and Dunlap J (Eds) p. 191-195

Kyle, M.M. (1995) Breeding cucurbits for multiple disease resistance. In: Cucurbitaceae'94, Proceedings of the Cucurbitaceae Conference, South Padre, Texas, 1-4 November 1994, Lester G and Dunlap J (Eds) p. 55-59

Gaba V, Elman C, Perl-Treves R, Gray J. (1996) A theoretical investigation of the genetic variability in the ability of *Agrobacterium* to transform *Cucumis melo* L. In: Proceedings the VI Eucarpia Meeting on Cucurbit Genetics and Breeding, Malaga, Spain 28 - 30 May 1996 p. 172 - 178

Meeting Abstracts

Silberstein L, Kovalski I, Huang R, Perl-Treves R (1996) Development of RAPD and RFLP markers for genome mapping in *Cucumis melo*. Annual Meeting of the Israeli Botanical Society and the Israeli Society for Plant Molecular Biology and Plant Tissue Culture, Beer Sheva 28.2.1996

Anagnostou, K., M.M. Kyle and R. Perl-Treves. 1996. Genetic relationships among resistance to zucchini yellow mosaic virus, watermelon mosaic virus, papaya ringspot virus and powdery mildew in melon (*Cucumis melo* L.). HortScience .

Journal Papers

Munger, Y. Zhang, S.L. Fenton and M.M. Kyle. 1995. Leaf blower adapted for large scale inoculation of plants with mechanically-transmitted viruses. HortScience 1266-1267.

Anagnostou K, , Kyle M, Perl-Treves R. Genetic relationships among resistance to zucchini yellow mosaic virus, watermelon mosaic virus, papaya ringpot virus and powdery mildew in melon (*Cucumis melo*). To be submitted soon

Silberstein L, Kovalski I, Huang R, Anagnostou K, Kyle MM and Perl-Treves R. Molecular variation in *Cucumis melo* as revealed by RFLP and RAPD markers. To be submitted soon

Stephanski A, Kovalski I, Schaffer A, Perl-Treves R. Patterns of variation in sugar metabolism within the *Cucumis melo* germplasm. In Preparation

CITED REFERENCES

Baudracco-Arnas S (1995) A simple and inexpensive method for DNA extraction from *Cucumis melo* L. Cucurbit Genet Coop Reports 18: 50-51

Baudracco-Arnas S, Pitrat M (1996) A genetic map of melon (*Cucumis melo* L.) with RFLP, RAPD, isozyme, disease resistance and morphological markers. Theor Appl Genet 93: 57-64

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA miniprep: version II. Plant Mol Biol Reporter 1: 19-21

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Gupta M, Chyi Y-S, Romero-Severson J, Owen JL (1994) Amplification of DNA markers from evolutionarily-diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89: 998-1006

Katzir N., Danin-poleg Y., Tzuri G., Karchi Z., Lavi U. and Cregan P.B. (1996) Length polymorphism and homologies of microsatellites in several Cucurbitaceae species. Theor Appl Genet (in press)

Kennard WL, Poetter K, Dijknizen A, Meglic V, Staub JE, Havey MJ (1994) Linkage among RFLP, RAPD, isozymes, disease resistance and morphological markers in narrow and wide crosses of cucumber. Theor Appl Genet 89: 42-48

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76: 815-829

McCreight, J.D., Bohn, G.W., Kinshaba, A.N. 1992. "Pedigree" P.I. 414723 melon. Cucurbit Genet Coop Reports 15: 51-52.

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88: 9828-9832

Miki T, Yamamoto M, Nakagawa H, Ogura N, Mori H, Imaseki H, Sato T (1995) Nucleotide sequence of a cDNA for ACC synthase from melon fruits. Plant Physiol 107: 297 - 298

Munger, H.M., Robinson, R.W. 1991. Nomenclature of *Cucumis melo* L. Cucurbit Genet. Coop. Reports 14: 43-44

Rosenman N, Kahana A, Xiang Y, Perl-Treves R. Expression of Agamous-like genes in male and female flowers of cucumber. Proceedings the VI Eucarpia Meeting on Cucurbit Genetics and Breeding, Malaga, Spain 28-30 May 1996

Schaffer AA, Aloni B, Fogelman E (1987) Sucrose metabolism and accumulation in developing fruit of Cucumis. Phytochemistry 26: 1883-1887

Swofford, L. (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 21: 4407-4414

Zietkiewitch E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat anchor polymerase chain reaction amplification. Genomics 20: 176-183

Clone Type	# probes assayed	# different clones	Polymorphic (8 varieties)	Polymorphic (2 parents)	Rated good for mapping
Melon <i>Pst</i> I Genomic clones	25	23	19 (83 %)	11 (48 %)	6
Cucumber floral cDNA clones	34	33	27 (82 %)	21 (64 %)	14

Table 1. Extent of polymorphism detected with different types of RFLP probes between melon varieties. Each probe was reacted with DNA from 8 melon accessions digested with 6 restriction enzymes. Number (and percentage) of probes detecting an RFLP between any of the 8 varieties, as well as polymorphism between the two accessions used as mapping parents (Cv. Topmark and var. momordica) are shown. Number of RFLP probes revealing easily scorable, co-dominant patterns between the two parents are indicated as well.

