Theor Appl Genet (2002) 104:1055–1063
DOI 10.1007/s00122-001-0808-x

Y. Brotman · L. Silberstein · I. Kovalski · C. Perin
C. Dogimont · M. Pitrat · J. Klingler · G.A. Thompson
R. Perl-Treves

Resistance gene homologues in melon are linked to genetic loci conferring disease and pest resistance

Received: 29 April 2001 / Accepted: 24 September 2001 / Published online: 8 February 2002

Abstract Genomic and cDNA fragments with homology to known disease resistance genes (RGH fragments) were cloned from *Cucumis melo* using degenerate-primer PCR. Fifteen homologues of the NBS-LRR gene family have been isolated. The NBS-LRR homologues show high divergence and, based on the partial NBS-fragment sequences, appear to include members of the two major subfamilies that have been described in dicot plants, one that possesses a TIR-protein element and one that lacks such a domain. Genomic organization of these sequences was explored by DNA gel-blot analysis, and conservation among other Cucurbitaceae was assessed. Two mapping populations that segregate for several disease and pest resistance loci were used to map the RGH probes onto the melon genetic map. Several NBS-LRR related sequences mapped to the vicinity of genetic loci that control resistance to papaya ringspot virus, *Fusarium oxysporum* race 1, *F. oxysporum* race 2 and to the insect pest *Aphis gossypii*. The utility of such markers for breeding resistant melon cultivars and for cloning the respective *R*-genes is discussed.

Communicated by J.S. Beckmann

Y. Brotman · L. Silberstein · I. Kovalski · R. Perl-Treves (✉)
Faculty of Life Sciences, Bar-Ilan University,
Ramat-Gan 52900, Israel
e-mail: perl@mail.biu.ac.il
Tel.: +972-3-5318249, Fax: +972-3-5351824

C. Perin · C. Dogimont · M. Pitrat
INRA, Station de Génétique et d'Amélioration des Fruit et Légumes,
B.P. 94, 84143 Montfavet Cedex, France

J. Klingler
Department of Plant Sciences, University of Arizona, Tucson,
AZ 85721, USA

G.A. Thompson
Department of Applied Science,
University of Arkansas at Little Rock, Little Rock, AR 72204,
USA

Keywords Melon · *Cucumis melo* · *R*-genes · Resistance gene homologues · NBS-LRR · RFLP · *Aphis gossypii* · *Fusarium oxysporum*

Introduction

The genetic mechanism that underlies plant defense against pathogen attacks has become the focus of intense molecular research, following the breakthrough cloning of the first resistance genes (*R*-genes: Hammond-Kosack and Jones 1997; Martin 1999). The best known model for plant–pathogen interaction is the "gene for gene" mechanism, that requires recognition of a pathogen-derived gene product by a plant *R*-gene product, in order to develop an incompatible response. As a result of such molecular recognition, a signal transduction cascade is initiated, and plant defenses are expressed. An important, rather unexpected, discovery was that most of the *R*-genes cloned so-far share structural features, in spite of the diversity of pathogens against which they act, which included bacteria, fungi and viruses, as well as insects and nematodes (Hammond-Kosack and Jones 1997; Martin 1999; Ellis et al. 2000). Thus, nearly all the cloned *R*-genes, involving the dominant, 'gene-for-gene' mechanism, may be assigned to two gene families. The Pto receptor-kinase family encodes intracellular serine-threonine kinases, while the LRR (leucine-rich repeat) superfamily encodes proteins with an LRR domain, that exhibits hypervariability and confers recognition-specificity. Most LRR proteins also contain an NBS (Nucleotide Binding Site) domain, and additional elements such as a coiled-coil domain or a Toll-Interleukin-like (TIR) domain (Bent 1996). Genes that belong to still other classes of *R*-genes include barley's *Mlo* (Buschges et al. 1997), that shows no homology to known genes, maize *HM1* (Johal and Briggs 1992), whose product de-toxifies a fungal toxin, and the recently cloned *RTM1* and *RTM2* genes from *Arabidopsis*, encoding a protein with a similarity to lectins (Chisholm et al. 2000) and one with a heat-shock protein domain (Whitham et al. 2000).

