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Cloning and mapping of resistance gene homologues in melon

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Abstract

Four families of resistance gene homologues containing the nucleotide binding site (NBS) motif were obtained in *Cucumis melo* using degenerate primers designed from two conserved regions of known disease resistance genes. The four homologues had high levels of amino acid identity to NBS-containing resistance genes found in other species. Three of these homologues (MRGH4, MRGH21 and MRGH63) were placed in the genetic map of melon. The RFLP detected with MRGH21 was located on the distal part of Linkage Group 7. Homologues MRGH63 and MRGH4 detected RFLPs that mapped to Linkage Group 4. The map positions of these loci relative to those of resistance genes mapped in melon and the potential use of these loci in marker-assisted selection and map-based cloning strategies is discussed.

Keywords: Disease resistance; RGH; Mapping; *Cucumis melo*

1. Introduction

Molecular markers have been used to facilitate the process of introgression of valuable genes into commercial cultivars [1]. One of the most widely used strategies to identify molecular markers in the neighbourhood of a particular monogenic trait using RAPD or AFLP markers is the 'bulked segregant analysis' (BSA) method [2]. When the trait of interest is a monogenic disease resistance, BSA has proved to be extremely efficient [3,4]. Cloning of resistance gene homologues (RGH) can also be used as a method to identify markers linked to disease resistance genes since they are often arranged in clusters in the plant genome [5–7]. New RGH loci are generated most probably by heterologous recombination creating new resistance genes with new pathogen specificities or without any function [8].

Disease resistance genes have been isolated and characterized at the molecular level in several plant species [9]. The major group of disease resistance genes that have been characterized encode proteins which contain NBS and LRR-type domains. Conservation of some protein motifs among disease resistance genes of different plant species provides a method for isolating additional homologous disease resistance genes in these or other species. With a PCR approach based on degenerate primers designed from conserved motifs in the NBS domain, several disease resistance gene homologues have been cloned in monocot and dicot species [10–13]. In all cases, some of the homologous sequences obtained with this procedure mapped close to known disease resistance loci.

We used this approach to clone disease resistance gene homologues in melon (*Cucumis melo*) using degenerate primers obtained from two conserved motifs in the NBS domain. Four resistance gene homologue (RGH) families were obtained and mapped using an F_2 melon population. The

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map position for three of the RGHs has been established and compared with the location of known disease resistance genes.

2. Methods

2.1. Plant material

A population used for the construction of a melon map [14] was used. This population consisted of 93 plants of the F_2 from the cross between the *C. melo* accession PI 161375 (of Korean origin) and line T111 of the Spanish melon type 'Piel de Sapo' (PS). This map was then employed to locate the RGHs developed in this study.

2.2. DNA extraction

Total genomic DNA was isolated from young leaves as described by Doyle and Doyle [15] with minor modifications (i.e. two consecutive chloroform–isoamyl extractions were performed to improve DNA quality). The extraction procedure yielded 0.2–0.5 mg of DNA/g of fresh tissue.

2.3. PCR with degenerate primers

Five degenerate primers (named Primers 1, 1′, 2, 3 and 4) were designed which corresponded to the GGVGKTT (P-loop), and the GLPLAL conserved motifs in the NBS domain (Table 1). These primers were obtained from sequences corresponding to NBS plant resistance genes [10] as *RPS2* [16], *N* [17], and *L6* [18] which confer resistance to *Pseudomonas syringae* in *Arabidopsis*, tobacco mosaic virus in *Nicotiana glutinosa* and *Melampsora lini* in flax, respectively.

PCR reactions were performed in a total volume of 50 μl with 1 U of Taq DNA polymerase in 10 mM Tris/HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.4 mM dNTPs, 0.4 μM of each primer and 50–100 ng of genomic DNA. Cycling conditions were: initial denaturation step for 2 min 30 s at 94°C followed by 35 cycles each with 15 s denaturation at 94°C, 45 s annealing at 46°C, and 30 s elongation at 72°C, with a final extension cycle at 72°C for 5 min.

