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BARD

FINAL REPORT

PROJECT NO. IS-2708-96

**Map Merging and Homology Studies in *Cucumis*
*species***

N. Katzir, R. Perl-Treves, J.E. Staub

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BARD Final Scientific Report

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Map merging and homology studies in *Cucumis species*

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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
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Cooperation Summary (numbers)

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Cooperation: briefly explain whether synergistic, complementary or supportive.

The cooperation and links developed between all four partners were most fruitful, with mutual visits and continuous correspondence and discussions. There was synergistic cooperation, especially in the development and application of the SSR markers in all the mapping projects. Details of the cooperation are described below.

Project title: Map merging and homology studies in *Cucumis* species

Abstract

List of original objectives

- (1) Construct a saturated map of melon, using RFLP, SSR, RAPD and Inter-SSR genetic markers.
- (2) Study the homology between the genomes of cucumber and melon.
- (3) Add to the *Cucumis* map, biologically important genes that had been cloned in other plant systems.

Background

Cucumber and melon are important vegetable crops in Israel and the US. Genome analysis of these crops has lagged behind the major plant crops, but in the last few years genetic maps with molecular markers have been developed. The groups that participated in this program were all involved in initial mapping of cucurbit crops. This grant was meant to contribute to this trend and promote some of the more advanced applications of genome analysis, i.e., map saturation and comparative mapping between cucurbit species.

Major achievements

The main achievements of the research were (a) the construction of melon maps that include important horticultural traits and Resistance Gene Homologues, (b) the development of approximately 200 SSR markers of melon and cucumber, (c) the preliminary map merging of melon maps and of comparative mapping between melon and cucumber.

Implications

As a result of this program, we have a good estimate of the applicability of different types of markers developed in one cucurbit species to genetic mapping in other species. Since the linkage groups of melon and cucumber can now be related to each other, future identification of important genes in the two crops will be facilitated. Moreover, the further saturation of the maps with additional markers will now allow us to target several disease resistance loci/horticultural traits for marker-assisted selection, fine mapping and positional cloning.

Achievements

The major objective of the proposal was the construction of a detailed map of melon (*Cucumis melo* L.). The achievement of this goal was planned through the development and merging of individual maps. Two maps were constructed as planned, using SSR, ISSR, RFLP and RAPD markers. One map was constructed at BIU using an F₂ progeny from the cross *C. melo* PI 414723 x Cv. Topmark. This map that began as part of a previous BARD project (IS-2129-92) and includes 110 markers, resistance to *Aphis gossypii* and several Resistance Genes homologues (Brotman et al. 2000 and Klingler et al 2000, Appendices E and F). The second map was constructed at Newe Ya'ar from a cross between PI414723 and Dulce. This map includes 22 SSR, 46 RAPD, 2 ISSR markers and 4 horticultural markers [female flower type (*a*), Fusarium resistance, striped epicarp (*st*), and fruit flesh pH (*pH*)] that were analyzed in an F₂/F₃ population to produce a map spanning 14 linkage groups (Danin-Poleg et al. 2000c Appendix C). Currently the two maps are being further saturated with additional markers SSRs, horticultural traits and R-Gene-Homologues.

The above two maps, as well as additional two maps developed by a French group and a Spanish group (headed by M. Pitrat and P. Arus, respectively), have been primarily merged using a common set of primers, (Brotman et al. 2000 and Danin-Poleg et al. 2000a, Appendices A and E). This was the first attempt to merge melon maps in order to achieve a reference map for melon. The primers used as anchor points for merging were mainly SSR markers developed through this BARD project. At Newe Ya'ar, 100 SSR markers have been developed and a similar set has been developed at Wisconsin University. The potential of SSR markers as anchor points for melon map merging and for comparative mapping with cucumber was demonstrated (Danin-Poleg et al. 2000 a and b, Appendices A and B). Mapping of all the available SSRs will enable further merging and comparative studies.

The comparative study of melon and cucumber was the second objective of the project. The US investigators have been developing a detailed cucumber map. The intensive work performed in cucumber is described in Appendix H. The feasibility of comparative mapping between the two *Cucumis* species has been demonstrated (Danin-Poleg et al. 2000 a and b, Appendices A and B). Conservation of SSR loci between melon and cucumber was proven

by sequence comparisons (Danin-Poleg et al. 2000 b). Common SSR markers are currently being mapped in cucumber at Wisconsin and in melon at Newe Ya'ar. It is expected that this comparative study will be completed within one year. In addition, a considerable number of sequence characterized cDNAs and genomic fragments of melon and cucumber has accumulated at BIU. Mapping these clones in both species will continue, and hopefully complement comparative mapping between the two species.

The third objective of the project was the mapping, on the *Cucumis* map, of biologically important genes that had been cloned in other plant systems. The BIU group has been adding Resistance Gene Homologues to the melon map (Brotman et al 2000, Appendices E and G). These include genes from two gene families: NBS-LRR and *Pto*-kinase. In addition, many of the RFLPs on the map have been sequence-characterized.

To summarize, the main achievements of the research were (a) the construction of melon maps that include important horticultural traits and Resistance Gene Homologues, (b) the development of approximately 200 SSR markers of melon and cucumber and (c) the preliminary map merging of melon maps and of comparative mapping between melon and cucumber. The significance of these scientific achievements stems from the strength of saturated maps as most important tools for both marker assisted selection (MAS) and map based cloning. The power of the two applications has been proven in recent years for several crops. The achievements of this BARD project have contributed most significantly for these applications in the two important *Cucumis* crops.