Melon (*Cucumis melo*) is an important agricultural crop in many countries. Several diseases and pests constrain melon production, affecting the yield and forcing massive use of chemical sprays. To assist the breeding of resistant varieties and to obtain a better knowledge on the genetics of this species, genetic maps of melon have been constructed that contain economically important traits and molecular markers (Baudracco-Arnas and Pitrat 1996; Wang et al. 1997; Oliver et al. 2001; Perin et al. 1998, 2000; Danin-Poleg et al. 2000). One approach to identify novel *R*-genes from additional plant species relies on homology to previously characterized genes from other plants. It has been shown in several crops that *R*-gene homologues (RGH) isolated by a PCR approach often map to disease resistance loci where they form gene clusters (e.g. Leister et al. 1996, 1998; Yu et al. 1996). To obtain a collection of RGH sequences from melon, we applied degenerate PCR primers designed to bind to the P-loop, Kinase-2 and the GLPL (also called 'Hydrophobic Domain') elements in the NBS-LRR gene family (Traut 1994; Meyers et al. 1999). We report the cloning and characterization of 15 NBS homologues. We show that multiple and diverse *R*-gene homologues exist in the melon genome, and some of these appear to be well conserved even across genera in the Cucurbitaceae. Most of these were localized as RFLP markers on molecular linkage maps of melon, to determine whether such sequences map to regions that harbor disease/pest resistance genes.

Materials and methods

PCR amplification of RGH sequences

Three melon genotypes provided template DNA for PCR amplification of RGH sequences: cultivar Top-Mark, the Indian accession PI414723, and the aphid-resistant breeding line AR5 (McCreight et al. 1984; Anagnostou et al. 2000). Plant DNA was prepared according to Baudracco-Arnas (1995). PCR reactions were performed in a total volume of 50 µl with 0.5 units of *Taq* polymerase in 10 mM Tris HCI, pH = 9, 1.5 mM $MgCl_2$, 50 mM KCI, 0.1 mM dNTPs, 0.1% Triton × 100, 0.2 mg/ml of BSA, 0.25 µM of each primer and 30–100 ng of template DNA. Degenerate primers (Table 1) were applied at annealing temperatures that varied between 38 and 50 °C. Amplification included initial denaturation for 5 min at 95 °C, followed by 35 cycles of 1-min denaturation at 95 °C, 1 min at the annealing temperature, 1′ 30″ elongation at 72 °C and a final extension step at 72 °C for 10 min. For RGH cloning using the reverse transcriptase-PCR method, total RNA was isolated from PI 414723 using the Tri Reagent (Molecular Research Center Inc.) and treated with DNAase I (Promega). First-strand cDNA was synthesized using 0.2 units of AMV-reverse transcriptase in a 25-µl volume, with 1 unit of RNAase inhibitor, 5 mM $MgCl_2$, 1 mM dNTP mix, and 1 × RT buffer (Promega) and 10 picomol of the reverse primer for 1 h at 45 °C. Eight microliters of the first-strand reaction were PCR-amplified in a total volume of 50 µl, using the same primer pairs and cycling conditions as above.