Due to the number of PCR products obtained with primers 1–4 and 1′–4 (Table 1) a nested PCR reaction was performed on these PCR products. A new internal primer (Primer 5) was designed from the kinase-2 motif (Table 1) and used with Primer 4. The same PCR conditions as above were used, except that the annealing temperature was reduced to 40°C.

2.4. Analysis of amplification products

Amplified fragments ranging 450–550 bp in size (P-loop/GLPLAL) and 275 bp (kinase-2/GLPLAL) were recovered from the agarose gel using the QIAEXII gel extraction kit (QIAGEN), cloned into the pGEM-T vector system (Promega) and transformed into DH5α competent cells. Clones were sequenced using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABI Prism 310 DNA sequencer (Applied Biosystems).

2.5. RFLP analysis

Genomic DNA (5 μg) from lines PI 161375 and PS was digested with the restriction enzymes *Mva*I, *Hin*dIII, *Dra*I, *Eco*RI and *Eco*RV, size fractionated, transferred to a nylon membrane (Hybond N+) and hybridised as described by Sambrook et al. [19]. Probes were labelled with ^{32}P-dCTP using the random priming method. Washes were performed at 65°C with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 20 min

Table 1
Degenerate primers designed from the P-loop (Primer 1 and 1′), the GLPLAL (Primer 2, 3, and 4), and the kinase-2 (Primer 5) conserved motifs of the resistance genes *RPS2*, *N*, and *L6*

	Motif[a]	Sequence[b]
Primer 1	G G V/I G K T T	GGIGGI(A,G) TIGGIAAIACIAC
Primer 1′	G M G G V/I G	GGIATGGGIGGI(A,G) TIGG
Primer 2	G L P L A/T L	IAGIG(C,T) IAGIGGIAGICC
Primer 3	G L P L A/T L	IAGIG(C,T) IAGIGGIAAICC
Primer 4	G L P L A/T L	IAGIG(C,T) IAAIGGIAGICC
Primer 5	L D D V D	TI(C/T)TIGA(C/T) GA(C/T)(A/G)TIGA(C/T)

[a] Conserved amino acid sequence used for designing the primer.
[b] Nucleotide sequence of the primer.

each. Membranes were exposed to AGFA Curix RP2 film for 2–3 days.

To map the polymorphic RGHs, DNA from 93 F_2 individuals from the mapping population was digested with *Eco*RI, *Bam*HI or *Mva*I and hybridised with the corresponding probes. The data obtained with the F_2 were processed in MAPMAKER 3.0 [20] to establish map positions for the RGHs. For MRGH18, which did not show polymorphism with the above mentioned restriction enzymes, we designed specific PCR primers based on its sequence. The PCR product obtained from the parental DNA was digested with a set of restriction enzymes with 4-base recognition sequences (*Alu*I, *Bsh*1236I, *Bsu*RI, *Dde*I, *Hin*fI, *Hin*p1I, *Hpa*II, and *Rsa*I) in order to detect polymorphism.

2.6. Sequence analysis

Sequence data were analysed using the Genetics Computer Group software (Madison, WI). DNA homologies were performed with the BLAST algorithm. Amino acid sequences deduced from the nucleotide sequences were aligned using the CLUSTALW program (http://www2.ebi.ac.uk/clustalw/). A genetic distance matrix was calculated from the alignment data and analysed with the neighbour-joining method [21]. The phylogenetic tree was represented using the TREEVIEW program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). MRGH DNA sequences appear in the EMBL database under the accession numbers AJ 251869, AJ 251870, AJ 251871, and AJ 251872.

