Genome Mapping of Melon (Cucumis melo L.) for Localizing Disease Resistance Genes

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ABSTRACT. Genetic maps are available for most crop species. The immediate application of a map is to localize genes of agronomic importance such as disease resistance. We have created a genetic map of melon (Cucumis melo L.) using over 200 AFLP (Amplified Fragment Length Polymorphisms) and other markers. New as well as mapped markers were used to localize Fon-2, a gene conferring resistance to fusarium wilt of melon caused by race 1 of Fusarium oxysporum f.sp. melonis. A previously identified RAPD (Random Amplified Polymorphic DNA) marker was found to cosegregate and two AFLP markers flank the gene. These markers are being converted to codominant PCR-based markers for marker-assisted selection (MAS). To facilitate identification of additional markers and map-based cloning, a BAC (Bacterial Artificial Chromosome) library is being constructed.

Melon (Cucumis melo L.) is a valuable cash crop grown in the United States and throughout the world. Farmers in the U.S. grow >120,000 acres of melons (musk-melon and honeydew) annually and produce nearly one million tons of melons with a total value of 500 million dollars (NASS, 1997). Production is mostly in California, Arizona, Texas, Georgia, Florida, South Carolina, Virginia, New Jersey, Maryland, and Indiana (listed in order of total production). The best quality melons come from the Southwest and California where light intensity is high, relative humidity is low and water is supplied by irrigation rather than rainfall. In the winter, the US imports melons from Mexico, Honduras, Guatemala, Costa Rica, and Dominican Republic (Thoman, 1997). In most cases, producers grow the netted, orange-fleshed muskmelon although the smooth-skinned, green-fleshed honeydews have increased in popularity over past 10 years or so. In addition, orange-fleshed honeydews and green-fleshed melons have been developed in the U.S. (Ng, 1993). The smooth-skinned, delicately fleshed Charentais types and the dark green smooth-skinned hami melons (Cucumis melo ssp. melo Cantalupensis Group) are favored in Europe and Asia, respectively.

Members of the Cucurbitaceae family, melons are thought to originate from Africa (Bates and Robinson, 1995). The genus Cucumis consists of two distinct groups differing in their origin and basic chromosome number. The larger group, which includes melons, originates from Africa with 2n = 24 chromosomes. The second group originates from South Asia and includes cucumber (C. sativus) which has a 2n = 14 chromosomes (Bates and Robinson, 1995). These groups appear to have diverged some 90 million years ago, the time the continents of Africa and Asia separated (Raven et al., 1981). Many of the family members have been domesticated and are agronomically important, including watermelon (Citrullus lanatus), squash (Cucurbita pepo), and pumpkin (C. maxima). Although these taxa are generally repro-
ductively isolated and have chromosome number (2n) ranging from 14 (C. sativus) to 40 (C. maxima and C. pepo) (Dane, 1991; Roy et al., 1991), they have a rather similar nuclear DNA content (Arumuganathan and Earle, 1991). The haploid DNA content of melon is estimated at 0.47 to 0.52 pg which corresponds to 5 × 10^8 bp (base pair)—a relatively small genome size about three times that of Arabidopsis, but very similar to that of rice (Arumuganathan and Earle, 1991).

This paper will focus on genetic mapping of melon using DNA markers, and its application for identifying markers linked to target genes, especially disease resistance genes such as Fom-2. Progress on the development of other valuable resources for melon improvement, particularly the construction of a large-insert bacterial artificial chromosome (BAC) library will be presented.

**Fusarium wilt of melon**

By any measurement diseases caused by fungi are major limiting factors in melon production worldwide, of which fusarium wilt is of major importance. Caused by *Fusarium oxysporum* Schlechtend. ex Fr. sp. *melonis* Snyder & Hans. (Fom), fusarium wilt occurs throughout North America, Europe and Asia. *Fom* only infects melons—plants are susceptible in all stages of development. The pathogen blocks the uptake of water and nutrients, killing the root and crown tissue. In infected fields, yield losses can be as high as 100% and once established, the pathogen remains indefinitely (Sherf and Macnab, 1986). Even following prolonged periods of cultivation of nonhost crops, fusarium wilt pathogens can still be recovered from the soil (Banihashemi and DeZeeuw, 1975).

