Identification of a Randomly Amplified Polymorphic DNA Marker Linked to the *Fom 2* Fusarium Wilt Resistance Gene in Muskmelon MR-1

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ABSTRACT

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Resistance to *Fusarium oxysporum* f. sp. *melonis* race 1 (causal agent of Fusarium wilt) in *Cucumis melo* (muskmelon) is controlled by the dominant gene *Fom 2*. Bulked segregant and random amplified polymorphic DNA (RAPD) analyses were employed to identify genetic markers linked to race 1 Fusarium wilt resistance. DNA was isolated from pooled (bulked) susceptible or resistant plant tissue from an F_2 population of a susceptible AY × resistant MR-1 cross that segregated for race 1 resistance. Bulks from resistant plants were composed of both homozygous and heterozygous individuals and also pure homozygous individuals, determined by analysis of F_3 populations. After screening the DNA from bulked resistant and susceptible plants by PCR with 320 decamer primers, one primer, 5′ CCC CTC GAA T 3′, produced a 1.6-kb fragment in the resistant bulks that was absent from the susceptible bulks. Using this primer to screen individual F_2 plants, the outcome of 92 of 94 individual host-pathogen interactions was correctly predicted. Nine susceptible and three resistant commercial cultivars also were screened with this primer. The 1.6-kb fragment was not amplified using DNA from any of these cultivars. This is the first report of a RAPD marker linked to resistance to Fusarium wilt in muskmelon.

Fusarium wilt of muskmelon, *Cucumis melo* L., caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *melonis* W.C. Snyder & H.N. Hans., occurs throughout North America (8,9,10) as well as in Europe and Asia (20). Disease outbreaks, though infrequent, can be devastating, with reports of crop losses as high as 100% (1,20). Four races of *F. oxysporum* f. sp. *melonis*—0, 1, 2, and 1-2—have been identified. Race 1-2 is divided into strains 1-2Y and 1-2W, which correspond to a yellowing and a wilting strain, respectively (23). Races were designated based on strains of the pathogen with the capability to infect muskmelon cultivars containing resistance genes. *Fom 1* and *Fom 2* are two independently inherited, dominant genes that control resistance to races 0 and 2 and races 0 and 1, respectively (15,16,17,18,20,23). When both genes are present, a high level of resistance to races 0, 1, and 2 is conferred (17). Race 0 can only incite disease in muskmelon plants that have no genes for resistance against *F. oxysporum* f. sp. *melonis*. No genes have been identified in muskmelon that confer resistance to either strain of race 1-2 (5,11,12,13,22,23).

Control of Fusarium wilt relies primarily on resistant cultivars. To speed up the breeding process, genetic markers associated with resistance to certain diseases, such as Fusarium wilt, could be used to rapidly screen large numbers of individual plants to ascertain introgression of resistance. Identification of such markers, as opposed to the time-honored but time-consuming method of artificial inoculation, holds promise as a method of shortening resistance screening by reducing the need for extensive inoculations with the Fusarium wilt pathogen.

A recently developed technique for rapid identification of genetic markers employs the polymerase chain reaction (PCR) in combination with bulking individuals from a segregating population to identify random amplified polymorphic DNA (RAPD) markers (14). Bulked segregant analysis creates near isogenic lines (NILs) from a segregating population by pooling DNA of individuals from an F_2 population segregating for a specific trait. In essence, two pools of DNA are obtained that are similar in all genetic respects, except for the phenotype of interest. In this study, the trait used was resistance to race 1 of *F. oxysporum* f. sp. *melonis*. By using arbitrary decamer primers and PCR to screen the susceptible and resistant pools of DNA, any differences due to chromosomal changes or base changes resulting in DNA fragments unique to the resistant pool would be linked to the locus for race 1 Fusarium wilt resistance (14,21).

