Marker Saturation of the Region Flanking the Gene *NSV* Conferring Resistance to the Melon Necrotic Spot *Carmovirus* (MNSV) in Melon

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ABSTRACT. The recessive allele (*nsv*) of the *NSV* gene confers resistance to the *Carmovirus* melon necrotic spot virus (MNSV) in melon (*Cucumis melo* L.). Using an F₂ population obtained from the cross between the resistant Korean accession PI 161375 and a susceptible line of 'Piel de Sapo', we have mapped the *NSV* locus to linkage group 11 (G11) of the melon genome. Additional markers closely linked to *NSV* were developed by bulked segregant analysis (BSA) using a doubled haploid progeny population derived from the same cross. A detailed map of the *NSV* region was constructed containing 10 markers spanning a distance of 17.7 cM. The nearest flanking markers to *NSV* were two amplified fragment length polymorphisms (AFLPs) (CTA/ACG-115 and CTA/ACG-120) and one random amplified polymorphic DNA (RAPD) (OPD08-0.80) separated by 5.9 cM. Two more markers, ACC/ACC-110 and OPX15-1.06, cosegregated with *NSV*.

Melon necrotic spot virus (MNSV) is a single-stranded RNA *Carmovirus* of the Tombusviridae family. MNSV infects cucurbit crop species such as melons, cucumbers and watermelons, grown under greenhouse conditions, and causes severe yield loss.

The virus was first described in Japan (Kishi, 1966) and later detected on greenhouse-grown melons in western regions (Gonzalez-Garza et al., 1979). MNSV can be transmitted mechanically, by zoospores of the fungus *Olpidium bornovanus*, by chewing insects and through the seed (Lecoq et al., 1998). Symptoms produced by MNSV in melon include local necrotic spots on inoculated cotyledons or large necrotic lesions on systemic leaves and necrosis on stems and petioles. Severity of the symptom depends on the growing conditions and the cultivar. For example, European MNSV isolates induce systemic symptoms in cucumber and melon only erratically. Mechanically infected melon cotyledons show necrotic spots of 2 to 3 mm in diameter 3 to 5 d after inoculation and cotyledons die 15 to 21 d after inoculation.

The spread of MNSV in cultivated melon can be controlled through the use of virus-free seeds or resistant varieties. There are at least two sources of resistance to MNSV in melon: the cultivar 'Gulfstream' and the Korean accession PI 161375. The resistance

is controlled by the recessive allele (*nsv*) of the *NSV* gene (Coudriet et al., 1981) and is effective against all known strains of the virus, except for a new strain recently described (Díaz et al., 2000). Several commercial cultivars have been released that are homozygous for the *nsv* resistance allele. The gene has been mapped to linkage group 7 of the first genetic map of melon (Baudracco-Arnas and Pitrat, 1996) with the nearest flanking markers at distances of 13.5 and 10.7 cM.

Although the pathological test used in breeding programs to detect MNSV (based on MNSV inoculation) is inexpensive and easy to perform, the availability of molecular markers closely linked to the *nsv* allele could speed up selection as has been shown for other traits (Tanksley et al., 1989). In melon, tightly linked markers are available for only one gene, *Fom-2*, that confers resistance to races 0 and 1 of *Fusarium oxysporum* f.sp. *melonis* (Wang et al., 2000; Zeng et al., 1999). Other important disease resistance genes such as *Fom-1* against *Fusarium oxysporum* f.sp. *melonis* races 0 and 2, *Pm-1* against the powdery mildew incited by *Sphaeroteca fuliginea* race 1, and *nsv* are not currently associated with any molecular markers.

In this paper we describe the development of molecular markers linked to the *NSV* gene. The chromosomal location of this gene was first determined in an F₂ mapping population. Additional markers closely linked to the gene were developed with amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA (RAPD) markers bulked segregant analysis (BSA) (Michelmore et al., 1991) in a population of doubled haploid lines (DHLs). A detailed map flanking the *NSV* region was constructed consisting of 10 markers spanning 17.7 cM.