3. Results

3.1. PCR amplification of melon resistance gene homologues with the NBS domain

We used the PCR primers described in Table 1 to amplify melon RGHs. Primer combinations 1–2 and 1–3 resulted in the amplification of unrelated sequences when using PI 161375 genomic DNA as template. Primer combination 1–4 amplified three main PCR fragments of approximately 900, 700 and 500 bp when using the same template. The brightest band was in the range of 500 bp, similar to the size of the fragment amplified between the conserved regions from the known NBS resistance genes from which the primers were designed, considering also that no introns have been reported in this region. The 500 bp band was cloned and 64 independent colonies were classified in families after digestion with the restriction enzyme *Alu*I. After sequencing some of these clones, we characterised three clone families that showed a high identity at DNA and protein levels with known resistance genes and resistance gene homologues present in the sequence databases examined. These three families were named MRGH18, MRGH21, and MRGH63 (MRGH are the initials of 'melon resistance gene homologues', the number that follows corresponds to the clone number).

A single PCR band of the 275 bp size was obtained, cloned and sequenced using a nested PCR approach with Primers 4 and 5 on the 500 bp PCR product from primer combinations 1–4 and 1′–4. This resulted in the isolation of 21 more members of the MRGH18 and MRGH63 families and five clones belonging to a new family named MRGH4.

3.2. Sequence analysis of the melon resistance gene homologues

MRGH4, MRGH18, MRGH21 and MRGH63 were 275, 513, 507 and 513 bp in size, respectively, and contained uninterrupted open reading frames of 91, 171, 169 and 171 amino acids respectively. The amino acid sequences of the four homologues were compared revealing similarities ranging from 75% between MRGH4 and MRGH63 to 45% between MRGH4 and MRGH21. Amino acid sequence comparisons between the four homologues and three disease resistance genes of the NBS type (Fig. 1) showed similarities ranging from 32.5% between MRGH4 and *L6* [18] to 56.3% between MRGH21 and *N* [17]. The P-loop, kinase-2 and the GLPLAL motifs that were used for PCR priming were not represented in the alignment.

We obtained several clones belonging to three of the NBS families. Eleven, ten and five clones were sequenced which belonged to MRGH63, MRGH18 and MRGH4 families respectively. Homologies within each family were high, ranging from a 97 to a 99% of DNA sequence similarity. Some of the clones contained several nucleotide substitutions that did not change the deduced polypeptide sequence.

Fig. 1. Alignment of the protein sequences deduced from the melon resistance gene homologues MRGH4, MRGH18, MRGH21 and MRGH63 and the NBS region of the disease resistance genes *L6* (accession number U 27081), *N* (accession number U 15605) and *M* (accession number U 73916). Only the region between the motifs GGVGKTT/GLPLAL and kinase-2/GLPLAL from where the homologues were cloned is represented. Identical amino acid residues are shadowed in black and similar amino acid residues are shadowed in dark or light grey.

A more detailed alignment was performed with NBS-type sequences present in the databases. Again, only the region between the P-loop and the GLPLAL motifs was used in the alignment. A total of 32 sequences were compared, of which 11 were known resistance genes and the rest RGHs obtained by PCR from different plant species (chickpea, lettuce, maize, melon, pea, pine, potato, rape, soybean, sunflower and tomato). The phylogenetic tree constructed with these data is presented in Fig. 2. Three of the melon RGHs were placed on a first cluster that contains RGHs from species belonging to the *Fabaceae* family as soybean, pea and chickpea, whereas the fourth (MRGH-21) was located on another cluster that contains RGHs from various families like *Cruciferae*, *Solanaceae*, and *Gramineae*, but separated from them.

3.3. RFLP mapping

DNA probes corresponding to the NBS homologues MRGH4, MRGH18, MRGH21, and MRGH63 were hybridised with genomic DNA from the parental lines PI 161375 and PS and then mapped in their F_2 progeny. Only one member of each clone family was used as DNA probe in the hybridisation experiments because of the high level of intra-family similarity.

MRGH21 showed two polymorphic bands of 7.5 kb (PI 161375) and 8.0 kb (PS) after digestion with the restriction enzyme *Eco*RI. The hybridisation pattern showed the presence of a single RFLP that mapped in the distal part of linkage Group 7, 4 cM apart from RFLP marker MC52A (Fig. 3).