In this investigation, we report the identification of a 1.6-kb PCR fragment associated with race 1 Fusarium wilt resistance in the multidisease resistant breeding line MR-1. Using this PCR-based marker, we were able to predict resistance with 98% accuracy in a breeding program using line MR-1 and the susceptible cultivar Ananas Yokneum (AY).

MATERIALS AND METHODS

Germ plasm. All crosses were performed by C. E. Thomas and staff at the U.S. Department of Agriculture, Agricultural Research Service, Vegetable Laboratory in Charleston, SC. Reciprocal crosses between MR-1 (resistant to races 0, 1, and 2) (23) and cultivar AY (susceptible to all races of *F. oxysporum* f. sp. *melonis*) were performed to produce the F_1 generation. F_1 were selfed to produce the F_2 generation. F_2 individuals resistant to race 1 *F. oxysporum* f. sp. *melonis* were selfed to produce F_3 lines to determine the F_2 genotype. Forty plants derived from each F_3 were evaluated for resistance to race 1 *F. oxysporum* f. sp. *melonis*. If none of the 40 plants developed symptoms after inoculation with the pathogen,

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then the F_2 line from which they were derived was considered to be homozygous for race 1 Fusarium wilt resistance.

Plant growth conditions. All plants were grown in 10-cm^2 plastic pots containing Redi-earth peatlite potting mixture. Greenhouse temperatures were 25 ± 6°C. Plants grown from November to March were supplemented daily with light provided by four high-intensity sodium lamps from 5:00 p.m. to 11:00 p.m.

Leaf sampling techniques. At the 5-leaf stage and prior to inoculation, the second true leaf from each plant was removed, individually packaged, and frozen at –80°C prior to drying in a Savant Speed Vac concentrator attached to a Savant refrigerated concentration trap (Savant Instruments, Farmingdale, NY) under 5 µm of mercury vacuum for 12 h. The freeze-dried tissues were stored in sealed plastic bags at room temperature.

Bulking of muskmelon tissues. For the bulked segregant analysis experiments, equal weights (200 mg) of freeze-dried muskmelon tissues from each plant in a segregating F_2 population were combined to form two "pools" of DNA. Plant tissues were bulked for DNA extraction after determination of whether F_2 plants were resistant or susceptible to *F. oxysporum* f. sp. *melonis* race 1. Both homozygous resistant bulks and a mixture of heterozygous and homozygous resistant bulks of F_2 individuals as well as homozygous susceptible bulks of F_2 individuals were prepared and used. The mixed resistant bulk contained tissue from 22 resistant plants. The homozygous resistant bulk contained tissue from 7 resistant plants. The susceptible bulk contained tissue from 11 plants.

Fungal culture maintenance. Cultures of *F. oxysporum* f. sp. *melonis* race 1 were maintained on potato-dextrose agar (PDA; 20 g/500 ml of distilled H_2O). Petri plates were sealed with Parafilm and placed in an incubator at 22 ± 1°C for 14 days with continuous fluorescent illumination to facilitate conidial development. Fresh working cultures of the *F. oxysporum* f. sp. *melonis* isolate were obtained from stocks every 6 months to avoid loss of virulence.

Host inoculation. Fourteen-day-old cultures of *F. oxysporum* f. sp. *melonis* race 1 were flooded with sterile distilled H_2O and scraped with a glass rod until a viscous, purple-pink spore suspension was obtained. This slurry was filtered through four layers of sterile cheesecloth, and the filtrate was adjusted with distilled H_2O to obtain a conidial suspension of 10^4 macroconidia per ml.

The day of or the day after collecting leaf tissue candidate plants were carefully removed from pots, and the lower 1 cm of the root-ball was cutoff using a sterile razor blade. The root-ball was dipped into the conidial suspension for 10 s and replaced in its pot. Control plants were inoculated in the same manner with sterile distilled H_2O. All plants were incubated in the greenhouse for the duration of the experiment.