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Materials and methods

PLANT MATERIAL. Two segregating populations of melons (*Cucumis melo* L.) were used for mapping. The 'Piel de Sapo' line T111 (PS) from Semillas Fitó S.A. and the resistant Korean accession PI 161375 were crossed to obtain 93 F_2 individuals used for the construction of a linkage map (Oliver et al., 2001). For the MNSV test in the F_2 population each individual was selfed to obtain F_3 seed for a progeny test. A population of 69 doubled haploid lines (DHLs) was generated in our laboratory (Dolcet et al., unpublished) from the F_1 hybrid between PS and PI 161375 by in vivo induced parthenogenesis and in vitro embryo rescue, after Sauton and Dumas de Vault (1987). In vitro chromosome doubling of the haploid plants and further selfing produced several homozygous lines.

MNSV RESISTANCE ASSAY ON F_3 AND DHLs. Isolate M-8-85 of MNSV was propagated on the susceptible melon line PS by mechanical inoculation of the cotyledons and the infected plants were kept as a source of virus. One gram of tissue disks from cotyledons containing fresh MNSV lesions was ground in 4 mL of 0.03 M Na_2HPO_4 , pH 8.5 that contained 0.2% DIECA and activated charcoal (75 mg·mL⁻¹). This extract was spread over the cotyledons of 2-week-old melon plants. The cotyledons were dusted previously with carborundum or in some cases it was added to the extract before inoculation. Finally the inoculated cotyledons were washed with water to remove excess of carborundum and charcoal.

Plants were visually scored as susceptible 3 to 5 d after the mechanical inoculation if they showed MNSV necrotic spots on the cotyledons. If no symptoms were detected 10 d after the inoculation plants were scored as resistant to MNSV (Pitrat and Lecoq, 1984).

The MNSV test was performed on the cotyledons of 20 F_3 seedlings of each of 91 of the 93 F_2 individuals and on 10 seedlings from each of 69 DHLs.

MARKER ANALYSIS. Total genomic DNA from F_2 individuals and DHLs was isolated from young leaves with the protocol described by Doyle and Doyle (1990) with some modifications (Garcia-Mas et al., 2000) to improve the DNA quality.

To generate DNA bulks for BSA, eight resistant and eight susceptible DHLs were used for AFLP analysis. A different set of 10 resistant and 10 susceptible DHLs were used to obtain the DNA bulks for RAPD analysis. For each bulk, genomic DNA from each DHL was mixed in equal concentration.

A total of 168 RAPD primers were tested on the DNA pools, which belonged to the Operon series A, B, C, D, E, F, J, T, U, and Z (Operon Technologies Inc., Alameda, California). RAPD reactions were performed as described by Williams et al. (1990) with some modifications: 10 to 20 ng of template, 1× buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl_2 and 0.001% gelatin), 0.44 mM MgCl_2 , 0.1 mM dNTPs, 1 unit of AmpliTaq (Perkin Elmer-Applied Biosystems) and 20 ng of decamer primer. Reactions were performed in a final volume of 25 μL in a Perkin Elmer 9700 thermocycler with an initial denaturing step of 1 min at 94 °C followed by 45 cycles of melting at 94 °C for 10 s, annealing at 35 °C for 10 s and extension at 72 °C for 1

min, and a final extension step at 72 °C for 2 min. RAPD fragments were separated on a 2.5% agarose gel at 120 V, and DNA bands were visualized by ethidium bromide staining. Primers showing no amplification or polymorphism were excluded from subsequent analysis. After this initial screening, tests with polymorphic primers were repeated. RAPD primers with reproducible polymorphisms were tested with the individual DHLs and the parental DNA. The nomenclature used for the RAPD markers consisted of the letters OP (for Operon Technologies), followed by the primer reference (i.e., D08) and the size of the amplified band in kilobases, preceded by a dash.