MRGH63 yielded a polymorphic pattern after digestion with the restriction enzyme *Mva*I, as shown in Fig. 4. The hybridisation pattern was in agreement with the presence of a single locus, with a 7.0 kb allele present in PI 161375 and a 7.5 kb allele present in PS. The RFLP detected with MRGH63 was positioned in linkage Group 4 flanked by markers MC276 (4 cM) and MC99B (34 cM).

MRGH4 was polymorphic after digestion with the restriction enzyme *Bam*HI and detected a single locus and minor hybridising bands with positions compatible with the MRGH63 hybridisation pattern. The polymorphic bands between PI 161375 and PS were 5.0 and 7.5 kb in size, respectively. The RFLP produced with MRGH4 mapped in linkage Group 4 at 1 cM from MRGH63.

NBS homologue MRGH18 was not polymorphic with any of the restriction enzymes used. Specific primers were designed from the MRGH18 DNA sequence to PCR amplify a fragment of 500 bp in both PI 161375 and PS. This DNA fragment was digested with eight 4-base cutter restriction enzymes but no polymorphism could be detected between both parents.

4. Discussion

Using a degenerate primer PCR approach from conserved motifs in the NBS class of disease resistance genes, we report herein the cloning of four resistance gene homologue families (MRGH4, MRGH18, MRGH21 and MRGH63). Similarity of DNA sequences among clones of the same family was very high (97–99%) and differences were due to a few nucleotide substitutions. These point mutations could be explained by Taq polymerase mistakes, by the detection of alleles of the same locus or by the presence of highly conserved duplicated loci. The deduced protein sequences of