Cloning and analysis of PCR products

PCR amplifications products were separated by electrophoresis on 1% TAE-agarose gels. Bands exhibiting the predicted size and additional larger ones were excised, and DNA was eluted using the DNA Isolation Kit (Biological Industries, Beth Haemek, Israel). PCR products were cloned using the pGEM-T-easy vector system (Promega) and transformed into the *Escherichia coli* XL1-blue MRF^- strain. To differentiate between individual clones, we digested DNA from 6 to 12 individual colonies from each ligation to release the inserts, and sorted them according to small size variations on 2% agarose gels. Sequencing was carried out using an ABI sequencer and the *Taq* dideoxy Terminator Cycle sequencing kit (Perkin-Elmer). Clones that exhibited homology to *R*-genes were fully sequenced from both strands, and sequences were deposited in the GenBank (accession numbers are given in Table 2). Sequence data were analyzed with GCG9 programs (University of Wisconsin Genetics Computer Group, Madison). Homology searches were conducted using the GenBank, OWL and EMBL databases with the BLAST algorithm and the Biccellerator package (Compugen, Israel). The predicted amino-acid sequences were aligned using the GCG PILEUP program, and the alignment was improved manually. Pairwise Pam-Dayhoff genetic distances were calculated by the program PROTDIST (PHYLIP package, Felsenstein 1993), and phylogenetic trees were constructed from these distances by the NEIGHBOR program (same package) using the UPGMA method. The trees were subjected to Bootstrap analysis using the SEQBOOT and CONSENSE programs of the same package.

Southern-blot analysis

For the cucurbit comparative blot we used DNA from *C. melo* PI 414723, *Cucumis sativus* cultivar Erez, *Citrullus lanatus* PI 296341 and the *Cucurbita maxima* × *Cucurbita moschata* cultivar hybrid 'Brava'. Genomic DNA was analyzed by Southern hybridization according to Kahana et al. (1999).

Table 1 Primers used in this study for the cloning of NBS homologous sequences. Key to nucleotide symbols: I, inosine; R – A and G mix; Y – C and T; W – A and C; S – G and C; N – G, C, T and A

Protein region	Primer	Peptide encoded	Primer sequence (5′ →3′)
Kinase-1a	15912	GGVGKTT	GGT GGG GTT GGG AAG ACA ACG
	15914	GGVGKTT	GGI GGI GTI GGI AAI ACI AC
	16410	GGLGKTT	GGI GGI YTI GGI AAR ACI AC
	16403	GGMGKTT	GGI GGI ATI GGI AAA ACI AC
	16409	GGSGKTT	GGI GGI WSI GGI AAR ACI AC
	689	GM(P/G)G(L/V)GK	GGI ATG SSI GGI STN GGN AAR
	PLP	GG(V/I/M)GKTT	GGI GGI RTI GGI AAR ACI AC
Kinase-2	310	VLDDVW	CCA IAC RTC RTC NAR NAC
	$antiK_2$	VLDDVW	CCA NAC RTC RTC IAR IAC
HD	$antiHD_1$	CKGLPL	ARN GGI ARI CCY TTR CA
	464	GLPL(I/A)LK	TTI ANI GYI ARI GGN ARN CC

Table 2 *R*-gene homologues cloned in this study. The length of each clone, the primers and annealing temperature used to generate it, as well as the genotype from which it originated, are indicated. In a few cases the same sequence was cloned from two genotypes. Clone NRT-A4 was generated by RT-PCR using total RNA as a template. The other clones represent genomic fragments. The nucleotide sequences of these clones have been deposited in the GenBank and given accession numbers AF354504–AF354517

Clone	Length	Annealing temp.	Primer combination	Genotype
NBS1	252 bp	45 °C	PLP+antiK2	AR5
NBS2	506 bp	48 °C	PLP+antiHD1	PI 414723,AR5
NBS3	264 bp	45 °C	PLP+antiK2	AR5
NBS5	244 bp	45 °C	PLP+antiK2	AR5
NBS7	507 bp	48 °C	PLP+antiHD1	AR5
NBS17-5	255 bp	45 °C	16410+310	PI 414723,AR5
NBS23-2	246 bp	45 °C	15914+310	PI 414723
NBS26-2	255 bp	45 °C	15912+310	PI 414723
NBS37-4	266 bp	38 °C	15914+310	PI 414723
NBS39-2	223 bp	38 °C	15914+310	PI 414723
NBS42-12	245 bp	38 °C	16409+310	PI 414723,AR5
NBS45-8	251 bp	38 °C	689+310	PI 414723
NBS46-7	249 bp	38 °C	16403+310	TopMark
NBS47-3	249 bp	38 °C	16409+310	TopMark
NBS-A4	250 bp	45 °C	16409+310	PI 414723