	No. Primers Assayed	No. Primers Polymorphic	No. Bands Scored	No. Bands Polymorphic
Two-Parent Survey	115	70 (61%)	616	148 (24%)
13-Varieties Survey	18	18 (100%)	176	96 (54.5%)

Table 2. Levels of RAPD polymorphism, expressed as the number and percentage of primers and bands that detect differences between cultivar Topmark and P.I. 414723 ("Two-Parent Survey") and among 13 *C. melo* varieties ("13-Varieties-Survey").

Type	Locus	F2 segregation					Chi Square Test			Linkage Group
		A (like TM)	D (A or H)	H	C (B or H)	B (like MOM)	Exp. ratio	P. Chi Sqr.	Chi. Prob.	
RAPD	R-13	14			21		3:1	4.2	0.123	
	R-20a	7			14		3:1	0.78	0.68	
	R-20b		14			7	3:1	0.78	0.68	
	D-8	4			34		3:1	4.25	0.12	
	R-19		47			12	3:1	0.68	0.71	
	B-20a		7			4	3:1	0.76	0.68	
	B-20b		10			1	3:1	1.48	0.48	
	A-2a	22			88		3:1	1.47	0.48	
	A-2b		2			108	3:1	314	0.00	1
	A-10	32			79		3:1	0.87	0.65	2
	A-12a		63			23	3:1	0.14	0.93	3
	A-12b		7			79	3:1	205	0.00	1
	A-16		38			13	3:1	0.01	0.99	3
	A-18a		6			89	3:1	239	0.00	1
	A-18b	17			76		3:1	2.24	0.33	(1)
	C-2		6			93	3:1	251	0.00	1
	C-6		56			18	3:1	0.02	0.99	
	C-8	16			73		3:1	2.34	0.31	
	R-10	25			54		3:1	1.86	0.39	
	B-11		64			13	3:1	2.71	0.26	4
	D-13		2			51	3:1	143	0.00	1
	D-12a	7			1		3:1	16.67	0.00	
	D-12b		1			7	3:1	16.67	0.00	1
	R-2a		37			8	3:1	1.25	0.53	(7)
	R-2b	4			41		3:1	6.23	0.04	1
RFLP	ACS	18		43	9	23	1:2:1	0.64	0.89	(7)
	CAG-1	26	1	24	9	13	1:2:1	8.9	0.03	
	CAG-3	9	4	33	10	14	1:2:1	2.68	0.44	
	AC-7	28	1	47	2	24	1:2:1	0.58	0.90	
	AC-11	1		13	11	81	1:2:1	185	0.00	1
	PT-4	2	1	8	3	87	1:2:1	217	0.00	1
	PT-12	25	3	48	1	20	1:2:1	0.63	0.89	(2)
	PT-13	32	18	40	4	6	1:2:1	17.4	0.00	5
	AC-13		33			33	3:1	14.0	0.00	1
	AC-36	58	9	22	1	2	1:2:1	94.1	0.00	5
	PT-7a	10	9	49		30	1:2:1	9.99	0.02	
	PT-7b	24		25	29	24	1:2:1	7.25	0.06	4
	AC-15	21		36	9	18	1:2:1	0.36	0.95	
	AC-12	20	1	43	1	15	1:2:1	1.46	0.69	2
	AC-19	25	6	29	12	17	1:2:1	4.18	0.24	5
	CC-3	0		6	3	85	1:2:1	227	0.00	1
	AC-5a	17	1	55		13	1:2:1	7.7	0.05	
	AC-5b	21		45	12	14	1:2:1	2.48	0.48	4
	AC-8	24		33	5	21	1:2:1	2.08	0.56	3
	AC-39	22		49		21	1:2:1	0.41	0.94	3
TRAIT	AC-38	17	6	21	2	8	1:2:1	3.87	0.28	
	PMR-1	16	4	26	2	13	1:2:1	0.49	0.92	
	WMVel	14	1	33	1	12	1:2:1	1.14	0.77	6
	ZYVel	11	1	33	1	13	1:2:1	1.29	0.73	6
	PRSV	13		25		9	1:2:1	0.87	0.83	
	sex	3		20	90		3:1	23.5	0.00	1