Details of cooperation

The cooperation and links developed between all four partners were most fruitful. The major cooperation was the common development and application of the SSR markers. These most valuable markers were first developed for *Cucumis* through this project. The 200 markers developed are currently the only available *Cucumis* SSRs. We have shown in these three years that the use of SSRs is an attractive technology that can be transferred across mapping population. The application of this common set of markers was a most effective link among

all participating research groups, in addition to the other major groups involved in *Cucumis* mapping: the French group headed by M. Pitrat and the Spanish group headed by P. Arus. Gennaro Fazio, a Ph.D student from Jack Staub's lab, visited Nurit Katzir's lab to learn the technology of SSR markers, including their development and application, as described in the appendix H. Dr. Leah Silberstein from R. Perl-Treves lab came to Newe Ya'ar and mapped a set of SSRs on the BIU map. All four participating authors met twice during the time of this project: once at Cucurbitaceae 1998, in Pacific Grove, CA, USA, and once at Cucurbitaceae 2000, in Ma'ale HaHamisha, Israel. During these meetings there were fruitful discussions of research plans. In addition, Nurit Katzir visited Jack Staub's lab in January 1998.

List of Publications

Brotman, Y., Silberstein, L., Kovalski, I., Klinger, J., Thompson, G., Katzir, N. and Perl-Treves, R. (2000). Linkage groups of *Cucumis melo*, including resistance gene homologues and known genes. (eds. N. Katzir and H.S. Paris). Acta. Hort. 510: 441- 448.

Danin-Poleg, Y., Reis, N., Baudracco-Arnas, S., Pitrat M. Staub J. E., Oliver M. Arus P. and Katzir, N. (2000a). Simple Sequence Repeats in *Cucumis* mapping and map merging. Genome (in press)

Danin-Poleg, Y., Reis N., Tzuri G., and Katzir N. (2000b). Development and Characterization of microsatellite in *Cucumis*. Theor. Appl. Genet. (in press).

Danin-Poleg Y., Tadmor Y., Tzuri G., Reis N., Hirschberg J. and Katzir N. (2000c). Construction of a genetic map of melon with molecular marker, horticultural traits and ZYMV resistance (Submitted to Mol. Breed.)

Danin-Poleg, Y., Tzuri, G., Reis, N., Karchi, Z. and Katzir, N. (2000d). Search for molecular markers associated with resistance to viruses in melon. Cucurbitaceae 2000: the 7th Eucarpia Meeting on Cucurbit Genetics and Breeding (eds. N. Katzir and H.S. Paris). Acta. Hort. 510: 399-403.

Klinger, J., Kovalski, I., Silberstein, L., Thompson, G.A. and Perl-Treves, R. (2000). Mapping of cotton-melon aphid resistance in melon. Accepted by Jour. Am. Soc. Hort. Sci.

Final Report
 BARD Project No. IS-2708-96
 USA cooperators Jack E. Staub, USDA, ARS, Madison, WI and James D. McCreight,
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- V. Development and evaluation of recombinant inbred lines in cucumber for the analysis of quantitative traits
- VI. Ongoing studies

I. Method for the Development and Characterization of Microsatellite Markers in Cucumber

Introduction

Genetic markers such as isozymes, RAPDs and RFLPs have been characterized in cucumber (*Cucumis sativus* L.) (Dijkhuizen et al., 1996; Serquen et al., 1997). However, critical documentation of these markers and their usefulness in marker-assisted selection (MAS) for applied breeding programs has been limited. This is at least partially due to the species' low polymorphism level. A higher level of polymorphism has been associated with SSR loci in preliminary studies with *C. sativus*, as well as in other genera (*Cucurbita* and *Citrullus*) of the Cucurbitaceae (Katzir et al., 1996). In this study 12 of 20 SSRs (60%) possessed two to five alleles per locus.

A moderately large molecular marker database now exists for cucumber (Meglic et al., 1996a and 1996b; Dijkhuizen et al., 1996). The number of polymorphic loci identified in these crops is useful for variety identification and seed purity testing, but is not robust enough to be used for legal applications or some phases of germplasm management (Staub et al., 1999). The identification and characterization of SSR markers would allow for more precision in the estimation of genetic similarities among cucumber cultivars, and thus provide cucumber researchers with additional markers for genetic analysis (Staub et al., 1996). Since their initial implementation, PCR markers based on

microsatellites have become the marker of choice for many genetic. Their codominant nature, and high polymorphism rate, and high through-put capacity are important methodological characteristics. The initial development of microsatellite markers requires the characterization of sequences flanking the repeat motif, followed by the design of flanking PCR primers. Many methods have been devised for the initial characterization of the flanking sequences (Litt and Luty , 1989). Other methods include: database search, construction of a genomic or cDNA library and screening with oligonucleotide probes, and construction of microsatellite-enriched libraries followed by PCR screening. We describe herein a novel method to capture sequences that contain microsatellite motifs.

Materials and Methods.