A total of 184 primer pairs were tested on the DNA pools, obtained with all the possible combinations between the AFLP selective primers *Mse*I (CTT, CTG, CTC, CTA, CAT, CAG, CAC, CAA, CGT, CGG, CGC, CGA, CCT, CCG, CCC, CCA, ACT, ACG, ACC, ACA, AAT, AAC, AAA) and *Eco*RI (AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG). AFLP markers were obtained as described by Vos et al. (1995). Genomic DNA (500 ng) was digested using *Eco*RI and *Mse*I restriction enzymes, and specific adapters were ligated to the resulting fragments. Five microliters of template DNA from a 1:5 dilution were used for preamplification with primers carrying one selective nucleotide. The preamplification products were diluted 1:15 and used as template for selective amplification. Primers with three selective nucle-

Fig. 1. Melon linkage group 11 (G11) showing the *Nsv* gene that maps at the same position as markers OPX15-1.06 and MC320, on the left. The detailed map of the *Nsv* region, obtained with the population of DHLs, is shown on the right. Flanking markers of the region interval are aligned with their positions in G11. Markers in bold on the map on the left are RFLP markers. Genetic distances are in centimorgans (cM).

Fig. 2. AFLP electropherogram obtained with the Genescan software (ABIPrism 310). A fraction of the AFLP pattern that includes AFLP markers CTA/ACG-115/CTA/ACG-120 and ACC/ACC-110, tested in DNA bulks and individual DHLs, is represented. Polymorphic DNA fragments are shown with arrows.

otides were used, with the *EcoRI* primer labelled with fluorescent yellow, green and blue dyes (Perkin Elmer-Applied Biosystems). The resultant labeled fragments were run by capillary electrophoresis on an Abi-Prism 310 automated DNA sequencer (Perkin Elmer-Applied Biosystems) and analyzed using GENESCAN Analysis Software 2.0. AFLPs were noted with the *MseI/EcoRI* selective primers followed by a dash and the size of the amplified fragment in base pairs.

The RFLP probe MC320 used in this study was a cDNA clone previously mapped in the melon genetic map by Oliver et al. (2001). Five micrograms of genomic DNA from the parents and DHLs were digested with the restriction enzyme *EcoRV*, run on a 0.9% agarose gel at 30 V for 12 h and blotted onto Hybond N+ membranes. The RFLP probe was labeled using the random priming method (Sambrook et al., 1989). Prehybridization of membranes was performed in 0.5 M Na_2HPO_4 , 0.5 M NaH_2PO_4 , 7 M SDS, 10 mg·mL⁻¹ BSA and 0.05 mg·mL⁻¹ of denatured salmon sperm DNA at 65 °C for 2 to 3 h and afterwards hybridized in the same buffer with the labelled probe at 65 °C overnight. Washes were carried out with 2× SSC, 0.1% SDS and 1× SSC, 0.1% SDS at 65 °C for 20 min each, and membranes were exposed to AGFA Curix RP2 film for 2 to 3 d.

MAPMAKER v. 3.0 (Lander et al., 1987) was used to locate the *NSV* gene in the F_2 map and for the construction of the map around the *NSV* region with the DHLs population. All markers were placed in the maps with a LOD ≥ 5.0 .

Results

NSV MAPPING IN THE F_2 POPULATION. Based on the F_3 progeny test data, the segregation of response to MNSV in the F_2 population was 22:49:20 (homozygous resistant : heterozygous susceptible : homozygous susceptible). This agrees with the expected 1:2:1 segregation ($\chi^2 = 0.62^{\text{NS}}$). These data were used to map the *NSV* gene to linkage group 11 in the vicinity of the morphological trait *P* (trimerous vs. pentamerous number of carpels) (Fig. 1). Two markers present in G11, MC320 and OPX15-1.06, cosegregated with *NSV*. The dominant RAPD marker OPX15-1.06 was amplified from the resistant parent PI 161375. Two AFLP markers, CAT/AAC-296 and CCT/AAC-183, which cosegregated with *P* and were located 9.2 cM away from the gene, were later used to orient the genetic map around the *NSV* locus.