Currently, four races (races 0, 1, 2, and 1,2) of the fungus are defined by their ability to incite disease in four differential varieties. 'Charentais T' and 'Top Mark' are susceptible to all races. 'Doublon' is resistant to races 0 and 2 (Fom-1). CM 17-187 is resistant to races 0 and 1 (Fom-2). All are susceptible to race 1,2. Race 2 is most widely distributed in the United States and was the only race known in North America until 1985, when race 1 was discovered in the North Atlantic states (Martyn and Gordon, 1996). Currently, only a few eastern-type melons are resistant to race 1 (Zuniga et al., 1997) and commercial varieties currently grown in California are susceptible (Gwynne et al., 1997). Several breeding lines have high levels of resistance to the disease, including the widely used line MR-1, which is one of the genotypes we are using for genetic map construction (Thomas and Jourdain, 1989; Thomas et al., 1990).

Developed from PI 124111 discovered 60 years ago, breeding line MR-1 carries resistance to races 0, 1, and 2 of fusarium wilt (Fom-1 and Fom-2), powdery mildew races 1, 2, and 3 (Pm-3, Pm-6 and an unknown gene for race 3), Alternaria leaf blight (Ac), and all five pathotypes of downy mildew (Pc-1 and Pc-2) (Thomas, 1986; Thomas et al., 1992; Zink and Thomas, 1990). However, because MR-1 lacks desirable fruit traits, progress in introgressing its resistance genes into commercially acceptable varieties has been particularly challenging and time-consuming. Clearly, if resistance can be identified more rapidly and accurately, considerably more progress can be made introgressing these traits into horticulturally acceptable cultivars.

Genetic control of resistance to fusarium wilt has been studied in some detail (Thomas et al., 1992; Zink and Thomas, 1990). Two single dominant genes *Fom*-1 and *Fom*-2 confer resistance to *Fom* races 0 and 2, and races 0 and 1, respectively. Resistance to race 1,2 appears to be polygenic (Blancard et al., 1994; Zink and Thomas, 1990).

**Genetic mapping in plants**

Linkage maps are vital tools in genetic studies whether they are constructed from phenotypic or molecular markers. A map serves several purposes. First of all, it serves as the basis to locate, or tag gene (or genes) in order to facilitate selection of traits. Markers linked to the target gene can be potentially used in marker-assisted selection (MAS). Secondly, a map can be used to identify markers for map-based cloning and finally, it provides useful clues to understand the biological basis of complex traits and phenomena (Lee, 1995). Extensive genetic maps have been developed for major crop plants including Gramineae (barley, oats, rice, wheat, sugarcane), Leguminosae (alfalfa, soybean, mungbean, cowpea, peanut, lentil,
common bean, pea, and Faba bean), Cruciferae (Brassica oleracea, B. rapa, B. nigra, and B. napus), Malvaceae (cotton), Solanaceae (tomato, potato and pepper), and woody species (USDA, 1995). Rice (Causse et al., 1994), maize (Causse et al., 1996) and tomato ( Tanksley et al., 1992) are among the most intensively studied.

Maps are developed from specific populations segregating for sufficient number of loci (genes of interest and molecular markers). However, the population may not segregate for all traits that are of interest to the researcher. This is why there are several maps for a number of crop plants. For example, there are at least six maps developed from different inter- or intraspecific mapping populations in soybean (USDA, 1995). Maps created from different populations of a species can be combined or integrated into one. This can be done by using markers common to all maps and has been done in barley (Qi et al., 1996) and rice (Antonio et al., 1996). In barley, four maps were integrated into one joined map using 22% of the markers that were common among the populations. The integrated map agreed well with individual ones in overall marker order. This is even more apparent in rice in which the final map developed from four component maps shared identical marker order, with only slight differences in genetic distances (Antonio et al., 1996). This suggests that it is possible to generate a unified map within a species across different mapping populations. There is no doubt of the utility of unified maps for gene localization and genome studies.