Table 3. Segregation and linkage group assignment of genetic markers. Twenty four RAPDs, 21 RFLPs and 5 traits were scored in the F2. A- homozygous for the Topmark allele; B- homozygous for the momordica allele; H- heterozygous; C - either B or H genotype; D- B or H. Pearson chi square value and probability of agreement with a 3:1 or 1:2:1 segregation are shown. Tentative assignment to linkage group is given. Figures indicate linkage with LOD score ≥ 3 ; figures in parentheses - linkage with smaller LOD (≥ 2). RFLP markers coded PT are melon genomic clones; those coded CC or AC are cucumber cDNAs. CAG-1 and CAG-3 are cucumber cDNAs encoding Agamous homologs, ACS encodes a melon fruit cDNA for ACC synthase (Miki et al. 1995).

Fig. 1. Representative RFLP patterns of 8 melon varieties obtained with cucumber cDNA probes AC10 (panel A) and AC15 (panel B). Varieties are indicated by codes: HON - cv Honeydew (USA), TM - cv Topmark (USA), MOM - PI 414723 (India), DUD - var dudaim (Cornell line 85895; Far East?), CHI - var Chito (PI 140471; Far East?), CON - var Conomon (Cornell line 85893; Far East?), FLX - var Flexuosus (from Lebanon), AGR (var Agrestis (den Nijs; Africa?).

RAPD patterns of melon varieties

Fig. 2. RAPD survey of 13 melon varieties (and a *C. metuliferus* accession, designated C.MET) with decamer-primer OPB-6. Melon varieties are as in Fig. 1, and, in addition: CAS - cv Rochet Pamal (Spain), CHA - cv Charentais (France), YC - breeding line derived from PI 124111 (India), GAL - cv Galia (Israel), END - cv Ein-Dor (Israel).

Fig. 3. Phylogenetic tree computed by the Kitsch program, PHYLIP software package, displaying clustering relationships between 13 *C. melo* accessions and a *C. metuliferus* accession, based on a data set of 176 RAPD bands. Genetic distance values between all pairwise combinations of accessions were computed as the proportion of different characters of the total number of characters (missing values ignored) and these were used as input to the clustering program that searched for the shortest tree under a molecular clock assumption. Varieties are indicated as in Fig. 2.

Fig. 4. Great morphological variation in *Cucumis melo* fruits grown at Rehovot in summer 1995.

Fig. 5. Inter-SSR PCR patterns of melon varieties. Amplification was carried out using BC primer # 891 (sequence 5'-HVH (TG)₇-3'). Twenty seven amplification cycles with an annealing temperature of 45°C were performed with 32-P labeling. Products were separated in an acrylamide-urea gel. Varieties are as in Fig. 2, and, in addition: DUG - var Dudaim (from Georgia, IPK no. CuM-296), GIN - Ginsen Makuwa (from Japan, PI 420176), COV - var Conomon (from Vietnam, IPK no CuM-24?), FLR - var Flexuosus (CuM-349 from Irak), KAK - PI 164493 (from India), AGA - var Agrestis (CuM 246? from Afghanistan), HCR - cv Hilo Carrete (Spain), CCA - Amarillo with orange flesh (Spain), IML - PI 476342 from Kazakhstan, MEC - CuM 255 from China, BNJ - Burpee's Netted Gem (USA).

pH versus total sugars

Fig. 6. Scatter plot of fruit pH and total sugars (sucrose, fructose and glucose, mg/ g fresh weight, measured by HPLC) in a set of melon varieties. Dots represent averages from 3 fruits. While a correlation between pH and sugars is usually observed, a few mildly-acidic varieties with high sugar are indicated.

INVERTASE (pH5) versus SUCROSE

Fig. 7. Scatter plot showing relationship between sucrose level (mg/g fresh weight) and acid-invertase activity (expressed in mg glucose per g fresh weight per hr) in fruits from different melon varieties (measurements are averages from 3 fruits). Low invertase is probably a "requirement" for significant sucrose accumulation.

Fig. 8. Preliminary linkage groups displayed by the MAPMAKER program for an initial set of markers mapped in our F2 population. Group 1 includes the sex gene A, 4 RFLPs and 8 RAPDs; 4 of the latter were confirmed as sex-linked in a second population. Groups 2 and 5 include 2 RFLPs and a RAPD marker each. Group 3 has 2 RFLPs and 2 RAPDs, and Group 5 has 3 RFLPs. Group 6 has the 2 viral resistances (here both the phenotypic and ELISA scores are shown), and Group 7 includes a RAPD and an RFLP of the melon ACC-synthase clone. It is apparent that RFLP data (after omitting markers with very skewed segregation) fit more readily into linkage groups, i.e. are more informative than RAPD data.