Linkage mapping of RGH fragments

The RGH probes were mapped as RFLP markers using two RIL mapping populations: the first was derived from the cross Vedrantais × PI 161375 and the second from Vedrantais × PI 414723 (Perin et al. 1998, 2000). Blots with parental DNA digested with four restriction endonucleases, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I, were used to screen for polymorphism. When polymorphism was present, blots with DNA from approximately 50–100 individual RILs were prepared using the appropriate endonuclease. Southern hybridization protocols were according to Kahana et al. (1999). Blots were exposed to X-ray film (FujiFilm) or, when the signals were weak, analysed by the Phosphor-Imager (BAS 1500, FujiFilm) for 1–5 days. The MAPMAKER software (Lander et al. 1987) was used. RGH markers were first linked to an existing linkage group based upon LOD scores ≥ 3, using the command GROUP. Localization to specific intervals in the reference map was achieved by the commands BUILD and TRY. Genetic distances were calculated by the Kosambi mapping function.

Results

A diverse family of NBS-LRR homologues from melon

In order to clone NBS-LRR homologues, we applied a total of 31 primers in 127 combinations, using DNA from three genotypes: PI 414723, cultivar Top Mark and breeding line AR5. Primers that successfully amplified NBS homologues are shown in Table 1. The large number of primer variants tested reflects the high variability of this super-family: the NBS region is the only one that harbors a few short motifs that are sufficiently conserved to allow the design of degenerate primers, but even this region is highly variable. It was therefore important to perform the appropriate PCR controls, and to define the optimal conditions for every primer combination. We varied the annealing temperature between 38 and 59 °C and ran every set of reactions with three controls: no template DNA, and two single-primer reactions. The latter proved to be important, as most of the PCR products in our reactions were produced by a single primer in the mix rather than the pair, even when the temperature was increased. In the example shown in Fig. 1, primers 15914 and 464 amplified several bands at annealing temperatures of 48 °C and 45 °C, but most bands are generated also by either primer 15914 or primer 464 alone. The approximately 500-bp band is, nevertheless, primer pair-specific and, upon cloning, was shown to contain an NBS-homologoue. Similarly, a 250-bp band, produced specifically by primers 15914 and 310, had the expected size, based on the corresponding region in other species,

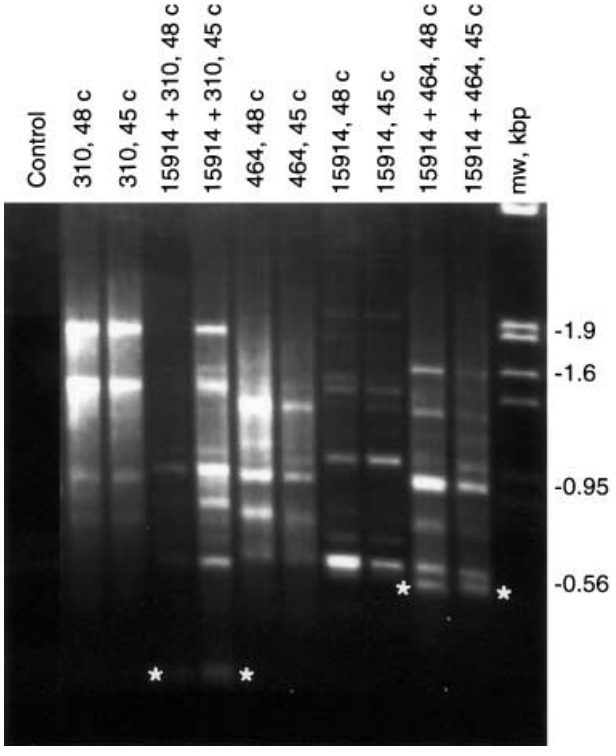

Fig. 1 Amplification of NBS-domain homologues using degenerate PCR primers. The PCR primers that were used are indicated above each lane. Reactions were performed at two different annealing temperatures, 45 °C and 48 °C. Reactions primed by a single primer were run as well. The 'Control' reaction lacked template DNA. The bands marked by *asterisks* were produced by the pair of primers rather than a single primer and had the expected size; they turned out to contain NBS homologues, along with unrelated sequences. *MW* = molecular-weight standards