Disease scoring for Fusarium wilt. Symptoms typically developed within 14 days in susceptible plants. At 21 days, plants were rated as dead or alive. Plants surviving after 21 days were reinoculated as described above and monitored for an additional 21 days before being rated as resistant. Crown sections from stems of all plants, both susceptible and resistant, were surface-sterilized and plated on PDA to verify Fusarium infection.

PCR. DNA was isolated following the protocol described by Dellaporta et al. (2), with the exception that freeze-dried material was used. For bulk segregant analysis experiments, 1 g of freeze-dried bulked leaf tissue was used. In contrast, 50 mg of freeze-dried leaf tissue was used for DNA isolation from individual plants. DNA quality and quantity was estimated by visualization on an agarose gel. PCR amplifications were carried out in 25-µl reaction volumes. Each reaction contained 2 µl of DNA (5 ng/µl of stock), 9.5 µl of sterile distilled H_2O, 2.5 µl of Perkin-Elmer Cetus (Norwalk, CT) 10× PCR buffer II, 4 µl of $MgCl_2$ (25 mM stock), 0.5 µl each of Perkin-Elmer dATP, dCTP, dGTP, and dTTP (10 mM stock), 0.125 µl of Perkin-Elmer Ampli*Taq* polymerase, 2.5 µl of nonacetylated bovine serum albumin (New England Biolabs, Beverly, MA), and 2.5 µl of 10-mer primer (10 mM stock). Primers were synthesized by Operon (Operon Technologies, Alameda, CA), the University of British Columbia (UBC), Vancouver, BC, Canada, Clemson University, Clemson, SC, or DNA International (Lake Oswego, OR). Primer kits utilized for these experiments were Operon OPD 1-20 and UBC sets 1, 2, and 6. PCR was run on a Coy model 60 (Coy Laboratory Products, Ann Arbor, MI) thermocycler or a Perkin-Elmer model 480 thermocycler. Cycle parameters were 5 min at 94°C, followed by 45 cycles of 1 min at 93°C, 1 min at 35°C, and 2 min at 72°C, with a 5 min 72°C extension. Amplified products were electrophoresed at 5 V/cm for 2 h in a 1.5% agarose gel and stained with ethidium bromide.

Genetic linkage assessment. To determine the degree of linkage of the RAPD marker to the *Fom 2* resistance gene, DNA was isolated from 94 F_2 individuals. These plants were inoculated with *F. oxysporum* f. sp. *melonis* race 1 and later scored for resistance. The DNA from each F_2 was amplified by PCR using a decamer primer, 5′ CCC CTC GAA T 3′, which yielded a unique 1.6-kb fragment in the resistant DNA pools from our bulk segregant analysis studies.

Southern blot and DNA hybridization. DNA for Southern blotting was transferred from agarose gels onto Hybond-N nylon membrane (Schleicher & Schuell, Keene, NH) by capillary action using standard protocols described by Sambrook et al. (19). DNA was fixed to the membrane by exposure for 5 min to UV light from a Foto/prep I transilluminator (Fotodyne, Hartland, WI).

PCR fragments or cloned products used as hybridization probes were radiolabeled with ^{32}P αdCTP (Du Pont Co., Wilmington, DE) using a Hexamer random primed DNA labeling kit (United States Biochemical Corporation, Cleveland) following the manufacturer's protocol. After overnight hybridization in standard buffer (6× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 sodium citrate, pH 7.0]) at 65°C, the membrane was passed through a series of washes of decreasing concentrations of SSC and sodium dodecyl sulfate (SDS) to a final wash concentration of 0.125× SSC containing 0.025% SDS for 20 min at 65°C, wrapped in plastic wrap, overlaid with Kodak X-OMAT autoradiograph film (Eastman Kodak Co., Rochester, NY) with intensifying screen, and left at –80°C until the desired signal strength was achieved. Film was developed according to manufacturer's specification with Kodak chemicals.