Genetic maps of different species can also be unified by common markers. In fact, the grass genomes (oats, wheat, barley, maize, sorghum, sugarcane, millet and rice) were unified using common RFLP markers (Devos and Gale, 1997). A unified map across different species is a powerful tool for genome analyses, especially for identifying novel genes in the parallel genomes (Bennetzen, 1996). In cross-species cloning, markers linked to a trait of interest in maize, for example, can be transferred to a small genome crop such as rice, facilitating chromosome walking and gene cloning. This suggests the possibility that maps of different cucurbits can also be unified based on molecular markers where sufficient mapping information is available for each species.

**Genetic mapping of melon**

Compared with other major agricultural crops, genetic mapping of melon is still in its infancy. Genetic studies of melon have identified >90 genes (Pitrat, 1994). However, before the advent of molecular markers (for a review of DNA marker systems, refer to Staub et al., 1996), linkage mapping was limited to known genes or phenotypic markers. Pitrat (1991) analyzed linkage in an F₂ population among 28 phenotypic markers involving disease resistance, flower and vegetative characters and found that 23 of the markers fell into eight linkage groups. This was probably the first attempt at creating a linkage map of melon. Baudracco-Armas and Pitrat (1995) developed a mapping strategy for melon using RAPD and RFLP markers. They assigned 77 DNA markers to 12 linkage groups with 2 to 12 markers in each group. The map was extended to 14 linkage groups covering 1390 cM with 102 markers, including five phenotypic markers (Baudracco-Armas and Pitrat, 1996). A genetic map of melon with over 200 markers was developed using predominantly AFLP (Vos et al., 1995) markers by Wang et al. (1997). The map spans 1930 cM in 14 major linkage groups. Staub et al. (1998) recently surveyed isozyme loci over 400 melon accessions and generated two linkage groups of six and five loci, respectively, out of 19 loci studied.

For the past few years, we have been working on genetic mapping of the melon genome and disease resistance gene identification using DNA markers (Wang et al., 1995, 1996, 1997; Wechter et al., 1995, 1998). The parents used are MR-1 and 'Ananas Yokneam' (AY). MR-1 contains genes conferring high levels of resistance to races 0, 1, and 2 of *Fom. AY*, a cultivar from Israel is susceptible to these pathogens (Thomas, 1986; Thomas et al., 1990).

To construct our initial linkage map of melon (Wang et al., 1997), 66 backcross (BC) individuals from AY x (MR-1 x AY) were used. All individuals used were derived from single fruit. The remaining seed from the fruit were grown to immortalize these lines for future evaluations and to consoli-
date the genetic map. Nuclear DNA from the 66 individuals was isolated and stored at 4°C. AFLP was selected as the method of choice for marker development. It combines the features of RFLP and PCR and is more reliable and stable across different populations in a species. This has been shown for several plant species including rice (Nandi et al., 1997), potato (Roupe van der Voort et al., 1997) and barley (Waugh et al., 1997). Both AFLP System I (three selective nucleotides in both primers) and System II (three selective nucleotides in one primer and two in the other) Kits (BRL-Life Technologies) were used and provided useful polymorphisms for scoring. We found that \(^{32}P\) labeled products were as easy to score as those labeled with \(^{33}P\) in terms of resolution and band sharpness.

Only major bands that could be reliably reproduced were scored. Data were analyzed by MapMaker 2.0 (Courtesy of S. Tingey, Du Pont). Markers were initially grouped using a LOD score of 4.0 and recombination ratio of 0.24. Each group was ordered using the first-order command using a LOD score of 6 and recombination ratio of 0.24. Orders were confirmed using the Ripple command. Occasionally the Ripple analysis suggested an alternative marker order was more likely. In these cases, the most likely order was established using the Try command, an analysis which places the marker in all locations and computes the most likely location. Of 228 markers, 204, including 196 AFLP, 6 RAPD, and 1 SSR markers, fell into 14 major and 6 minor linkage groups (Wang et al., 1997). The total map distance is 1942 cM. About 14% of the scored markers deviated from the expected distribution of 1:1 (p = 0.05).

As mentioned above, a number of genetic maps exist for melon, consisting of RFLP (Baudracco-Arnas and Pitrat, 1996), SSR (Katzir et al., 1997), isozyme (Staub et al., 1998) and AFLP (Wang et al., 1997) markers. Each of the maps is far from saturated, but if these maps can be unified this will of immense value to the melon and cucurbit community and will lay the groundwork for long term genome research for cucurbits.