BULKED SEGREGANT ANALYSIS OF DHLs WITH RAPD AND AFLP MARKERS. DHLs were tested for resistance to MNSV. Of 69 DHLs 32 were resistant and 37 susceptible, corresponding to the 1:1 ($\chi^2 = 0.36^{\text{NS}}$) expected segregation. From 184 AFLP primer combinations tested we identified 10 reproducible DNA fragments present in only one of the

DNA pools. These fragments were also tested in PI 161375, PS and the 16 DHLs used to build the DNA pools. Five markers, CTA/ACG-115, CTA/ACG-120, CGA/ACA-74, ACC/AAC-144 and ACC/ACC-110 were selected for further assays because they correlated with either the presence or absence of the resistance allele in the pooled individuals. AFLP markers CTA/ACG-115 and ACC/

Table 1. Sequences of the RAPD and the AFLP primers used to detect markers linked to the *NSV* gene. Selective nucleotides for AFLP primers are in bold.

Marker	Primers	Sequence
OPD08-0.80	OPD08	GTGTGCCCCA
OPX15-1.06	OPX15	CAGACAAGCC
CTA/ACG-115	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACTA
ACC/ACC-110	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAAACC
CGA/ACA-74	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAAACGA
ACC/AAC-144	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTC AAC GATGAGTCCTGAGTAAACC
CAT/AAC-296	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTC AAC GATGAGTCCTGAGTAAACAT
CCT/AAC-183	<i>EcoRI</i> - <i>MseI</i> -	GACTGCGTACCAATTC AAC GATGAGTCCTGAGTAAACCT

Fig. 3. Ethidium bromide stained agarose gel showing RAPD OPD08-0.80 in parental lines (PI = PI 161375; PS = 'Piel de sapo'), DNA bulks resistant (R) and susceptible (S) individuals. The polymorphic band of 800 bp present in the susceptible individuals is shown with an arrow. Size marker is pUC mix (MBI Fermentas).

ACC-110 were associated with a DNA fragment present in the susceptible DNA pool and in PS (Fig. 2), whereas CTA/ACG-120, ACC/AAC-144 and CGA/ACA-74 revealed a DNA fragment present in the resistant DNA pool and in PI 161375. The primer combinations used to obtain the AFLP markers are shown in Table 1. Primer combination CTA/ACG showed two polymorphic DNA fragments of 115 and 120 bp in size (CTA/ACG-115 and CTA/ACG-120) (Fig. 2). When tested in the 16 DHLs used to obtain the DNA pools, CTA/ACG-115 and CTA/ACG-120 fragments were present only in the susceptible or in the resistant individuals, respectively.

Pooled DNA samples were screened with 168 RAPD primers, and 13 polymorphic DNA fragments were identified. When tested with the parental and individual DHLs, only RAPD OPD08-0.80 was reproducibly linked to *NSV*. Marker OPD08-0.80 was present in PS and the susceptible DNA pool (Fig. 3). Resistant individuals tested did not show the 800 bp band corresponding to marker OPD08-0.80. Instead they had a lightly stained fragment of 850 bp.

GENETIC MAP AROUND THE *NSV* GENE. Markers linked to the *NSV* gene were scored in the DHL population to construct a linkage map of the region surrounding the resistance gene. Seven AFLP markers (CTA/ACG-115, CTA/ACG-120, CGA/ACA-74, ACC/AAC-144, ACC/ACC-110, CAT/AAC-296, CCT/AAC-183), two RAPD markers (OPD08-0.80 and OPX15-1.06) and one RFLP marker (MC320) were mapped in this region (Fig. 1). Two markers, ACC/ACC-110 and OPX15-1.06 cosegregated with *NSV*. The closest flanking markers were CTA/ACG-115/CTA/ACG-120 and OPD08-0.80, which were located 1.5 cM and 4.4 cM from *NSV*, respectively. The most distant markers MC320 and CAT/AAC-296/CCT/AAC-183 delimited a region of 17.7 cM around *NSV*. The location of four of these markers (MC320, OPX15-1.06, CAT/AAC-296 and CCT/AAC-183) plus the *NSV* gene was in agreement with the map obtained with the F_2 population.

