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ORIGINAL PAPER

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A physical map covering the *nsv* locus that confers resistance to *Melon necrotic spot virus* in melon (*Cucumis melo* L.)

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Abstract *Melon necrotic spot virus* (MNSV) is a member of the genus *Carmovirus*, which produces severe yield losses in melon and cucumber crops. The *nsv* gene is the only known natural source of resistance against MNSV in melon, and confers protection against all widespread strains of this virus. *nsv* has been previously mapped in melon linkage group 11, in a region spanning 5.9 cM, saturated with RAPD and AFLP markers. To identify the *nsv* gene by positional cloning, we started construction of a high-resolution map for this locus. On the basis of the two mapping populations, F_2 and BC1, which share the same resistant parent PI 161375 (*nsv*/*nsv*), and using more than 3,000 offspring, a high-resolution genetic map has been constructed in the region around the *nsv* locus, spanning 3.2 cM between CAPS markers M29 and M132. The availability of two melon BAC libraries allowed for screening and the identification of new markers closer to the resistance gene, by means of BAC-end sequencing and mapping. We constructed a BAC contig in this region and identified the marker 52K20sp6, which co-segregates with *nsv* in 408 F_2 and 2.727 BC1 individuals in both mapping populations. We also identified a single 100 kb BAC that physically contains the resistance gene and covers a genetic distance of 0.73 cM between both BAC ends. These are the basis for the isolation of the *nsv* recessive-resistance gene.

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Introduction

Melon (*Cucumis melo* L.) is an economically important cucurbit cultivated in tropical and subtropical climates. It is a diploid species ($2n = 2x = 24$) which has an estimated genome size of 450 Mb (Arumuganathan and Earle 1991). Several melon genetic maps have been constructed based mainly on RAPDs, RFLPs, AFLPs and SSRs, and some important agronomic traits have been positioned in these maps (Gonzalo et al. 2005; Perin et al. 2002). Among the important agronomic traits, virus resistance is a major melon-breeding objective, as several diseases caused by viruses have great economic impact in melon-growing regions worldwide. Significant examples include *Cucumber mosaic virus*, *Watermelon mosaic virus*, *Zucchini yellow mosaic virus*, *Cucumber vein yellowing virus*, *Cucurbit yellow stunting disorder virus* and *Melon necrotic spot virus* (Abou-Jawda et al. 2000; Célix et al. 1996; Luis-Arteaga et al. 1998; Mansour and Musa 1993; Provvidenti 1996).

Melon necrotic spot virus (MNSV) is a *Carmovirus* within the family *Tombusviridae* (Hibi and Furuki 1985), which is present in cucurbit crops worldwide. MNSV can be transmitted mechanically, by the zoospores of the fungus *Olpidium bornovanus* and through the seed. The

symptoms induced by MNSV in melon include local necrotic spots or large necrotic lesions on leaves, and necrosis on stems and petioles (Matsuo et al. 1991). The MNSV host range is restricted to members of the *Cucurbitaceae* family, where severity of the symptoms depends on the growing conditions, and the species and cultivar used. The MNSV genome is a single-stranded (+)-sense RNA molecule of 4.3 kb with at least five open reading frames (Díaz et al. 2004a; Riviere and Rochon 1990). The spread of MNSV in cultivated melon can be controlled through the use of virus-free seeds, control of the fungal vector and the use of resistant cultivars. There are at least two sources of resistance to MNSV in melon: the cultivar 'Gulfstream' and the Korean accession PI 161375, both determined by the recessive *nsv* gene (Coudriet et al. 1981). Breeding for MNSV resistance in melon is restricted to the *nsv* gene, which is effective against all known strains of the virus, except for the recently described strain MNSV-264 that is able to overcome this resistance (Díaz et al. 2004a). Many seed companies use *nsv* in their breeding materials, and the majority of commercial melon hybrids already contain this type of resistance. The resistance conferred by *nsv* is active at the single cell level (Díaz et al. 2004a). Recent studies performed with chimeras between resistance-breaking and non-breaking MNSV strains have shown that the avirulence determinant corresponding to *nsv* is located in an untranslated region of the 3′-end of the viral genome (Díaz et al. 2004a).

The *nsv* gene has been mapped to linkage group XII of the melon genetic map from the cross between the line 'Védrantais' and PI 161375 (Perin et al. 2002), and to the corresponding linkage group 11 of the genetic map from the cross between PI 161375 and the line 'Piel de sapo' (Oliver et al. 2001). More recently, additional markers closely linked to the gene were developed using bulked segregant analysis (BSA) (Michelmore et al. 1991), with 184 AFLP primer combinations and 168 RAPD primers in a population of 69 doubled haploid lines (DHLs) from the PI 161375 x 'Piel de sapo' cross (Morales et al. 2002). A detailed map flanking the *nsv* region has been constructed, consisting of 10 markers spanning a 17.7 cM interval, with two markers, the AFLP CTA/ACG-115 and the RAPD OPD08-0.80 flanking the gene over a 5.9 cM interval and two more markers, AFLP ACC/ACC-110 and RAPD OPX15-1.06, co-segregating with the resistance gene (Morales et al. 2002). As a first step towards the map-based cloning of the *nsv* gene, we report a high-resolution mapping of the *nsv* locus. This high-resolution map was obtained with more than 3.000 individuals of two different mapping populations, using AFLP and RAPD markers from Morales et al. (2002), which were converted into SCAR and CAPS markers for the efficient screening of recombinant individuals in this region. Two melon BAC libraries from the resistant genotype PIT92 (van Leeuwen et al. 2003) and the susceptible cultivar WMR29 (Bendahmane, unpublished) allowed the screening and the identification of new markers closer to the resistance gene, by means of BAC-end sequencing and mapping. We then constructed a BAC contig around this locus and identified a BAC clone that physically contains the *nsv* gene.