**Gene tagging**

High-density genetic maps facilitate identification of genes of interest, including disease resistance genes. In crop plants, this is greatly assisted by bulked segregant analysis (BSA) (Michelmore et al., 1991). In this technique, individuals segregating for a trait of interest are combined into two groups or pools, creating the equivalent of near-isogenic DNA pools. The resulting two bulks are then used for screening available markers. The procedure circumvents the need to create isogenic lines that differ only in the trait of interest and often take many years to develop. For single gene traits, such as *Fom-2* and *Fom-1* genes that confer resistance to races 1 and 2 of *Fom*, markers closely linked to genes of interest can be efficiently identified. A very similar technique was independently developed by Giovannoni et al. (1991) using mapped RFLP markers as the basis for pooling individuals rather than phenotype scoring such as disease resistance or susceptibility.

As described above we have adopted AFLPs for creating a linkage map in melon. The combination of AFLP markers with BSA provides a very powerful tool to rapidly identify tightly linked markers. For example, in barley, among 38 markers found by BSA linked to *Mlo*, a gene whose recessive form confers resistance to the barley powdery mildew pathogen *Erysiphe graminis* f.sp. *hordei*, one was within the gene and two others were very closely linked (0.2 and 0.4 cM) (Bhshges et al., 1997). In tomato, among three markers found in bulks linked to *Cf-9*, a gene that confers resistance to leaf mold pathogen *Cladosporium fulvum*, two were within 10 kb of the gene (Thomas et al., 1995). In a similar way, Meksem et al. (1995) identified 29 AFLP markers linked to the *R1* locus in potato for race-specific resistance to *Phytophthora infestans*; eight were within 0.4 cM and two were inseparable from the gene. Also in potato, Brigneti et al. (1997) screened both resistant and susceptible bulks with 408 AFLP primer combinations and found two markers inseparable from *Ky* gene which is responsible for resistance to potato virus Y using 351 F₂ progenies.

Markers for single gene resistance, as is the case for resistance to fusarium wilt, have the potential to be of immediate use for MAS in breeding programs. MAS offers the advantage that the environmental component of heritability is elimi-
MAS has been applied to pyramiding qualitative bacterial blight resistance genes in rice at IRRI (International Rice Research Institute) (Huang et al., 1997) and is becoming a routine research tool for backcrossing, mapping and selection for single gene traits such as disease resistance (Smith and Beavis, 1996). MAS is particularly suited to breeding for disease resistance because disease evaluation always presents a considerable challenge. In selection for nematode resistance in potato, MAS was shown to be fast and reproducible in selection of maize for drought tolerance at CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo) (Ribaut et al., 1997).

Markers linked to *Fom-2*

We identified previously a RAPD marker (596-1) closely linked to *Fom-2* using BSA (Wechter et al., 1995). In this study, two pools of resistant individuals were used. One pool consisted of seven *F₂* homozygous resistant individuals and another pool consisted of 22 *F₂* heterozygous resistant individuals. The homozygous susceptible bulk consisted of 11 *F₂* individuals. Zygosity was determined by evaluating the resistance of *F₃* plants to race 1 of the fusarium wilt fungus. Screening of 320 random oligonucleotide primers (10-mer) against the bulks identified one primer that produced a 1.6-kb fragment in both homozygous and heterozygous resistant bulks, but not in the homozygous susceptible bulk. Analysis of *F₂* individuals with known resistance phenotypes using the primer correctly identified the disease outcome for 92 of 94 individuals. This translates to 2 cM between 596-1 marker and *Fom-2*. Sequence-specific primers were designed based on the terminal sequences of the 1.6-kb fragment. These primers yielded the specific amplification product in a number of resistant commercial cultivars (Wechter et al., 1998).

To identify more markers linked to *Fom-2* gene, we screened over 400 AFLP primer pairs (204 EcoRI/MseI and 240 PsiI/MseI primer pairs) against the bulks. From the EcoRI/MseI primer pairs, 15 markers were identified—two of them (AGG/CCC, ACT/CAT1) were found to flank the *Fom-2* gene (1.7 cM away) when mapped using 60 backcross progenies. No stable polymorphic bands were, however, identified with PsiI/MseI primer pairs. This situation most likely reflects the genome structure or nucleotide composition around the target regions. Similar kind of findings were reported by Moreau et al. (1998) in tomato. RAPD marker 596-1 was found to cosegregate with the gene in these experiments. From our previous linkage studies, 596-1 was assigned to linkage group III (Wang et al., 1997). The additional new markers (AGG/CCC, ACT/CAT1) caused a rearrangement of other markers (TG/CAAA, AAC/CAT1) that previously flanked 596-1 (Wang, Thomas, Dean, unpublished data).