Cloning and sequencing of the RAPD band. The Promega pGEM-T vector systems kit (Promega, Madison, WI) was used to clone the linked RAPD product. The manufacturer's protocol was followed for ligation, plating, and screening for transformants. An appropriate clone, identified by colony lift and Southern hybridization using the 1.6-kb ^{32}P-labeled PCR product was restriction mapped with a combination of several single and double restriction endonuclease digests.

The nucleotide sequence of the termini of the cloned RAPD fragment was determined using Applied Biosystems *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and the supplied protocol. Samples were run on an Applied Biosystems model 373A automated DNA sequencing system. M13 forward and M13 reverse primers from Applied Biosystems were used for sequencing.

RESULTS

Genetic variability between MR-1 and AY. To determine the amount of difference between the parental lines MR-1 and AY, DNA was amplified using five arbitrary 10-mer primers. The results showed 26 shared bands and 11 unshared bands, representing a 30% overall genetic difference between the parents for the primers tested. This is a high degree of genetic difference and satisfactory for the use of bulked segregant analysis

Detection of a RAPD marker in bulked DNA pools. One polymorphic marker linked to resistance and one putative polymorphic marker linked to susceptibility were identified after screening

320 arbitrary oligonucleotide primers. The latter was not characterized further. The primer, UBC #596 (5′ CCC CTC GAA T 3′), yielded a 1.6-kb band in resistant bulks that was absent from the susceptible bulks (Fig. 1). This band was seen in F_2 bulks in which the resistant pool was composed of both homozygous and heterozygous individuals and also in pure homozygous bulks. Amplification of parental DNA produced the same 1.6-kb band in MR-1 but not in AY (Fig. 2). Ninety-eight percent (315/320) of the primers used in these experiments produced distinct amplified products when both heterozygous and homozygous bulks were used. If a primer produced no amplification in either bulk or only in one bulk or if a suspected polymorphism was present, the reaction was repeated up to three times to verify these results.

Genetic linkage between the 1.6-kb RAPD marker and *Fom 2*. The outcome of the host-pathogen interaction could be correctly predicted in 92 of 94 plants (Fig. 3) using UBC primer #596. This equates to 2 recombinants of 94. A genetic distance of 2.2 centimorgans between the RAPD marker and *Fom 2* was estimated using Mapmaker 1.9 (7). This represents a close linkage between the marker and the locus for resistance to race 1 Fusarium wilt in MR-1.

Twelve commercial cultivars, three resistant to *F. oxysporum* f. sp. *melonis* race 1 and nine susceptible to *F. oxysporum* f. sp. *melonis* race 1, failed to amplify the 1.6-kb fragment in any of the lines (Fig. 4). Southern hybridization of the PCR products of these cultivars with the 1.6-kb fragment yielded no detectable hybridization signal (Fig. 5). The additional hybridizing bands that occurred when using MR-1–derived DNA probably resulted from the low annealing temperature (35°C) used for PCR in these experiments.

Nucleotide sequence of RAPD fragment. Four hundred, seventy-six nucleotides of the terminal ends of the 1.6-kb RAPD fragment were sequenced: 198 nucleic acids from one end, using the M13 forward primer, and 278 nucleic acids from the other end, using the M13 reverse primer. A comparative search of the nonredundant DNA databases accessible through the National Center for Biotechnology Information, Bethesda, MD, was performed using the Blast algorithm. No significant matches were found. The sequences have been deposited with GenBank: accession number U29517 for the 198-base sequence (melon seq-F) and accession number U29518 for the 278-base sequence (melon seq-R).

DISCUSSION

Recent studies have shown that some domesticated crops that have been placed under intensive breeding programs have become genetically similar to the point where little if any genetic variation can be detected (4). PCR amplified products from AY and MR-1, using a limited number of decamer primers, showed a 30% genetic dissimilarity between the parents. These results indicated that a significant genetic difference does exist in these lines. Because of this, the use of RAPD to identify a specific genetic difference associated with resistance by direct comparison of these two lines would be extremely difficult. The use of bulk segregant analysis allowed us to obtain NILs for this analysis that facilitated the search for genetic markers linked to the *Fom 2* resistance gene.