DNA extraction, restriction and size fractionation. DNA from cucumber breeding line G421 was extracted from young leaves and apical meristems according to the nuclear DNA extraction protocol outlined in Maniatis (1982). Extraction products were treated with RNase ONE (Promega, Madison, WI). DNA was quantified using a TKO 100 fluorometer (Hoefer). In separate reactions 100 mg of DNA were restricted using EcoR I restriction enzyme (Promega, Madison, WI) and Acs I (Boehringer Mannheim) which recognizes the sequence (A or G)/AATT(T or C) and generates compatible ends with EcoR I. Restricted DNA was size fractionated using a low pressure 75cm long 16mm diameter chromatography column (Fisher) filled with Sephacryl S-500 (Pharmacia). The column was packed according to instructions for Sephacryl S-500 and equilibrated with 200 ml elution buffer at 0.3 ml/min (0.1 M Tris-HCL pH 8.0, 0.15 M NaCl, 0.001 M EDTA). Both samples of restricted DNA were fractionated separately and eluted DNA was collected at 2 ml aliquots. Eluted DNA was precipitated, washed and resuspended in TE. Elution aliquots were sized by agarose gel electrophoresis. Fractions between 200 bp to 1200 bp were combined for ligation to vector.

Library construction, mass excision and plasmid DNA extraction. Fractionated DNA was ligated to Ziplox EcoR I arms (Life Technologies, Gaithersburg, MD) and packaged in a lambda vector with Gigapack III Gold packaging extract (Stratagene, La Jolla, CA). The resulting library was titered and efficiency of packaging was determined by blue/white colony screening. Primary Ziplox libraries were mass excised *in vivo* into

the pZL1 plasmid vector (Life Technologies, Gaithersburg, MD) in DH10B Zip (Life Technologies, Gaithersburg, MD) strain of *E. Coli*. The mass excision and expansion of the plasmid library were performed at the same time in a semisolid media to minimize representational biases that can occur during expansion in liquid media. Plasmid DNA was extracted by a plasmid maxi-prep procedure (Qiagen, Valencia, CA).

Capture of plasmids containing microsatellites. In preparation for the GeneTrapper™ (Life Technologies, Gaithersburg, MD) reaction, several oligonucleotides (20-30 bp) coded for common microsatellite sequences (CT, TG, CTT, TCC, ATT) were biotinylated (biotin-14-dCTP) with a terminal transferase enzyme (Life Technologies, Gaithersburg, MD). The “capture” steps are described in detail in the GeneTrapper kit (Life Technologies, Gaithersburg, MD) and graphically represented in Figure 1.

1. The plasmid DNA was treated with Gene II protein and Exonuclease III to obtain single stranded circular plasmid DNA.
2. Single stranded DNA was hybridized with the biotinylated oligonucleotides. During the hybridization, the single stranded oligonucleotides hydrogen bonded with their complementary microsatellite sequences.
3. Para-magnetic beads bound to streptavidin were used to capture the microsatellite containing single stranded plasmid molecules. The product of the capture was washed several times to eliminate unbound single stranded DNA.
4. The final product of the capture was then treated with a buffer to release the captured single stranded DNA. The single stranded DNA was subsequently primed and repaired to form double-strand DNA.
5. The double-stranded plasmids were then used to transform DH10B *E. coli* that had been grown on ampicillin selective media. It was assumed that only *E. coli* harboring the intact pZL1 (putatively containing a microsatellite region) coming from the cucumber DNA library survived the selection media.
6. Single colonies were picked, named sequentially, and then amplified prior to plasmid DNA extraction. Confirmation of the presence of a microsatellite region was obtained by subjecting plasmid DNA to PCR using the standard forward or reverse M13 primer and a primer coding for the captured microsatellite type.

7. Plasmid DNA from positive clones was sequenced at the University of Wisconsin Biotechnology Center to identify the microsatellite type and length.

Sequence analysis and primer design. All sequences from positive clones were analyzed and compared to each other using the assembly feature in the GeneTool software package (Biotoools, Edmonton, Canada) in order to discard duplicates and identify overlapping DNA regions. The resulting unique sequences were entered into the primer design software OLIGO 6.0 (Life Science Software, Long Lake, MN).

Results and Discussion

The ease of application of the GeneTrapper™ System (Life Technologies) made it possible to isolate and characterize several cucumber microsatellite loci in a relatively short time without the use of radiolabeled products. These results were obtained in 1998 independently of a similar technique developed by Paetkau (1999).

This methodology has been repeated five times with success and reproducibility of several types of microsatellites (di-, tri-, and tetra-nucleotides) (Figure 1). Nevertheless, this methodology continues to be further refined and is still considered to be in its developmental stages. The first capture was performed with a (CT)12 oligonucleotide, and the procedure yielded 457 clones. The PCR assays identified the size of the insert and its relative position. Based on these assays, 300 of the 457 clones were discarded as suspected duplicates. Sixty-five clones were selected for the primer design step of the SSR construction (Table 1). The insert sequences were entered into the Oligo 6.0 primer design tool (Molecular Biology Insights Inc., Long Lake, MN).

Other captures using (TG)12, (CTT)8, (TCC)8, (ACTC)6, (ATT)9 have been successfully performed, and are currently being characterized as with the CT repeat capture. All primer pairs that have been designed are being tested on *C. sativus* lines G421 and H-19 using a thermal gradient PCR programmed on an Eppendorf Mastercycler Gradient thermal cycler (Hamburg, Germany) to establish the optimum annealing temperature range for each SSR primer. This information will be used later in the development of PCR procedures for multiplexing SSR markers. The SSR markers being developed in cucumber are being tested on *C. melo* parent lines MR-1 and Topmark to establish the extent of their cross-compatibility and potential use in melon.