Material and methods

Plant material and DNA preparation

We used two mapping populations to obtain the high-resolution genetic map of the *nsv* region. The first segregating population was an F_2 of 408 individuals derived from the cross between the Korean accession PI 161375 (*nsv*/*nsv*) and the line T111 (*Nsv*/*Nsv*) from the Spanish melon *inodorus* type 'Piel de sapo'. The 91 recombinant F_2 individuals identified between the *nsv* flanking markers were individually selfed to obtain F_3 families for the MSNV progeny test. The second mapping population was a backcross population of 2.727 individuals derived from the susceptible Charentais type line 'Védrantais' and the same resistant accession PI 161375 ('Védrantais (*Nsv*/*Nsv*) x PI 161375 (*nsv*/*nsv*)) x PI 161375 (*nsv*/*nsv*).

DNA was extracted from the 408 F_2 individuals, in Eppendorf tubes, following the protocol previously published (Garcia-Mas et al. 2000). DNA preparation for high-throughput PCR analysis in the BC1 population was carried out as follows: plants were grown in 104-well plates (3×3 cm wells, Ets Baan, France) for 2–3 weeks. Young leaves were placed in 96-well plates (2-mL wells, Costar, Corning, NY, USA) containing three glass beads per well (3-mm diameter, PolyLabo, Strasbourg, France), and lyophilized. The plates were sealed with Easy Peel sealers (Abgene, Epsom, UK) and the lyophilized leaves were ground with the beads in a shaker. DNA was extracted with 600 µL of extraction buffer containing 0.5 M NaCl, 0.1 M Tris, 50 mM EDTA, and 20 mM sodium metabisulfite (added just before use). The plates were sealed with thermo-well sealers (Corning, NY, USA) and incubated in a water bath at 95°C for one hour, then centrifuged at 3000 rpm for 20 min. The supernatant (280 µL) was transferred to 96-well plates (0.65-mL wells, ABgene, Epsom, UK) containing 35 µL of 10 M ammonium acetate, 280 µL of isopropanol, and mixed. The plates were centrifuged at 3000 rpm for 30 min, the supernatant decanted by inverting the plate, and the DNA pellets washed with 70% ethanol and air-dried by incubation at 60°C for about 20 min. DNA pellets were re-suspended in 50 µL TE-buffer pH 8.0, containing 25 µg/mL of RNase A, with slow shaking at 37°C for 1 h.

MNSV resistance tests

MNSV resistance tests were performed by mechanical inoculation with fresh necrotic lesions from susceptible melon lines on extended cotyledons of two-week-old melon plants. Usually 1 g of tissue disks from

Table 1 Main markers used to construct the genetic map from the *nsv* locus

Marker	Primer	Primer sequence	Polymorphism used for mapping
M29	M29-F	CATCAATTAAGACGACACACAGG	*Afl* III
	M29-R	TCGAGAACCTTTTTATTATTGATGG	
M132	M132-F	TCTGGAAAGGTCTACCCCCTCAT	*Taq* I
	M32-R	GGGTTGTTTTGGTTCTTGAATTGTATT	
D08	D08-F	GTGCCCCAACGACTAAAAAG	*Eco*RV
	D08-R	GCTTCCTGACAAGTAGTGGC	
X15	X15-F	CAGACAAGCCCAGATAATTAACA	Allele-specific PI
	X15-R	CAGACAAGCCTAGGAGTTGTGGG	
5A6sp6	5A6SP6-F	GTCCTATGAGAACGACCGAGA	*Taq*I
	5A6SP6-R	CGGACAGTTTTGTTAACCCATA	
52K20sp20	52K20SP6-F	GCAATGTATCCTTGCACAAATGC	SNP G-A (PI-PS)
	52K20SP6-R	TCCAGCTTCTATCCATCAATCTG	
1L3	1L3-F	GTCCTCAGTGGGAGTGACAGAAGTG	SNP A-G (PI-PS)
	1L3-R	CTTGATAGCACTATGCAGTCATATC	
ns1(M29)	ns1-F2c	GATTTCTTTTGAAATTTATATATATATCAGC	Allele-specific Vedrantais
	ns1-R2b	AAGAATCTTCTTGAAGACCGTATGT	
ns2(5A6sp6)	ns2-F1b	CAGATTATAAGTTTCAGGTGATATTA	Allele-specific Vedrantais
	ns2-R1b	TGATCCTGAAATTTATAATTAGGCATC	