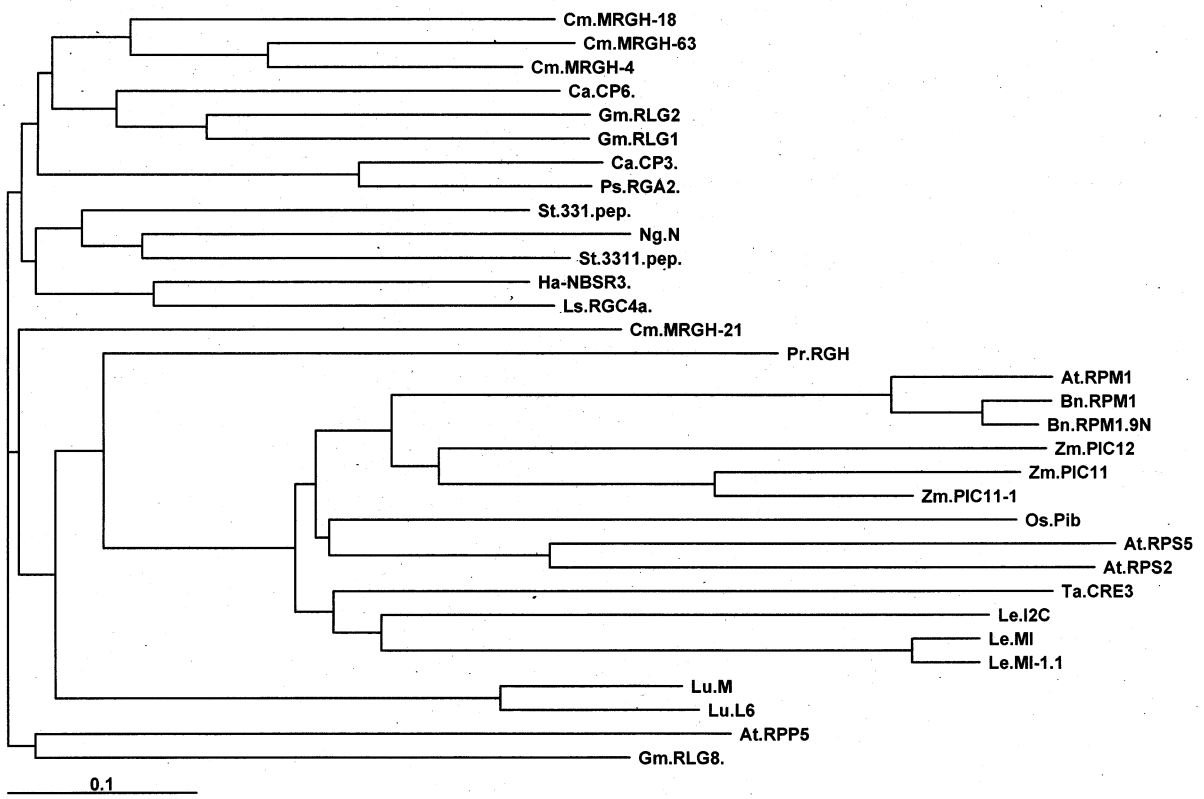

Fig. 2. Phylogenetic tree representing the melon RGHs and a group of R-genes and RGHs from other plant species. The amino acid sequence comprised between the P-loop and the GLPLAL domains of all the RGHs was aligned using the CLUSTALW program. The tree was constructed with the TreeView program. Sequences represented were as follows: At.RPM1, A. *thaliana*, disease resistance protein. AC Q39214; At.RPP5, A. *thaliana*, downy mildew resistance protein. AC O04264; At.RPS2, A. thaliana, resistance to pseudomonas syringae protein 2. AC Q42484; At.RPS5, A. *thaliana*, resistance to pseudomonas syringae protein 5. AC O81402; Bn.RPM1.1A, *B. napus*, disease resistance gene homolog 1A. AC Q9ZSH2; Bn.RPM1.9N, *B. napus*, disease resistance gene homolog 9N. AC Q9ZSH1; Ca.CP3, *C. arietinum*, homolog resistance gene. AC AAF36333.1; Ca.CP6, *C. arietinum*, homolog resistance gene. AC AAF36336.1; Cm.MRGH-18, *C. melo*, putative resistance gene homologue. AC CAB88868; Cm.MRGH-21, *C. melo*, putative resistance gene homologue. AC CAB88869; Cm.MRGH-4, *C. melo*, putative resistance gene homologue. AC CAB88871: Cm.MRGH-63, *C. melo*, putative resistance gene homologue. AC CAB88870; Gm.RLG1, *G. max*, disease resistance protein homolog RLG1, AC T08819; Gm.RLG2, *G. max*, disease resistance protein homolog, RLG2. AC T08820; Gm.RLG8. *G. max*, disease resistance protein homolog RLG8, AC T08835; Ha-NBSR3. *H. annuus*, candidate resistance gene sunflower downy mildew. AC AD00827; Le.I2C, *L. esculentum*, resistance complex protein. AC O24016; Le.MI, *L. esculentum*, root-knot nematode resistance protein. AC O81137; Le.MI-1.1, *L. esculentum*, disease resistance gene homologue MI. AC O81136; Ls.RGC4a. *L. sativa*, resistance protein candidate. AC AAC02205; Lu.L6, *L. usitatissimum*, rust resistance gene. AC Q40254; Lu.M, *L. usitatissimum*, rust resistance protein. AC P93244; Ng.N, *N. glutinosa*, tobacco mosaic virus resistance gene. AC Q40392; Os.Pib, *O. sativa*, MRNA for NBS type resistance gene. AC BAA93618; Pr.RGH *P. radiata*, resistance gene homolog. AC O49121; Ps.RGA2. *P. sativum*, disease resistance protein AC AAD52715.1; St.331.pep. *S. tuberosum*, disease resistance protein homolog. AC T07766;St.3311.pep. *S. tuberosum*, disease resistance protein homolog. AC T07772;Ta.CRE3, *T. aestivum*, cyst nematode resistance gene candidate. AC O65113;Zm.PIC11, *Z. mays*, disease resistance gene analog PIC11. AC Q9ZTJ5; Zm.PIC11-1, *Z. mays*, disease resistance gene analog PIC11-1. AC Q9ZTJ4; Zm.PIC12, *Z. mays*, disease resistance gene analog PIC12. AC Q9ZTJ3.