We found one polymorphism for resistance and one associated with susceptibility for 320 analyzed decamer primers. The marker associated with resistance was linked very tightly to the *Fom 2* locus and will be extremely valuable for breeding programs using MR-1 as the source of resistance to *F. oxysporum* f. sp. *melonis* race 1. Although this marker does not appear to be associated with resistance in the other cultivars tested, MR-1, which possesses multiple disease resistance, is a widely used breeding line. The source of resistance in other cultivars tested is unknown. Utilizing this PCR-based marker in a MR-1 breeding program would identify introgression with at least 98% accuracy at the F_2 level. At this level, current screening procedures are both labor-intensive and time-consuming and afford a possibility for escapes in the inoculation process. Genetic analysis of the marker linked to susceptibility was not pursued because it would not be useful for introgression of resistance. It is noteworthy that the marker we detected is very tightly linked. Indeed, we rejected other candidate markers that may have been weakly linked if we detected faint bands in the susceptible bulk.

The number of polymorphisms linked with resistance in our work was lower then reported for other plant systems. Michelmore et al. (14) found three polymorphisms associated with resistance to *Bremia lactucae* in lettuce using 100 decamer primers, Kesseli et al. (6) found four polymorphisms for resistance to *Plasmopara*

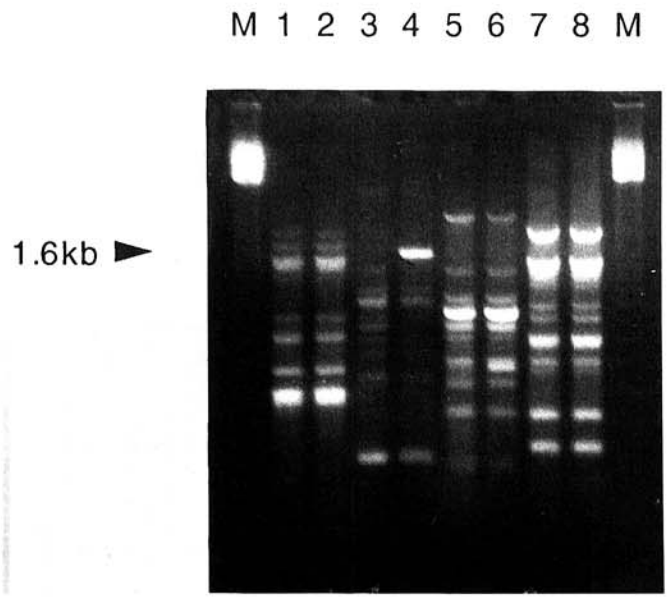

Fig . 1. Ethidium bromide-stained gel of polymerase chain reaction amplification products from muskmelon using decamer primers from the University of British Columbia, Vancouver, BC, Canada, and bulked Fusarium wilt resistant and susceptible F_2 muskmelon DNA. Lane 1 is primer 595, susceptible F_2 DNA. Lane 2 is primer 595, resistant F_2 DNA. Lane 3 is primer 596, susceptible F_2 DNA. Lane 4 is primer 596, resistant F_2 DNA. Lane 5 is primer 597, susceptible F_2 DNA. Lane 6 is primer 597, resistant F_2 DNA. Lane 7 is primer 598, susceptible F_2 DNA. Lane 8 is primer 598, resistant F_2 DNA. M is *Hin*dIII-digested λ DNA marker.