For each marker, the primer sequences used to amplify it and the method of detection of the polymorphism are detailed. ns1 and ns2 correspond to allele-specific markers developed from M29 and 5A6sp6, respectively

cotyledons containing fresh MNSV lesions was ground in 4 mL of 0.03 M Na_2HPO_4 pH 8.5, 0.2% DIECA and 75 mg/mL of activated charcoal for the F_2 or 0.5 g of activated charcoal and 0.4 of carborundum for the BC1. This extract was rubbed onto the cotyledons of 2-week-old melon plants, dusted previously with carborundum. In some cases carborundum (0.1 g/mL) was added to the extract before inoculation. Finally, the inoculated cotyledons were washed with water to remove excess carborundum and charcoal. Plants were visually scored as susceptible, 3 to 5 days after the mechanical inoculation, if they had MNSV necrotic spots on the cotyledons. If no symptoms were detected 10 days after the inoculation, plants were scored as resistant to MNSV.

Twenty F_3 individuals corresponding to each out of the twelve recombinants were tested for MNSV resistance. All the 2.727 BC1 individuals were tested for MNSV resistance.

AFLP and RAPD marker conversion

RAPD and AFLP markers flanking the *nsv* gene were converted into STS markers for analysis of the F_2 population. DNA from RAPD markers OPX15-1.6 and OPD08-0.80 was separated, and then cut from the agarose gel, eluted with the QIAEX II kit (QIAGEN, Hilden, Germany), cloned into the pGEM-T vector (Promega, Madison, USA) and sequenced. Specific primers were designed from the sequences, yielding markers X15 and D08 (Table 1). Bands of the AFLP markers CTA/ACG-115 and ACC/ACC-110 were separated on a denaturing polyacrylamide gel with [$\gamma-^{33}$P]ATP primer labeling, eluted from the dried gel, reamplified, cloned into the pGEM-T vector (Promega, Madison, USA) and sequenced. Genomic regions flanking the AFLP fragments were recovered using the Universal GenomeWalker Kit (Clontech, Palo Alto, USA) and sequenced. Specific primers were designed from the sequences, yielding markers M29 and M132 (Table 1). PCR for markers X15, D08, M29 and M132 was performed in a total volume of 25 µL of 10 mM Tris/HCl pH 8.3, 0.001% gelatine, 2.0–3.5 mM $MgCl_2$ (D08: 2.0 mM; X15: 2.5 mM; M29 and M132: 3.5 mM), 50 mM KCl, 0.4 mM of each dNTP, 0.4 µM of each primer, 5 U of Taq DNA polymerase and 50 ng of genomic DNA. Cycling conditions were an initial denaturation step for 2 min at 94°C followed by 35 cycles, each of 30 s of denaturation at 94°C, 30 s of annealing at Ta, and 60 s (X15, D08 and M29) or 90 s (M132) of elongation at 72°C, with a final extension cycle at 72°C for 5 min. Ta was 53°C for M132, 60°C for D08, 61°C for M29 and 63°C for X15. The PCR products were digested with the restriction enzymes *Eco*RV (D08), *Afl*III (M29) or *Taq*I (M132) to detect polymorphism between the parental lines.

BAC library screening with PCR markers

All the PCR markers shown in Fig. 2 were used to screen the PIT92 melon BAC library containing 23.040 clones with an average insert size of 141 kb and 6x coverage of the melon genome. The BAC library is organized in 60 384-well plates, and DNA from each six plates is organized in a single super-pool, giving a total of 10 DNA super-pools available for PCR screening (van Leeuwen et al. 2005). Some of the markers were also used to screen the WMR29 melon BAC library that contains 32.632 clones, with an average insert size of 100 kb and 7x coverage of the melon genome (Bendahmane, unpublished). This BAC library (obtained from the American cantaloupe type WMR29) is organized in 86 384-well plates, and DNA from each plate is available for PCR screening.

Each marker was screened in the BAC libraries using its particular PCR conditions, with DNA from PI 161375, 'Piel de sapo', 'Védrantais' or WMR29 as positive controls. BAC clone sizes were determined by PFGE as described previously (van Leeuwen et al. 2003).