Discussion

The *NSV* gene was mapped to linkage group G11 of the map constructed by Oliver et al. (2001) and cosegregated with RAPD OPX15-1.06 and RFLP MC320. The RAPD OPX15-1.06 marker was scored as dominant, and therefore positioned with a relatively low precision when compared to the codominant markers such as MC320. Flanking this region on one side and 9.2 cM apart was a cluster of markers including two codominant RFLPs (MC50A, and

MC255B), two dominant AFLPs (CAT/AAC-296 and CCT/AAC-183) and the dominant carpel number (*P*) gene. On the opposite side, at a distance of 18 cM, was the codominant RFLP marker MC330. Baudracco-Arnas and Pitrat (1996) placed the *nsv* allele on their linkage group 7, also on the vicinity of *P*. The populations used for the construction of both maps had one parent in common (PI 161375), that was also the source of the MNSV resistance. A comparison between these maps based on common RFLP and SSR markers (Danin-Poleg et al., 2000) confirmed that G11 of our map corresponded to linkage group 7 of the map constructed by Baudracco-Arnas and Pitrat (1996).

A population of DHLs derived from the same cross was used for further marker saturation of this region using a BSA strategy. DHLs were more suitable for this purpose than the F_2 population because 1) their complete homozygosity would allow detection of dominant marker alleles together with either the resistance or the susceptibility allele of the *NSV* gene, making the test more efficient, and 2) the MNSV test can be performed with several replications, whereas F_2 population analysis require F_3 progeny tests for a similar level of accuracy. This approach enabled us to detect six additional markers in the *NSV* region, and to locate with more precision the four markers already mapped with the F_2 population. Thus, we were able to place 10 markers in a relatively short (17.7 cM) chromosomal region that includes *NSV*, five of which were located within a 5.9 cM genetic interval flanking this gene. Two of the markers (ACC/ACC-110 and OPX15-1.06) cosegregated with *NSV*.

Five of the markers obtained were AFLPs and one a RAPD, indicating that in this study AFLPs were more efficient than RAPDs for the detection of linked markers with BSA. The RAPD method used to amplify longer DNA fragments than AFLPs and their conversion into polymorphic and codominant PCR-based markers, like sequence characterized amplified regions (SCARs) or cleaved amplified polymorphic sequences (CAPSs), is usually easier than that of AFLPs. Thus, the use of both kinds of markers is justified for the saturation of specific genomic regions.

The discovery and mapping of markers closely linked to *NSV* will facilitate selection of the resistant allele in melon breeding programs. Conversion of these markers into sequence-specific codominant PCR markers will further improve their efficiency. Two of these markers, CTA/ACG-115 and CTA/ACG-120, located at a distance of 1.5 cM from *NSV*, were obtained with the same primer combination, one linked to the susceptible allele and the other to the resistant allele with very similar sizes of their amplified products (115 and 120 bp). It is possible that these two markers are allelic and that CTA/ACG yields a codominant AFLP. Cloning and sequencing of both DNA fragments would be required to confirm this.

The resolution of the current map is still low (1.5 cM/recombination event) because the size of the DHL population was small. Two of the markers, OPX15-1.06 and ACC/ACC-110, could not be separated from *NSV* and their distance to the gene is less than 1.5 cM. We are currently converting the closest flanking markers (OPD8-0.80, CTA/ACG-115 and CTA/ACG-120) into codominant PCR-based markers to allow the screening of a large F_2 population to search for recombinant individuals. Such recombinants will allow us to refine the position of all markers and to initiate a second round of BSA that will permit the characterization of additional and more tightly linked markers. On average a genetic distance of 1 cM corresponds to a physical distance of 380 kb if we consider the melon genome to contain 454 Mb of DNA (Arumuganathan and Earle, 1991) and a genetic distance of 1197 cM (Oliver et al., 2001). To isolate *NSV* by positional cloning, we will need to identify tightly

linked flanking markers. A bacterial artificial chromosome (BAC) library has already been reported for melon (Luo et al., 2001), and one more is also available to us obtained in the Institut de Biologia Molecular de Barcelona (P. Puigdomènech, personal communication). Availability of such libraries will facilitate our ultimate objective of cloning this gene.

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