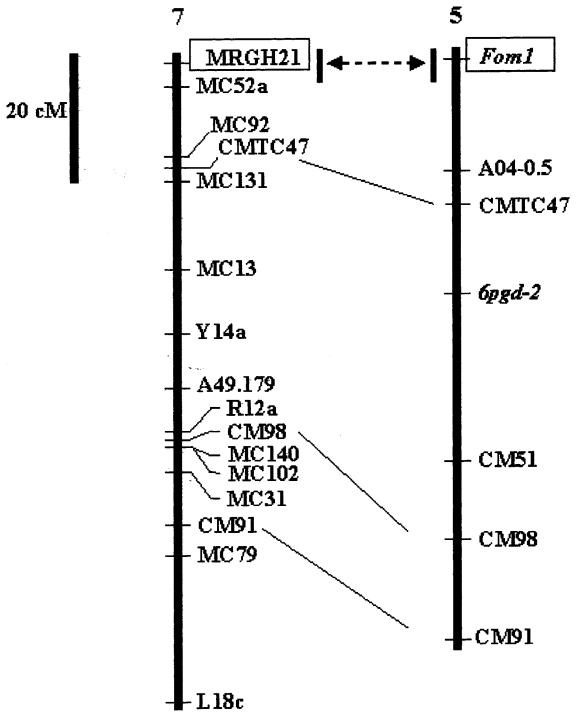

Fig. 3. Representation of Linkage Group 7 from the melon genetic map of Oliver et al. [14] and linkage Group 5 from the melon genetic map of Baudracco-Arnas and Pitrat [23], showing common RFLP markers CM98 and CM91 and SSR marker CMTC47 as bridges between both maps. Resistance gene *Fom*-1 and MRGH21 are represented with other markers as RFLPs (all markers with the MC or CM notation), AFLPs (A49.179), RAPDs (A04–0.5, Y14a, R12a and L18c), SSRs (CMCT47) and isozymes (*6pgd-2*). Vertical bars and the dashed arrows indicate similar positions between both maps where *Fom*-1 and MRGH21 are located.

the four RGH families were generally more similar to each other (similarities ranging from 45–75%) than they were from known resistance genes from other species, such as *N*, *RPS*2 and *L6* (32.5–56.3%). No cross-hybridisation among the four RGH families was observed when using stringent hybridisation conditions, but faint hybridising bands were present with probe MRGH4 that were compatible with the hybridisation pattern of MRGH63. This result is consistent with the close similarity (75%) between the amino acid sequences of these two clone families.

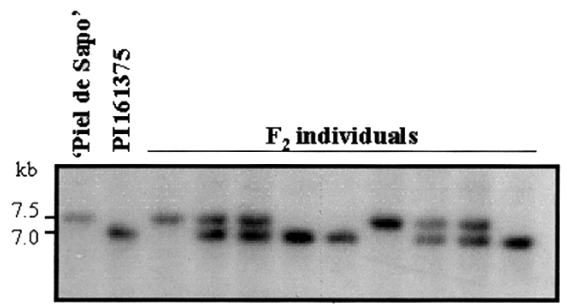

Fig. 4. DNA blot hybridisation with probe MRGH63 on genomic DNA digested with *Mva*I of the melon genetic map parental lines ('Piel de Sapo' and PI 161375) and nine individuals of the derived F_2 population. The band sizes (in kb) are also indicated.