To date, approximately 80% of the SSR markers that have been constructed have amplified in both *C. sativus* and *C. melo*. The development of SSR markers is an important step toward the construction of a syntenic map and the assessment of genetic diversity in the *Cucumis* species. Furthermore, these markers will be useful for mapping, and introgressing economically important traits into cultivated varieties.

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II. Response to Phenotypic Selection for Multiple Lateral Branching in Cucumber (*Cucumis sativus* L.)

Introduction

The acreage planted for mechanically-harvested cucumbers in the United States has increased 30 to 40% in the last 20 years. Because of rising labor costs and contract disputes this trend is projected to continue (Merchandising Guide, 1988). In the northern United States, acreage dedicated to once-over mechanical harvest is significant. For example, in Michigan, Wisconsin, Delaware, New York, Washington and Oregon mechanically-harvested acreage ranges between 30 to 45%.

Curiously, the yield of pickling cucumber (gynoecious or G, and monoecious or M) has plateaued in the last 15 years. Studies by Widders and Price (1989) and Staub et al. (1989 and 1992) suggest that this recent plateau may be associated with net photosynthetic capacity. Resource limitations may explain why fruit developing from the first pollinated flower on each lateral branch inhibits the development of subsequent fruits (Denna, 1973; Fuller and Leopold, 1977).

To overcome the yield plateau and to respond to the need for cultivars suitable for mechanical harvesting, our breeding program is manipulating cucumber plant architecture to develop high yielding genotypes. Standard cucumber varieties (G x M or G x G hybrids) possess an indeterminate (*De*) plant habit and few lateral branches (~1 to 2). We are developing all female genotypes which are short in stature (determinate; *de*) and possess a multiple lateral branching habit (~5 to 7 branches). This plant type can be sown at relatively high densities (compared to standard indeterminate types), and allows for an increase in early, concentrated yield (Staub et. al., 1992).

Information on quantitatively inherited traits related to yield components, however, is sparse. Serquen et al. (1997) suggest that few genes (perhaps 5 to 8) control days to anthesis, sex expression, mainstem length, numbers of multiple lateral branches, and fruit number and weight. We are particularly interested in multiple lateral branching since this trait is highly correlated to yield response (). We present

herein response to phenotypic selection in populations segregating for multiple lateral branches in the cross between line G421 (G, *de*) x H-19 (M, *De*). This experiment is part of a larger experiment that aims to compare the efficiency of marker-assisted selection of MLB to phenotypic selection in this cross.

Materials and Methods

The gynococious determinate cucumber inbred line G421 possessing normal-sized leaves and low lateral branch number (approximately 1) was crossed with the monoecious indeterminate little leaf inbred line H-19 possessing high lateral branching (approximately 8) in the winter of 1997 to produce F1 progeny. In the spring of 1998, 15 F1 plants were used as males (after sex expression change) to pollinate 200 G421 plants to generate BC1 seed (greenhouse in Arlington, Wisconsin).

A total of 4,000 BC1 progeny, parental lines, and F1 progeny were evaluated for multiple lateral branching in the summer of 1998 were evaluated at the University of Wisconsin, Hancock, Wisconsin (Typic Udipsament; sandy mixed, mesic). BC1 plants were segregated in plots according to the F1 hybrid by maternal parent. Seed was 0.6 m apart on rows spaced 1.5 m apart.

Data on MLB was collected 40 days after sowing when approximately 50 % of the plants had reached anthesis. MLB number was recorded when laterals reached a length of 25 cm or more. Data on MLB was analyzed and information on population mean, standard deviation and distribution was used to determine the threshold value (truncation point) for phenotypic selection.

To change sex expression, apical meristems of selected plants were treated 3 times in 5 day intervals with 2-3 ml aerosol solution of 6 mM silver thiosulfate. Selected plants were then selfed and backcrossed to field-grown G421 plants (recurrent parent).

In the summer of 1999, seed of selected BC2 (3233) and BC1S1 (1,367) from summer 1998 as well as parents, F1 hybrids (36), F2 (400), and BC1 (397) progeny seed lots were planted at Hancock, Wisconsin (Table 1). Parents, F1, F2, BC1, BC1S1 and BC2 families were arranged in a randomized complete block design with three replications of 12 plants each (total of 36 plants per family). Seed was planted 0.45 cm apart on rows positioned 1.5 m apart. Data collection, sex conversion, and selection were performed similar to summer 1998. Selections were backcrossed to G421.

Expected gain from selection was calculated according to Falconer and Mackay (1996) as $R = 1/2 i h^2 \sigma_P$. The intensity of selection, i was adjusted to 1/2 of its tabulated value to account for the fact that no selection was applied to the females (G421). The mean h^2 (0.45) used for calculation was taken from a previously published study that estimated heritabilities for this trait in two locations (Wisconsin and Georgia) (Serquen et al., 1997).