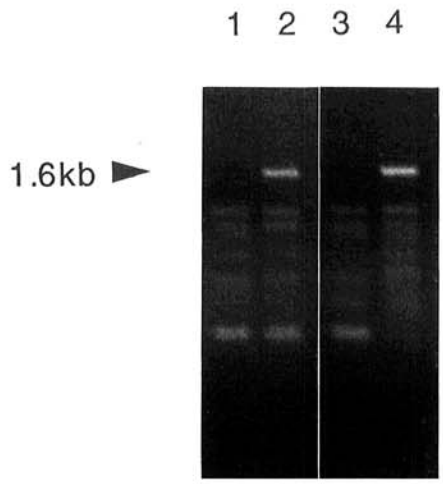

Fig. 2. Ethidium bromide-stained gel of DNA polymerase chain reaction amplification products from muskmelon using primer 596 from the University of British Columbia, Vancouver, BC, Canada. Lane 1 is from bulked Fusarium wilt susceptible F_2 DNA. Lane 2 is from bulked homozygous resistant F_2 DNA. Lane 3 is from AY DNA. Lane 4 is from MR-1 DNA.

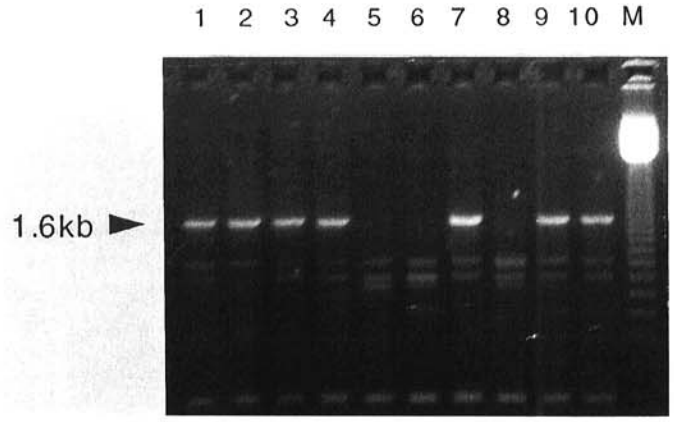

Fig. 3. Ethidium bromide-stained agarose gel of DNA polymerase chain reaction amplification products of 10 F_2 muskmelon individuals using primer 596 from the University of British Columbia, Vancouver, BC, Canada. Lanes 1–4, 7, 9, and 10 Fusarium wilt resistant individuals. Lanes 5, 6, and 8 susceptible individuals.

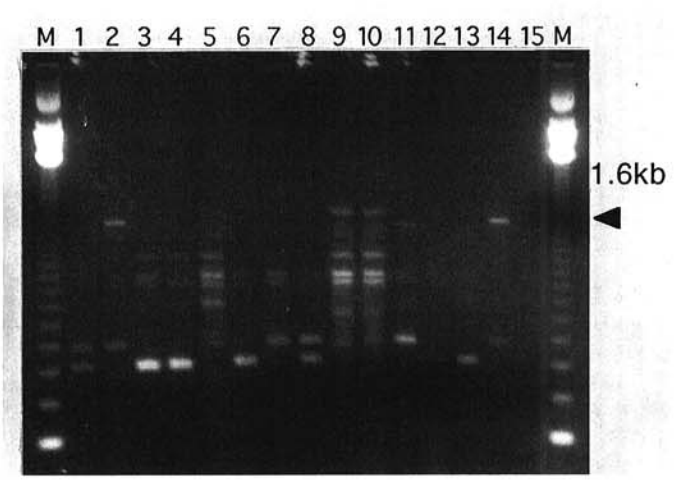

Fig. 4. Ethidium bromide-stained agarose gel of DNA polymerase chain reaction amplification products of 11 commercial muskmelon cultivars using primer 596 from the University of British Columbia, Vancouver, BC, Canada. Arrow indicates 1.6-kb band associated with race 1 Fusarium wilt resistance in bulk segregant analysis studies. Lanes 1 and 2 are susceptible and resistant F_2 individuals, respectively. Lanes 3–12 are cultivars Sweet Supreme (susceptible), Opera (resistant), Hy-mark (susceptible), Charentais T (susceptible), Supermarket (susceptible), Dorado (susceptible), Topmark (susceptible), PMR-45 (susceptible), CM 17-187 (resistant), and Sierra (resistant), respectively. Lanes 13 and 14 are AY and MR-1, respectively. Lane 15 is empty. Lane M is a 123-bp ladder.

lactucae-radicis in lettuce using 100 decamer primers, and Haley et al. (3) found 14 polymorphisms associated with resistance to *Uromyces appendiculatus* in common bean using 306 decamer primers. We have no obvious explanation for the low number of markers found linked to Fusarium wilt resistance.