Marker development from BAC-end sequences

For both BAC libraries used in this study, once a clone was identified as positive with a marker, BAC DNA was extracted and sequenced at both ends (van Leeuwen et al. 2003). Specific primers were designed from the BAC-end sequences and used to amplify DNA from the parental lines. These PCR products were purified, sequenced, and compared, in the search for polymorphisms between the parental lines amenable for genotyping the recombinant individuals (Morales et al. 2004). Marker 5A6sp6 was developed from a BAC-end sequence of clone 5A6 (Table 1). It was amplified with the specific primers 5A6sp6-F and 5A6sp6-R in a total volume of 25 µL of 10 mM Tris/HCl pH 8.3, 0.001% gelatine, 2.5 mM $MgCl_2$, 50 mM KCl, 0.4 mM of each dNTP, 0.4 µM of each primer, 5 U of Taq DNA polymerase and 50 ng of genomic DNA. Cycling conditions were with an initial denaturation step for 2 min at 94°C followed by 35 cycles, each with 20 s denaturation at 94°C, 20 s of annealing at 48°C, and 40 s of elongation at 72°C, and a final extension cycle at 72°C for 5 min. PCR products were digested with the restriction enzyme *Taq*I to detect polymorphism between the parental lines PI 161375 and 'Piel de sapo'. Marker 52K20sp6 was developed from a BAC-end sequence of clone 52K20 (Table 1). It was amplified with the specific primers 52K20sp6-F and 52K20sp6-R with the same conditions described for 5A6sp6 marker, with the exception that the annealing temperature was 65°C and the elongation time 50 s. The SNP G-A identified between PI 161375 and 'Piel de sapo' and 'Védrantais' was genotyped using the ABI Prism SNaPshot ddNTP Primer Extension kit (Applied Biosystems, Foster City, USA) with the internal primer 52K20sp6-SNP (CTTTCT AATTATGTACTAATTG) as previously described (Morales et al. 2004). Marker 1L3 was developed from a BAC-end sequence of clone 1-21-10 (Table 1). It was amplified with the specific primers 1L3-F and 1L3-R, as described before, with 30 s of denaturation and annealing, 60 s of elongation, and 60°C annealing temperature. The SNP A-G identified between PI 161375 and 'Piel de sapo' and 'Védrantais' was genotyped using the ABI Prism SNaPshot ddNTP Primer Extension kit (Applied Biosystems, Palo Alto, USA) with the internal primer 1L3-2-SNP (GGTTGAGAGTGTTAAGGTATG) as previously described (Morales et al. 2004).

Allele-specific markers for BC1 screening

CAPS markers M29 and 5A6sp6 developed in the F_2 population were converted into allele-specific markers for screening the BC1 population. PCR products for M29 and 5A6sp6 in PI 161375 and 'Védrantais' were sequenced, compared, and new primers designed near polymorphic sites, in order to amplify only the 'Védrantais' alleles. Marker ns1 (from M29) was amplified with primers ns1-F2c and ns1-R2b (Table 1), and marker ns2 (from 5A6sp6) with primers ns2-F1b and ns2-R1b (Table 1) in a total volume of 25 µL of 10 mM Tris/HCl pH 8.3, 0.001% gelatine, 2.5 mM $MgCl_2$, 50 mM KCl, 0.4 mM of each dNTP, 0.4 µM of each primer, 5 U of Taq DNA polymerase and 50 ng of genomic DNA. Cycling conditions were an initial denaturation step for 2 min at 94°C followed by 35 cycles, each with 10 s (ns1) or 15 s (ns2) denaturation at 94°C, 15 s (ns1) or 30 s (ns2) annealing at Ta, and 30 s (ns1) or 40 s (ns2) of elongation at 72°C, and a final extension cycle at 72°C for 5 min. Ta was 57°C for ns1 and 50°C for ns2.

Results

AFLP and RAPD marker conversion and mapping in the F_2 population

The *nsv* gene has previously been mapped at low resolution in the melon linkage group 11 of the melon genetic map (Oliver et al. 2001), with flanking markers that covered a region of 5.9 cM around the resistance gene (OPD08-0.80, CTA/ACG-115), and two additional co-segregating markers (OPX15-1.06, ACC/ACC-110) (Morales et al. 2002). The RAPD and AFLP markers OPX15-1.06, OPD08-0.80, CTA/ACG-115 and ACC/ACC-110 were converted into the SCAR marker X15 and the CAPS markers D08, M29 and M132, respectively, for easier screening (Fig. 1). Marker X15 produced a band of 1.1 kb in the PI 161375 parent and no amplification in the 'Piel de sapo' parent. After digestion of the PCR product of the D08 marker (750 bp) with the restriction enzyme *Eco*RV, the PI 161375 allele gave two bands, of 560 and 190 bp, whereas the 'Piel de sapo' allele remained uncut. For the M29 marker, the digestion of the 360 bp PCR product with *Afl*III gave two bands of 191 and 174 bp in the resistant allele, and the susceptible allele was not digested. Flanking markers D08 and M29 were used for recombinant screening of the 408 individuals of the F_2 population (Fig. 2). Ninety-one individuals which were identified contained a recombination event between D08 and M29.

CAPS marker M132, after digestion with the restriction enzyme *Taq*I, gave two bands of 750 and 450 bp in the resistant allele, whereas the susceptible allele had three bands of 750, 300 and 150 bp. When tested in the 91 M29/D08 recombinants, a total of 26 were detected between M29 and M132, and 65 between M132 and D08. Markers M29 and M132 delimited an interval that covered a genetic distance of 3.2 cM (Fig. 2).