Results and Discussion

The mean MLB number of the 126 selected plants in the BC1 population was 4.90. Since approximately 4.4 % (corresponding to a calculated selection intensity of 1.058) of the BC1 population was selected, the expected gain from selection was calculated to be 0.57 units (Figure 2). The realized gain from selection based on the difference of the generation means was approximately 0.4 units. The replications of the base BC1 population across years were similar. Means, standard deviations and population numbers are given in Table 1. The distributions of multiple lateral branches differ in BC1 (MLB mean = 2.2), BC2 (MLB mean = 2.6), and BC2S1 (MLB mean = 3.4) populations. The progressive changes (increase in MLB) in these generations are the result of selection and increased homozygosity (BC1S1; fixation) of loci affecting this trait.

A subset of approximately 200 BC2 plants (MLB mean = 5.16) of the BC2 population were selected to produce BC3 families (Table 2). As a result of this selection experiment we now possess approximately 150 BC3 families representing independent recombination events. These families will be used to generate either nearly isogenic lines (Bernacchi et al., 1998) or congenic lines (Hill, 1998) for detailed QTL mapping and to increase our understanding the genetic control of MLB in cucumber.

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III. Confirmation of RAPD-SCAR-SSR marker -QTL linkage for multiple lateral branching in cucumber

Introduction

The objective of this segment of the investigation was to determine whether certain markers that were found to be significantly linked to the multiple lateral trait in cucumber were confirmed independently of populations that were useful in detecting linkage for this trait. Backcross populations that were used for the phenotypic study (Section II above) were also used in this evaluation. The null hypothesis that was tested was:

H_0 : Under phenotypic selection markers that were linked to the trait would not be present in the selected individuals with higher frequency than expected if the markers had been segregating randomly.

This work is still in progress, and will be completed by 6/2001.

Materials and methods

Plant material. DNA was extracted from a subset of 75 individuals in the 5% upper tail of the BC2 population described in Section II were selected for this study. These individuals had undergone two cycles of phenotypic selection for multiple lateral branching, and had a minimum of five lateral branches each (donor and recipient parents possessed an average of 8.5 and 1.5, respectively). These plants were backcrossed to G421 (recurrent recipient parent) to generate a BC3 population.

DNA markers. Five markers were selected from the pool of polymorphic markers available in this particular cross. These markers were linked to the multiple lateral branching trait and were:

1. OP-W7-2 a dominant RAPD marker that was identified by Serquen et al (1997) and mapped to linkage group D.
2. CSWCT13 a codominant SSR marker developed in our lab.
3. OP-AO8 a codominant RAPD marker.
4. BC 551 a dominant RAPD marker mapped to linkage group D.
5. L18-2/H19 a SCAR marker.

Four neutral markers (unlinked) were included as controls as follows:

6. W7-1 a dominant RAPD band mapped to linkage group F used as neutral control.

7. N6 a codominant SCAR mapped to linkage group H used as neutral control.
8. OP-H5 a dominant RAPD marker mapped to linkage group C used as neutral control.
9. G14 a codominant SCAR used as neutral control.

In the case of L18-2/H19, the original polymorphism produced by RAPD primer L18 was a dominant band representing only the G421 recurrent parent allele in the population and was therefore not useful in a backcross. The band was sequenced and SCAR primers were produced which amplified both alleles with a size difference of two base pairs. The H-19 allele of this SCAR was sequenced and new primers specific for the donor parent H-19 were designed to make the marker more compatible with high throughput PCR and resolution in agarose gels. The resulting SCAR PCR primers amplified only the H19 allele. The initial RAPD band was identified by Serquen et al. (1997), and explained ~30% of the phenotypic variation for multiple lateral branching.

Analysis. The expected segregation ratio of a marker allele derived from the non-recurrent parent in a random BC2 population is 0.25. That is, 25 % of the BC2 population should possess the donor parent allele at any given locus. Deviation from this expected value should theoretically be the result of either non-random effects such as selection or random effects such as drift or sampling error. The BC2 population employed was derived from 126 BC1 parents which excludes random drift as a significant source of deviation from the expected ratio (Falconer & Macay, 1996) in the BC2 progeny. A Chi square test (one degree of freedom) was performed on all of the markers to determine if they fit the expected segregation ratios.

Results and discussion

The results of Chi square tests are shown in Table 3. The markers that had been previously identified as linked to the multiple lateral branching trait showed a significant segregation distortion in the 75 BC2 plants selected for hypothesis testing. However, the markers that were considered “selectively neutral” did fit the expected segregation ratio of 0.25. Since the amount of deviation of a particular marker from the expected value is the cumulative result of the effect of a QTL, the distance between the marker and the QTL, and environmental variance, it may be possible to rank the importance of these markers for later use in a marker assisted selection program.

IV. Marker-assisted selection for multiple lateral branching in cucumber

Introduction

An evaluation of the efficiency of marker-assisted during introgression in backcross populations derived from inbred lines is critical for determining the value of markers in a plant improvement program. This was studied by introgressing the regions of the cucumber genome that were found to be affecting the multiple branching trait described in Section III (work in progress to be completed 6/2001).

Evaluation of gain from selection can be accomplished solely through phenotypic selection. However, gain from selection is typically slow since only one generation per year can be realized unless winter nurseries are available for cycle increase. Thus, it would be important to increase the efficiency of selection by employing genetic markers linked to economically useful traits. The utility of marker-assisted selection (MAS) can be determined in cucumber if quantitative traits (yield components such as multiple lateral branching) can be evaluated in field and if plant can be classified by DNA genotyping.