As in any linkage map construction, the relative map location of individual markers may change slightly with different or expanded populations. In addition, when smaller populations are used, misscoring of DNA genotypes/phenotypes or disease phenotypes has a greater impact on marker order than in studies with relatively larger populations. The use of smaller populations offers, however, the advantage that more markers can be mapped rapidly. Markers that appear to be linked from these studies can then be more precisely mapped when a larger population is available. For MAS it may not be necessary to exhaustively fine map linked markers. However, mapping to a large population is required if the gene is to be cloned.

The two flanking AFLP markers and the cosegregating RAPD marker were used to survey a number of melon genotypes representing diverse geographic origins and genetic backgrounds. As expected, 596-1 predicts correctly the disease phenotypes for all 22 genotypes tested while AGG/CCC missed one and ACT/CAT1 missed four out of the 22 (Wang, Thomas and Dean, unpublished). Work is underway to convert the AFLP and RAPD markers to codominant PCR markers for easy use in MAS. Additional progenies are being used to further refine the map order of the linked AFLP markers. To identify more markers linked to *Fom-2*, KpnI/MseI primers will be evaluated. We have also initiated studies to identify markers linked to *Fom-1*.

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Large-insert library construction

A large-insert genomic DNA library will be of great utility to melon researchers, and will facilitate studies in a number of areas including marker identification and map-based cloning. Obviously, the larger the insert size the fewer clones are needed to represent a plant genome, which can be very large. With DNA fragments of 150 kb, only 3,000 clones are needed for a single genome coverage of melon. Typically, to ensure this library is representative, 20-fold genome coverage is sought. A number of cloning systems are available to clone DNA fragments greater than 100 kb, including PACs and YACs. At this time the most popular are BAC libraries as they offer several advantages, most notably their ease of handling. BACs are essentially very large plasmids and are readily maintained in *E. coli*. BAC libraries have been created for many crop plants including corn, sorghum, wheat and rice. The Clemson University Genomics Institute has made the majority of these libraries and is a national resource for their deposition and distribution.

We have recently initiated a project to create a BAC library of melon MR-1. BAC library construction is technically challenging and dependent on the plant in question. The first step involves isolation of high molecular weight DNA. We have developed a protocol to isolate nuclei from melon (Wang et al., 1997). The nuclei are then mixed with molten low melting-point agarose and formed into plugs. Plugs are then subjected to partial digestion with *Hind* III and the DNA is size separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Fragments greater than 350 kb are subjected to a second size selection by CHEF gel electrophoresis before being electroeluted and ligated into the BAC vector pBeloBAC11 (Shizuya et al., 1992).

The vector contains the *LacZ* gene for the identification of recombinant clones using the blue-white screening method. There are three unique cloning sites: *Bam* HI, *Sph* I, and *Hind* III, flanked by the T7 and SP6 promoters. These promoters facilitate generating RNA probes for chromosome walking and DNA sequencing of the insert fragment at the vector-insert junction. A number of other convenient restriction sites are available for making left and right end specific probes by inverse PCR or plasmid rescue. The G-C rich restriction sites (*Not* I, *Eag* I, *Xma* I, *Sma* I, *Bgl* I, and *Sfi* I) can be used to excise the inserts of BAC clones.

Following dialysis the ligation mix is electroporated into commercially prepared DH10B *E. coli* cells. We have been successful in obtaining BAC clones but at the time of writing we have not been able to obtain clones with inserts larger than 100 kb. We found the DNA plug, once chopped up for partial digestion, appeared to be sticky (presumably due to polysaccharides). This may affect the separation of DNA fragments from each other or from polysaccharides resulting in the trapping of smaller fragments which are preferentially cloned. Further improvements are being developed to solve this problem. Once the library is constructed, we plan to make it available to the cucurbit research community.

Literature cited


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