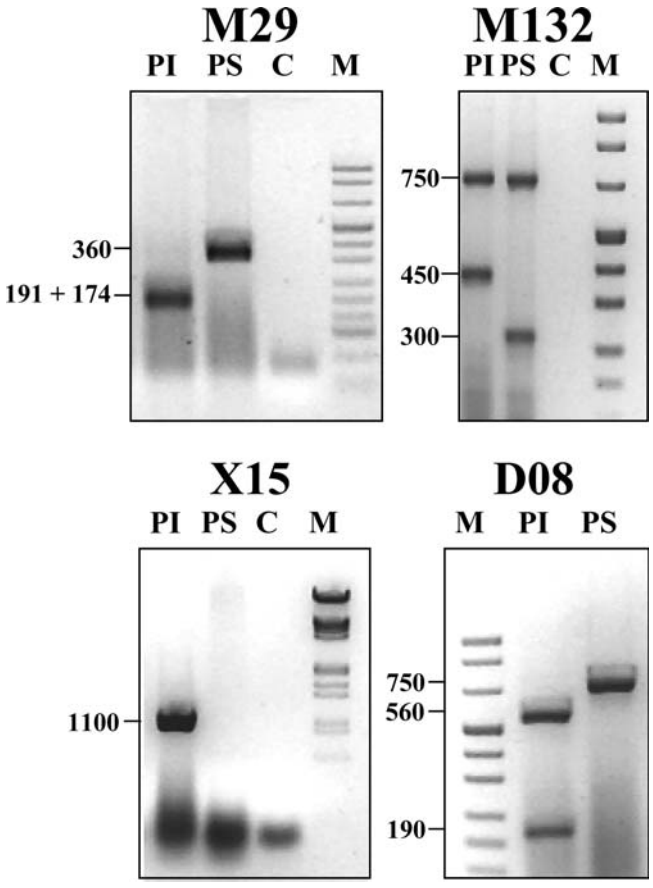

Fig. 1 CAPS and SCAR markers, developed from AFLPs and RAPDs, used to find recombinant individuals in the F_2 mapping population. CAPS markers M29, M132 and D08 were obtained after digestion of the PCR product with *Afl*III, *Taq*I and *Eco*RV, respectively. The digested fragments were separated in 1.5% agarose gels. The SCAR marker X15 amplified a fragment of 1.1 kb in the resistant parent PI 161375. The size of the fragments, in base pairs (bp), is indicated on the *left* of each panel. *PI*: PI 161375; *PS*: 'Piel de sapo'; *C*: control; *M*: molecular size markers pUC-Mix (MBI Fermentas) or λ DNA digested with *Eco*RI and *Hind*III

A resistance progeny test was performed in 12 recombinant families out of the 26 recombinants in the interval (the remaining 14 recombinants did not yield F_3 seed when selfed), and *nsv* was located between M29 and M132 at 7 and 5 recombination events, respectively (Fig. 2). Although only 12 recombination events delimited the interval M29/ *nsv*/M132, the additional 14 recombination events between M29 and M132, which were not phenotyped, were still useful for mapping new markers in this region (Fig. 2).

SCAR marker X15 did not have enough resolution in this F_2 map because it was dominant (Fig. 2).

BAC contig and BAC-end mapping

Screening the PIT92 melon BAC library with markers M29 and M132 allowed the detection of positive BAC clones 7K20 (95 kb) and 38B12 (100 kb) (Fig. 2), respectively. The dominant SCAR marker X15 was also used to screen the BAC library and allowed the identification of BAC clone 5A6 (140 kb) (Fig. 2). PCR amplifications of these markers on the BAC library were sequenced, to verify that they corresponded to the original marker sequence. The ends of these three BACs were sequenced, yielding new PCR markers that allowed their physical orientation. Some of these new markers, such as 5A6U, 5A6sp6, 38B12U and 7K20U, with polymorphism between PI 161375 and 'Piel de sapo', were also positioned in the genetic map. Marker 5A6sp6 gave a PCR fragment of 650 bp which, when cut with *Taq*I, gave two bands of 390 and 260 bp in the resistant genotype, and three bands of 390, 130 and 130 bp in the susceptible genotype. CAPS marker 5A6sp6 was mapped at only 1 recombination event from *nsv* on the M132 side. When considering all 26 recombinants in the M132/ M29 interval, BAC 5A6 spanned 5 recombination events. BAC 7K20 was too far on the M29 marker side, so this contig was not further extended, while BAC 38B12 delimited the right border of the BAC contig.

Successive BAC screening and BAC-end mapping, starting from marker 5A6sp6, allowed the construction of a contig with BAC clones 30L11 (120 kb), 5B3 (145 kb) and 52K20 (110 kb). The BAC ends 30L11sp6, 5B3sp6 and 52K20sp6 were successively positioned in the genetic map in the *nsv* direction, each one separated by recombination events from the original 26 recombinants in the M29/M132 interval. BAC 52K20 allowed the identification of marker 52K20sp6, which co-segregated with *nsv* (Fig. 2). Marker 52K20sp6 contained an SNP G-A between the resistant genotype PI 161375 and the two susceptible genotypes 'Piel de sapo' and 'Védrantais', which was genotyped in the recombinant individuals using the internal primer 52K20sp6-SNP (data not shown). As shown in Fig. 2, additional BAC clones were also included in the contig as a result of the BAC library redundancy. Their positions were verified by PCR with some of the BAC ends. The redundancy of the BAC library in this region is 4.6 and the BAC average size is 116 kb. The BAC contig spans a region of around 450 kb.