Cucumber is amenable to the cultivation in greenhouses during the fall and winter months, thus increasing the number of generations per year that can be used to introgress or backcross a new trait into an elite cultivar. MAS becomes more efficient than other selection methods where the target trait cannot be measured accurately (i.e., heritability approaches zero). Such is the case when greenhouse-grown processing type cucumbers are selected for target traits that are influenced by growing environment.

Measurement of efficiency of MAS can only be evaluated by comparing cycles of selection. In the intent of these experiments was to determine if breeding that would normally require three years of summer field evaluations (i.e., field selection followed by controlled matings) could be performed in one year. This can be accomplished by moving the introgression (selection) of a target trait forward with markers in one season of greenhouse selection. Thus, experiments were performed using markers linked to multiple lateral branching (quantitatively inherited; least effective factors = 5, Serquen et al., 1997) to determine if MAS could be effective as an indirect selection tool in a U.S. processing cucumber population. The strategy employed was to attempt to introgress

QTL regions with the largest effect on the target trait greenhouse using linked markers in processing cucumber population grown in a greenhouse. The intent was to obtain a large population (~100 selections) of selected plants whose progeny could be evaluated in a field nursery for gain from selection. MAS was practiced on a BC1 population to produce BC2 families and in a BC2 population to produce BC3 families (all progeny originated from H-19 x G241 matings and line G421 was used as the recurrent parent).

Materials and methods

Plant material and DNA extraction. The gynoeocious determinate processing cucumber inbred line G421 possessing normal-sized leaves and low lateral branch number (approximately 1) was crossed with the monoecious indeterminate little leaf inbred processing line H-19 possessing high lateral branching (approximately 8) in the winter of 1997 to produce F1 progeny. In the spring of 1998, 15 F1 plants were used as males (after sex expression change) to pollinate 200 G421 plants to generate BC1 seed (greenhouse in Arlington, Wisconsin). During the fall of 1999 and winter 2000 960 BC1 and 960 BC2 seedlings (G421 is the recurrent parent) were grown in the greenhouse in 4x12 flats with cells 5 cm wide x 7 cm long x 7cm deep filled with peat moss. Two flats corresponded to one 96 well (8x12) microtiter format plate for marker genotyping. Tissue for DNA analysis was collected by detaching 1 leaf measuring less than 1 cm² from a 2 week old seedling and introducing it into the corresponding 0.5 ml well of the microtiter plate. The tissue was lyophilized and DNA extractions were performed using the Aquapure genomic DNA extraction Kit (Bio-Rad Laboratories, Hercules, CA) in conjunction with the high throughput microtiter format.

Marker assisted selection. Aided by the use of multi-channel pipettes and in 96 well microtiter format all 960 BC1 and 960 BC2 were genotyped for the following marker loci: W7-2, AO8, CSWCT13, BC-551, and L18-2/H19. The genotyping for each population lasted for one week allowing for recovery and transplanting of selected plants into 8 inch pots of the 3 to 4 week old seedlings. These plants possessed all five markers from the donor parent (H-19). The selected seedlings were gynoeocious and were treated with silver thiosulfate to change sex expression to pollen producing male flowers. Pollen from the selected plants was used to pollinate G421 thus generating BC2 and BC3 progeny from BC1 and BC2 selected plants.

Results and discussion

The MAS process yielded 55 BC1 plants and 34 BC2 plants. Progeny families from these plants are currently being evaluated in field nursery at Hancock WI and are being compared to other BC2 and BC3 families derived from the phenotypic selection process described in Section II (Table 2). These comparisons will establish the practical usefulness of the molecular information (new marker systems and merged maps) that has been gathered during this and other project.

V. Development and evaluation of recombinant inbred lines in cucumber for the analysis of quantitative traits

Introduction

Rigorous evaluation of MAS requires the assessment of the multi-year, multi-location testing of populations that will allow for precise determination of gene action and the placement of QTL on molecular maps. Thus, we have developed a highly integrated strategy for testing the efficacy of the SSR and other previously documented markers (i.e., RAPD and SCAR). This strategy employs three primary steps using recombinant inbred lines (RIL), F2 progeny, and strategic backcrossing. A set of 165 F₈ RIL has been developed from G421 x H19 matings. In the summer of 1999 these lines were planted in a replicated experiment in Hancock (Wisconsin), Clinton (North Carolina), and Spanish Fork (Utah). RIL were primarily evaluated for yield components in cucumber (multiple lateral branching in all locations; sex expression, flowering date, and fruit number). These same lines are being evaluated as a part of a multi-year experiment in Hancock (Wisconsin) in the summer of 2000. The same lines are being genotyped using the combination of SSR, SCAR, and RAPD markers that have been developed as a result of this and other ongoing projects.

Multiple lateral branching is conditioned by at least 5 and perhaps as many as 8 loci. (Serquen et al., 1997). Both variance component analysis of this trait and that of QTL placement during map construction. Other yield components (e.g., fruit number and weight, sex expression, days to anthesis) have been assessed, and the genes controlling these traits are relatively low (perhaps between 4 to 10). These traits are being reevaluated and mapped using RIL in this experiment. The intent is to apply

the QTL mapping information gathered in this experiment to marker-assisted-breeding of other quantitatively inherited yield component traits in cucumber.