One modification reported in this investigation to existing extraction and bulking protocols for plant DNA was that all tissues used were freeze-dried first. Other procedures use fresh tissues. In our studies, the tissue, once dried, was stored for up to 3 years in a resealable plastic bag in a laboratory drawer without apparent loss of extractable DNA. Another modification made in our protocol that differs from that of Michelmore et al. (14) is the actual bulking procedure. Michelmore et al. extracted DNA separately from each plant then bulked the resulting DNA (14). In our protocol, freeze-dried plant tissues were pooled by equal weight, homogenized in a spice mill, and the DNA was extracted from the bulked tissue. This may be valuable in laboratories that want to utilize bulked

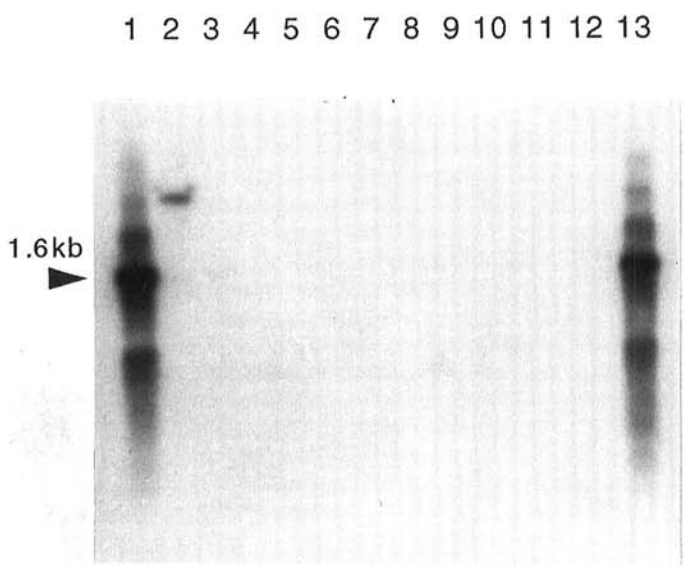

Fig. 5. Southern blot of polymerase chain reaction-generated products from DNA of commercial muskmelon cultivars. Lane 1 is a Fusarium wilt-resistant F_2 individual. Lane 2 is Sweet Supreme (susceptible). Lane 3 is Opera (resistant). Lane 4 is Hy-mark (susceptible). Lane 5 is Charentais T (susceptible). Lane 6 is Supermarket (susceptible). Lane 7 is Dorado (susceptible). Lane 8 is Topmark (susceptible). Lane 9 is PMR-45 (susceptible). Lane 10 is CM 17-187 (resistant). Lane 11 is Sierra (resistant). Lane 12 is AY. Lane 13 is MR-1.

segregant analysis but that are not equipped with a microfluorometer.

This is the first report of a RAPD marker for resistance to Fusarium wilt in muskmelon. Our results confirm the utility of using bulk segregant and RAPD analyses to quickly identify tightly linked DNA markers that may readily be used in breeding programs. Additionally, the identified marker will assist greatly in cloning the *Fom 2* gene.

Future work will include screening more primers to identify other Fusarium wilt-resistance markers. Also, complete sequencing of the 1.6-kb fragment is currently being performed. This sequence data will be used to design specific primers to generate sequence characterized amplification regions. The use of these primers in a screening/breeding program will eliminate the need to discern the fragment of interest among many fragments generated with single-primer RAPDs. This, in addition to the reduced possibility of artifact fragments associated with RAPD, will yield a more reproducible and specific assay technique.

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