An alignment of the four MRGH sequences with a group of 28 known resistance genes and RGH gene families representing different plant species, has shown that three of the four melon RGHs detected in this study fall into a small group of sequences which only contains RGHs from species belonging to the *Fabaceae* family: pea, chickpea and soybean. This conservation of sequence homology between *Fabaceae* and *Cucurbitaceae* RGHs agrees with the results presented by Chase et al. [22], comparing highly conserved protein sequences, indicating that these two plant families are among the most closely related phylogenetically within the dicots.

RGH-derived markers have been placed in the neighbourhood of disease resistance genes in several species [10]. For the moment, we do not have data on disease resistance gene segregation in our mapping population, but our map contains 27 anchor loci, 14 RFLPs and 13 SSRs, with the melon genetic map published by Baudracco-Arnas and Pitrat [23]. These markers allow the identification of eleven homologous linkage groups between the two maps [24]. The map of Baudracco-Arnas and Pitrat [23], constructed with a population that shares with ours one of the parents, PI 161375, includes the position of four resistance genes: two of them that confer resistance to *Fusarium oxysporum* f.sp. *melonis* (*Fom*-1 and *Fom*-2), a gene for resistance to melon necrotic spot virus (*nsv*), and the gene *Vat* for resistance to the aphid *Aphis gossypii* [25]. Each of these genes is placed on a different linkage group of this map.

Using this information it has been possible to assign preliminarily the position of three of our RGHs in the genomic region where two of these genes are placed. MRGH21 maps in Linkage Group 7 of our map. This group has three anchor loci (2 RFLPs and 1 microsatellite) with Linkage Group 5 of Baudracco-Arnas and Pitrat [23] that includes gene *Fom*-1 (Fig. 3). The closest common marker to MRGH21 and *Fom*-1 is the microsatellite CMTC47 [24], that is located 25 cM from

MRGH21 and 20 cM from *Fom*-1 on the same side. Both MRGH21 and *Fom*-1 are the most distal markers in their respective maps. The gene *Vat* is located in Linkage Group 2 of Baudracco-Arnas and Pitrat's map. MRGH63 and MRGH4 detect RFLPs that map to its homologous group in our mapping population (Linkage Group 4). In this case only one marker, the microsatellite CMAT35, is present in both groups (24; M. Pitrat, pers. comm.) and therefore can be used as bridge point between them. Thus, we know that *Vat* and these RGH loci are in the same chromosome, but we cannot determine with precision their relative position. Mapping additional transportable markers, like RFLPs and SSRs, in these regions of both maps, and the study of the co-segregation between the MRGH markers and resistance genes, in these or other melon populations, would determine the usefulness of the RGHs detected in this study for marker-assisted selection.

Some resistance genes are organized in clusters, and the same genomic regions have been identified carrying genes that confer resistance to virus, bacterial, fungal diseases or insects [26]. This is also the case for the *Fom*-1 region of melon, that includes the disease resistance gene *Prv* against papaya ringspot virus [27] and a QTL for resistance to the To72 strain of cucumber mosaic virus [28]. Other resistance genes have also been identified near the *Vat* locus: the *Pm-w* gene that confers resistance to *Sphaerotheca fuliginea* race 2 from the melon line WMR29 [29], and another gene of resistance to *Aphis gossypii* (*Agr*) from the melon accession PI 414723 [30]. The latter gene maps in a similar position as *Vat*, but a different mapping population was used to map it. Markers tightly linked to one of these genes may also be useful for the selection of the others or for the development of specific markers for different resistance genes contained in a small chromosomal region.

The cloning of NBS-containing sequences can accelerate the process of identification of markers for known disease resistance genes. These RGHs might be employed in a marker-assisted selection program to identify the presence of a tightly linked disease resistance gene. Furthermore RGHs may act as the starting point for map-based cloning using RGH as markers to construct a detailed map of the genomic region of interest. The possibility of a given RGH to coincide with the resistance gene itself is remote if we consider the complexity of some disease resistance gene clusters and the number of RGHs that they may contain [31]. However, Feuillet et al. [32] reported the cloning of a disease resistance gene (*Lr10*) in wheat with the use of a degenerate primer approach.

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