Materials and methods

RIL (165; F8) derived from the cross H19 x G421 as well as parental lines, F1, and 22 control lines for a total of 190 entries were planted in a randomized complete block design with three replications in three locations: 1) the University of Wisconsin research station in Hancock, Wisconsin; 2) the North Carolina State University research station in Clinton, North Carolina, and; 3) the Brigham Young University research station in Spanish Fork, Utah. Plants were spaced 12cm apart in 1.5 meter long plots placed on rows 1.5 meters apart. Measurements were taken on all the following traits except for the Spanish fork location where only multiple lateral branching data was collected:

Plant habit. Determinate (*de*) plant habit was determined by observing plants at ~7 to 10 nodes after sowing. Plants that possessed short vines with the main stem terminating in a flower cluster were classified as determinate.

Leaf type. Plants were classified as little leaf, standard leaf or intermediate leaf types by visual examination at least by one week before harvest. Extreme parental leaf types will be easily separated by general inspection (little = 30 to 40 cm² and standard = 80 to 100 cm²).

Days to anthesis. Individual plots were examined to determine the number of days from planting to the time the corolla of at least three fully expanded flowers were present on 50 % of the plants in the plot.

Number of female nodes. Starting with the first flowering node, the sex expression was recorded on 10 nodes in each of 3 consecutive plants in a plot in all replications. A second measurement was taken counting all female nodes in the first 6 nodes of the lateral branches.

Number of fruit and harvest date. Plots were harvested at least twice (once when fruits are immature and once when fruit are mature). Immature fruits > 20 mm in diameter were hand-harvested from all plants in a plot when 2-3 fruit > 51 mm in diameter (oversized) are observed within a plot (first harvest). The second harvest was performed

when 3 fruits were observed to be oversized. The third harvest was simulated by counting all fruits greater than 20 mm in diameter on dead vines.

Fruit length. After the first harvest and second harvest, 5 mature fruit (2B-3A grade; 25 to 30 mm in diameter) from each row were randomly selected and their lengths averaged. When plants produced fewer than 10 mature fruit, all those available were measured.

Fruit length/diameter ratio (L/D). Fruit diameter was measured using the same fruit used in length determinations. Mean L/D was calculated from length and diameter determinations.

Lateral branch number. The total number of primary lateral branches was recorded from all plants within a plot at or before anthesis.

Results and discussion

Even though the relative values of the traits varied among test locations, the relative performance of ranking of RIL did not change across locations. Typical frequency distributions of values are presented Table 2 and Figure 2 for the Hancock location for number of multiple lateral branches. As predicted, selection for donor (H-19) parent characteristics while backcrossing to the recurrent low lateral branch number parent resulted in different response to selection depending on the generation evaluated (BC1, BC2, and BCS1). BCS1 plants possessed a higher mean number of branches when compared to other backcross populations.

DNA has been taken from individuals of each backcross generation, and is currently being analyzed for specific polymorphisms linked to each trait.. It is hoped that the frequency of alleles for high lateral number is higher in BCS1 populations when compared to the other BC populations tested. This would indicate that marker-assisted selection has potential for increasing the efficiency of cucumber breeding programs.

Literature cited

Serquen, F.C., J. Bacher, and J.E. Staub. 1997. Mapping and QTL analysis of horticultural traits in a narrow cross in cucumber (*Cucumis sativus* L.) using random-amplified polymorphic DNA markers. *Mol. Breed.* 3:257-268.

VI. Ongoing studies

Field developments. A second year trial of the recombinant inbred lines is now underway at Hancock (Wisconsin). This will be used to corroborate the data collected in 1999. A second year of data will enable us to better characterize QTL x year interactions.

Evaluation of BC and F2 populations is continuing in 2000. Progeny of plants obtained through marker-assisted selection are being compared to plants obtained through phenotypic selection.

Laboratory developments. All 165 RIL are being genotyped with all the polymorphic SSR, SCAR and RAPD markers available. These data, in combination with the phenotypic data gathered in 1999 and 2000 will enable our laboratory to identify QTL linked to important yield components.

Table 1. Simple sequence repeat primers constructed for hypothesis testing and marker analysis.