Construction of a genetic map in the BC1 population

During the mapping procedure previously described, due to the limited size of the PI 161375x'Piel de sapo' F_2 mapping population, we decided to screen a larger population in order to increase the mapping resolution by identifying more recombinant individuals. For this purpose a backcross population ('Védrantais'xPI 161373)xPI 161375 of 2.727 individuals was obtained. Before mapping in the BC1 population, we confirmed that the *nsv* flanking markers obtained in the F_2 map, M29 and 5A6sp6, also flanked the resistance gene in 163 individuals of an RIL population, that was derived from the same cross 'Védrantais' x PI 161375 (Perin et al. 2002).

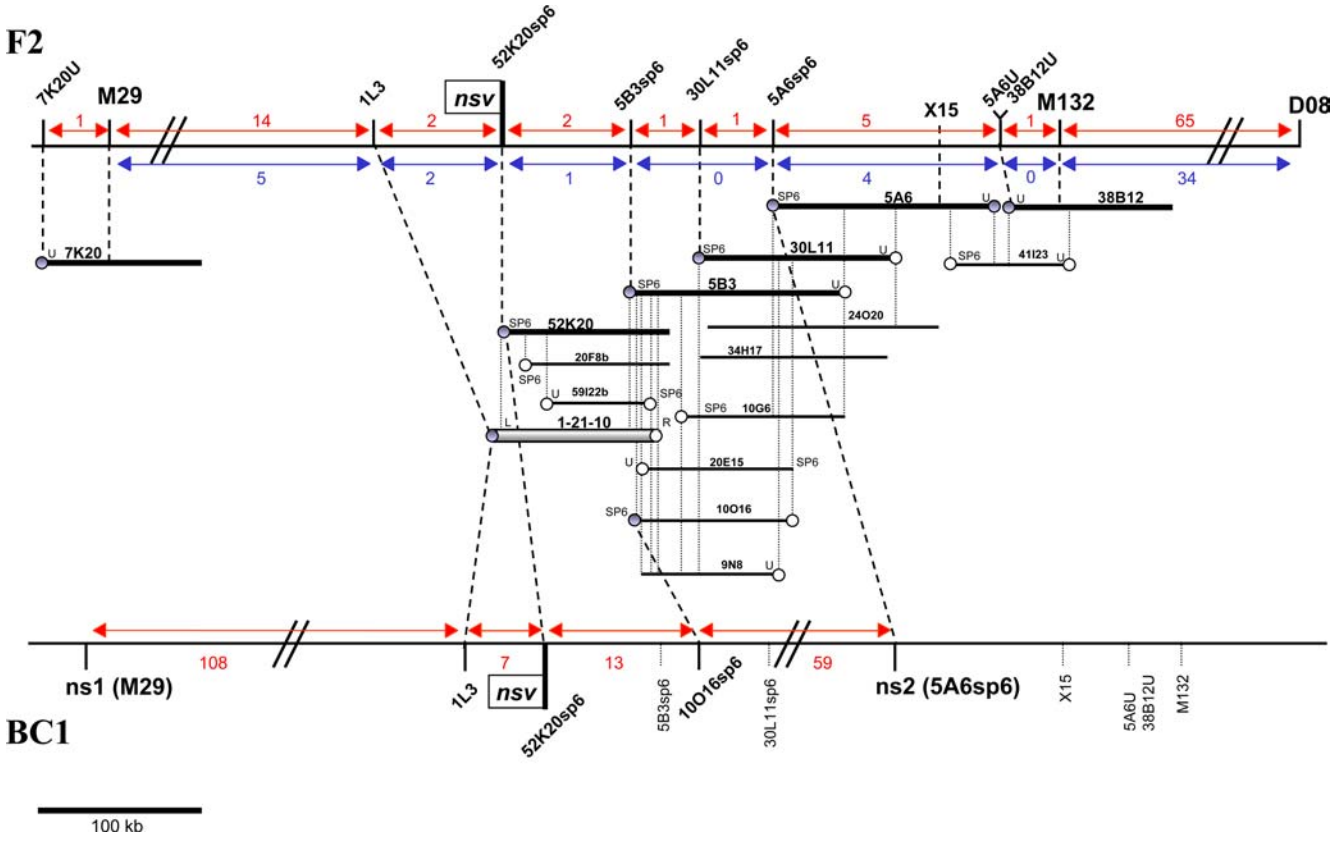

Fig. 2 High-resolution genetic maps near the *nsv* locus in the F_2 (PI 161375xPiel de Sapo) (*top*) and BC1 ((Védrantais x PI 161375) x PI 161375) (*bottom*) populations, and BAC contig in this region. *F_2 map*: flanking markers M29, M132 and D08 were used to screen 408 F_2 individuals and delimited a region spanning 26 recombinants, between M29 and M132. Numbers above the *line* are recombination events between markers. Numbers below the *line* are recombination events between markers in 12 individuals tested for MNSV resistance. *BC1 map*: flanking markers ns1 (M29) and ns2 (5A6sp6) were used to screen 2.727 BC1 individuals and delimited a region spanning 187 recombinants. Numbers below the *line* are recombination events between markers. The position of some markers (*vertical legend*) in the genetic map is inferred from the F_2 map. Recombination events between markers are not to scale. BACs with ends that were converted into markers in the genetic map are shown with *thick bars*. Other BACs in the region are shown with *thinner bars*. *Dark circles* represent BAC ends that were mapped, and are connected to the genetic maps with *thick dashed lines*. *White circles* are BAC ends used to construct the contig and are connected with *thinner dashed lines*. The physical map is to scale. BAC 1-21-10 from the WMR29 library that contains *Nsv* is represented in *grey*

The closest markers to *nsv* from the F_2 map at that time, M29 and 5A6sp6 (Fig. 2) were converted into allele-specific markers for the BC1 population, named ns1 and ns2, respectively. Markers ns1 and ns2 amplified fragments of 237 bp and 492 bp, respectively, in the susceptible genotype 'Védrantais'. The 2.727 BC1 individuals were inoculated with MNSV and the ns1 and ns2 flanking markers were tested in all the BC1 individuals to search for recombinants. *nsv* was positioned in an interval delimited by 187 recombination events between ns1 and ns2 (Fig. 2), with 115 recombinants from ns1 (M29) and 72 recombinants from ns2 (5A6sp6). Additional BAC-end markers, 30L11sp6, 10O16sp6 and 5B3sp6 (Fig. 2), from the F_2 population, were tested in the BC1 population parents in search for polymorphism. The BAC-end marker 10O16sp6, which was not polymorphic in the PI 161375x'Piel de sapo' segregating population, was mapped in the BC1 population on the 5A6sp6 side with only 13 recombination events from *nsv* (Fig. 2). The other two F_2-derived markers were monomorphic between PI 161375 and 'Védrantais', but their approximate position was inferred from the F_2 map.