SSR designation	Sense primer 5' to 3'	Antisense primer 5' to 3'	Expected size (bp)
CSWCOM01	AATTGCTGCCCTTTCACTG	GGCGCTTTAACCATTTCAG	139
CSWCOM02	TTAAAGGCCTTTTATAACACT	ATGACAGTTTAAAGCTGACA	82
CSWCT01	TTCTGATCAACGACGAAG	GAAACAAAAGCCTCCATTG	242
CSWCT02A	CAAATAAGGGCTGCTTAGC	TAATGAAGGCAAAGACTT	452
CSWCT02B	TTCTGCATACCCTCTCCT	CACACTTCCAGATGGTTG	203
CSWCT03	TTCTCAGAACTGCCACTG	CACTCTTGAGGGGAAAAA	390
CSWCT04	ACTATTGGGTCTCTCCTA	GACCCGAGGTTATTATT	556
CSWCT05	CTTGTTCCCACTCACA	GGTTCCGATTTATTACTT	470
CSWCT06A	TTTAAATTCTTCCTAACC	TTTGCTTTGCATTTGGAT	200
CSWCT06B	GAGACCTGAGTTTCATCG	TGATTTTGCTTTGC	163
CSWCT07A	GAATAAATAATTAGGTTT	TTTTTATAGTTCCATCTC	583
CSWCT07B	GAATAAATAATTAGGTTT	TATAAAAATAAAATTCCA	308
CSWCT08	ATTTCTTAAACCACCACA	ACCGTGTGTTTAGAATGC	594
CSWCT09	TATCCTTTAATGCTTTTCG	CTAGCCTTGCATTTTGT	273
CSWCT10	AGATCGGAATTGAAAAAG	AAAGGGGCTTCCTCTCTA	183
CSWCT11	ATAGGCAATATGGCTTCT	CACTTCAATGGAGTTTCG	226
CSWCT12	GCGGGGCCGTTTTACAT	GGCAAAGGGAAGAAGAAG	501
CSWCT13A	AGAATCAATTTCAAACCA	GAATTATGGGTTCATTTT	788
CSWCT13B	TGTGATCAACCAACTTCA	CACTGGGATCCTAACAAC	256
CSWCT13C	CATCATGCAGGCTTACAC	ATAGGGCAATTTGTCTCT	219
CSWCT14	TCCAATCGACCTAAATCCAAA	TGGCCCTAACTGTTGATGC	343
CSWCT15	GGAGGGTCAGGTAGTCAA	CGAGTTTGTGATCTTCAT	130
CSWCT16	TCTACGTCGATGAATCA	CTCAGATAACCCAAAATA	409
CSWCT17	TTGAATTATGGGTTCATTTT	GACAATGATAAACTTCCCTGA	193
CSWCT18A	AAGTAGTCCTCAATGCCACAT	TGCTTGCCTTCCTCCTC	338
CSWCT18B	AAGTAGTCCTCAATGCCACAT	GGAGAGAAATGGGGAAAA	182
CSWCT19A	GCATTGGCCCTTTTTGACCCG	GAATTCCACAAATTAAGTC	257
CSWCT19B	TCGACGAGAAAGATGAACCTA	GAATTCCACAAATTAAGTC	112
CSWCT20	GGAAACCTAATAAGAATATGG	GAATTCGGTGATAGACATTGA	286
CSWCT21	AGGGATGGTCAGTAGGTA	TGCTTCTTTTGTGCTC	299
CSWCT22A	GGGAGTATCGAAACAAAAGC	TTCTGATCAACGACGAAGTAA	229
CSWCTT01	CAGACTGTGGAACAAAGG	GAATTCGGAATGAAAGG	170
CSWCTT02A	AGATAACGCGGGTCAAAT	GTAGGTTAATTACCAAAA	640
CSWCTT02B	AAGCTAGTTCTTCTCTTC	GGTTCGGCCTCACACTC	74
CSWCTT03	GCATATCGATTTAGGTCTAAT	GGATGGAGCGTCTCTTA	184
CSWCTT04	GCGGAGATATGCCAATTTCAA	TTCAAGCGCGACGACAAT	231
CSWGAAA01	TTAGACTTTTATCTATGATTG	TTTATTTTATAACAGATTTCA	218
CSWGAT01	ACCCGTTCTGTCTGTCTCT	CCGACCTCTAGGGGATAA	235
CSWGATT01	ATAAATCCGCCCTCTCA	GGCTGGGCTGGTTTTGTA	651
CSWGTTT01	ATATGCCCAGGAGTCAGTTT	AGACCTTTGGCACCGTTCTAA	287
CSWTA01	AACATTCTCTATAAACATCTT	GGTTTCCCTAATGATATAA	254
CSWTA02	TCCTTCTTTTCTCGCTCTG	TGCCCTAAAATCCTCACACTC	229
CSWTG01	ATTGTCTGATTGGGTATG	GGCAGCAAGAGCACTACC	178
CSWTG02	CTTTTGGACCGTTTTTGG	AAGAACCGCTCGGAGATG	240
CSWTT01	CTTGGCGCGACCTAC	GTAAGCGCGAGTCATCAG	372
CSWTT02	ATTTTTGCTCTGCGATGT	AATTCTCCAAGCGTCCTT	193

Table 2. Generation means for multiple lateral branching in cucumber.

Generation	Year	Mean	SD	N
BC1	1998	2.23	1.20	2985
G421	1999	1.75	0.84	150
H19	1999	7.9	1.60	36
F1	1999	4.61	0.98	36
BC1	1999	2.21	1.14	397
BC2	1999	2.60	1.21	3233
BC2S1	1999	3.38	1.616	1367
F2	1999	3.58	1.38	400

Table 3. Chi square analyses of RAPD and SCAR markers for fit to expected ratios in the BC2 generation (i.e., present at a frequency of 0.25).

Marker name	Neutral Yes/No	Total plants tested	Number of plants expected with donor parent allele	Number of plants obtained with donor parent allele	Chi square value	P value
OP W7-2	No	75	19	39	21.87	0.001
CSWCT13	No	75	19	47	42.56	0.001
OP AO8	No	75	19	44	34.003	0.001
BC551	No	74	19	48	45.63	0.001
L18-2/H19	No	75	19	58	82.16	0.001
OP W7-1	Yes	75	19	18	0.03	0.001
N6	Yes	74	19	21	0.27	0.001
OP H5	Yes	75	19	20	0.083	0.001
G14	Yes	75	19	22	0.56	0.001

Figure 1. Methodology for the development of SSR markers

Figure 2. Distributions of multiple lateral branching among F2 and backcross cucumber populations.

Figure 3. Frequency of sex expression traits in RIL of cucumber.

Figure 4. Frequency of morphological traits in RIL of cucumber.