Marker 52K20sp6, which also contained an SNP G-A between PI 161375 and 'Védrantais', was mapped in the BC1 population using the single primer extension method. As in the F_2 map, 52K20sp6 co-segregated with the *nsv* resistance gene in 2.727 individuals.

Identification of a BAC clone spanning the *nsv* locus

Markers 52K20, 5A6sp6, 30L11sp6, 10O16sp6 and 5B3sp6 were used to screen the WMR29 melon BAC library yielding additional positive BAC clones such as 1-21-10 (100 kb) and 31-19-12 (100 kb, not shown in Fig. 2). As we knew the order of these markers in the F_2 map region, we concluded that the BAC clone 1-21-10 was located at the left end of the contig nearest to *nsv*. PCR markers were developed from BAC 1-21-10 ends

(1L3 and 1R3) and 1L3 was confirmed as the most distal marker in the contig (Fig. 2). An SNP A-G between PI 161375 and 'Piel de sapo' and 'Védrantais', which could be genotyped using the single primer extension method with the internal primer 1L3-2-SNP, was also identified, and 1L3 was mapped at 2 and 7 recombination events on the M29 side of *nsv* in the F_2 and the BC1 populations, respectively (Fig. 2).

As BAC-end 1R3 amplified in BAC clones 5B3, 52K20, 20E15, 9N8 and 10O16, we confirmed that the *Nsv* allele was physically contained in BAC 1-21-10, as markers 5B3sp6 (from BAC 5B3) and 10O16sp6 (from BAC 10O16) were genetically separated from the resistance gene in the F_2 and the BC1 populations at 1 and 13 recombination events, respectively (Fig. 2).

Discussion

A high-resolution genetic map of the *nsv* locus has been obtained using two different mapping populations; an F_2 and a BC1 population. A BAC contig was also constructed in this region by means of BAC-end sequencing and mapping BAC end-based markers in this genetic interval. As the allele that confers resistance in *nsv* is recessive, we wanted to characterize the *Nsv* allele from a susceptible genetic background. Thus, we screened a BAC library developed from the MNSV susceptible genotype WMR29, where we have identified a 100 kb BAC clone, 1-21-10, with the resistance gene. The other BAC clones from the *nsv* contig have been obtained from a BAC library from the resistant genotype PIT92, a DHL derived from the cross PI 161375x'Piel de sapo' and should contain the *nsv* allele (van Leeuwen et al. 2003).

The same genetic and physical map order was found using two different populations: 408 F_2 and 2.727 BC1 individuals, with genetic resolutions of 0.12 and 0.036% recombination frequency, respectively. These data confirm the precise order of the markers in two independent populations. The use of the F_2 mapping population resulted in the same genetic information as the one obtained using the BC1 population, because in the F_2 population the ends of BAC 1-21-10 were already genetically separated from *nsv* (Fig. 2). However, the more higher resolution of the BC1 population may allow further definition of the region where *nsv* is located. In the BC1 genetic map we identified 20 recombination events between the closest flanking markers, 10O16sp6 and 1L3. Because BAC 1-21-10 spans 100 kb, on average there is one recombination event for every 5 kb in these region, which is probably enough to allow selection of the appropriate ORF. For the barley *Mlo* gene, isolation by a map-based cloning procedure and an analysis with flanking markers of 2022 F_2 segregants was enough to position the *Mlo* gene in a region of 30 kb, where only one ORF was identified (Buschges et al. 1997).

On average, a genetic distance of 1 cM corresponds to a physical distance of 380 kb; we consider the melon genome size to be 454 Mb (Arumuganathan and Earle 1991) with a total genetic distance of 1197 cM for the melon genetic map (Oliver et al. 2001). From the data obtained in this report, we can calculate that in BACs 1-21-10 and 5A6 the physical/genetic relationships are 137 and 229 kb/cM, respectively. In the *nsv* locus the recombination frequency is slightly higher than the one estimated for the whole melon genome. Data obtained from the *Fom-2* region in melon linkage group 5 suggested that, for this locus, the average physical/genetic relationship was 227 kb/cM, with some variation in the recombination frequency in different regions in the locus (Joobeur et al. 2004). We found some differences in the estimated genetic distances between the F_2 and the BC1 populations, usually with smaller distances for the F_2 population. Markers M29 and 5A6sp6 are located at 1.96 and 0.49 cM from *nsv* in the F_2 population, and these distances increase to 4.2 and 2.6 cM in the BC1 population. However, the closest marker, 1L3, is located at 0.25 cM from the resistance gene in both populations. These data reflect the variation in the recombination frequency when using different types of crosses and in different regions of the same locus.

The high level of polymorphism found between the resistant parental accession PI 161375 and the susceptible parents 'Piel de sapo' and 'Védrantais' has been very useful to map all new markers in this region. PI 161375 belongs to the melon *conomon* group, which is genetically very distant from the susceptible parental lines 'Piel de sapo' and 'Védrantais', from the *inodorus* and the *cantalupensis* groups, respectively (Monforte et al. 2003). For this reason the majority of markers in the *nsv* locus are polymorphic in either the F_2 or the BC1 population, allowing them to be mapped. As we have reported previously (Morales et al. 2004), markers in melon can be efficiently mapped using either CAPS technology or the single primer extension method, among others. In particular, the single primer extension method has proven to be very efficient for mapping SNPs for markers 52K20sp6 or 1L3 between PI 161375 and 'Piel de sapo' and 'Védrantais'.

The vast majority of dominant disease-resistance genes that have been characterized in plants are members of a few families of proteins with several protein domains, such as NBS-LRRs, protein kinases, eLRRs or *Xa21*-like proteins (Hammond-Kosack and Parker 2003). We suspect that the recessive nature of the *nsv* phenotype is due to a natural mutation or loss-of-function of the wild-type allele *Nsv*, probably a host factor necessary for MNSV infection. Several recessive disease-resistance genes have been characterized to date (Díaz et al. 2004b). The *mlo* allele that confers resistance against powdery mildew in barley probably encodes a negative regulator of the induction of cell-death (Buschges et al. 1997). Recently, the *pvr2* resistance gene against *Potato virus Y* in pepper (Ruffel et al. 2002), *mo1* against *Lettuce mosaic virus* in lettuce (Nicaise et al. 2003) and *sbm1* against *Pea seed-borne mosaic virus* in pea (Gao et al. 2004) have been characterized. The three

viruses belong to the family *Potyviridae*, and in all cases the recessive resistance gene encodes the eukaryotic initiation factor 4E (eIF4E), a protein from the host translation complex, able to interact with the VPg potyvirus-encoded protein (Léonard et al. 2004). Mutations in the eIF4E may prevent correct interaction with the viral VPg, leading to virus resistance. Recently, the avirulence determinant for the *nsv* resistance gene has been shown to be located in a non-translated RNA region at the 3′-end of the virus genome (Díaz et al. 2004a). MNSV, a member of the family *Tombusviridae*, does not encode a VPg protein. Therefore, the *nsv*/MNSV experimental system appears to have distinct features compared to other systems analyzed to date, so is an interesting model for new insights into the mechanisms of recessive resistance to viruses.

Although the susceptibility test used in breeding programs 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). Some of the markers mapped in the *nsv* locus can potentially be used in marker-assisted selection programs to help introduce the *nsv* allele into elite lines. The CAPS markers M29, M132 or 5A6sp6 are located in the F_2 mapping population at 1.96, 1.2 and 0.49 cM from *nsv*, respectively, and could be useful for this purpose although we have not tested their efficiency in melon germplasm other than the parents of the mapping populations. The best markers for selection of *nsv* would be the closest markers, 52K20 (co-segregating with *nsv*) and 1L3 (0.25 cM from *nsv*), but they have been genotyped using an SNP detection method, which is still not suitable for high-throughput use in marker-assisted programs, due to economic restrictions. 52K20sp6 and 1L3 markers need to be converted into CAPS or SCAR markers for a more convenient use in breeding programs for MNSV resistance. But then, once the *nsv* gene is isolated, a marker based on the resistance gene sequence itself will be the best marker for marker-assisted selection purposes.

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