and gave rise to a positive clone. Thirteen bands that exhibited larger-than-expected size (selected assuming possible introns) were cloned, but none of these contained an RGH sequence.

Using the above approach, we cloned 58 PCR products and sequenced 118 representative clones. Twenty three clones turned out to be NBS-homologues that could be classified into 15 different sequences (Table 2); the remaining eight clones were redundant, being isolated more than once with different primer pairs. By sequencing several representative clones from some PCR bands, following their sorting according to slight size variation, it became clear that gel-eluted PCR bands represented mixtures of sequences. The same band often contained RGH as well as unrelated sequences. Increasing the stringency of PCR conditions (by applying higher annealing temperature "hot start" or "touch-down" protocols) to obtain more specific amplification products was not successful. In addition, we applied RT-PCR to target expressed RGH sequences resulting, so far, in the cloning of an NBS cDNA fragment, designated NRT-A4. The decoded amino-acid sequences of the 15 NBS sequences are presented in Fig. 2A. Two of the sequences, NBS37-4 and NBS7, are interrupted by multiple stop codons and must represent pseudogenes. Two clones, NBS2 and NBS7, were amplified using the 'Kinase-2' and 'Kinase-1a' and 'Hydrophobic Domain' motifs, resulting in approximately 500-bp fragments, while all the others were produced by primer that matched the 'Kinase-2' and 'Kinase-1a' motifs and were approximately 250-bp long. We have also tested primers that matched the 'Kinase-3A' as well as additional motifs located further downstream, but no RGH was isolated using such primers. Cluster analysis was performed on the aligned amino-acid sequences of the 15 melon fragments, along with representatives of known NBS-LRR resistance genes from tomato, tobacco, flax, *Arabidopsis* and rice (Fig. 2B).

The melon NBS sequences exhibit very high sequence divergence. The most-closely related sequences are NBS46-7, NBS2 and NBS23-2 (62–74% amino-acid identity, 74–80% similarity). Another close pair is NBS42-12 and NBS17-5 (60% identity, 68% similarity), but homology between most sequence-pairs is low: e.g. NBS5 and NBS17-5: 27% identity, 46% similarity; NRT-A4 and NBS37-4: 15% identity. The high divergence among our clones did not allow for a very consistent grouping of sequences into subclusters (Fig. 2B); only a few of the nodes generated by the NEIGHBOR-JOIN program withstood the Bootstrap test. Such nodes were marked by asterisks. The other, unmarked nodes, were not present in the majority of Bootstrap replicates (that are generated by random samplings of half of the data) because they are supported by only a few residues in the alignment.

The dendrogram (Fig. 2B) divides the sequences into two broad subfamilies: the first, rather-tight cluster, includes ten melon gene fragments as well as the tobacco *N* gene and flax *L6* gene; and the second, much looser

A.

```
                Kinase-1a
NBS2        ....GGMGKT TLAKALYNKI ADDF..EGCC FLPNIREASN ..QYGGLVQL
NBS7        ....GGVGKT TLAKALYSRI ADSL..EGCC FLANIRVEA. LN*YHCLVQL
NBS23-2     ......VGKT TLAKALYNKI TYEF..EACC FLSNVREASE ..QFNGLVQL
NBS46-7     .....GMGKT TLAKALYNKI ASQF..EGCC FLSNVREASK ..QFNGLAQL
NBS3        ....GGVGKT TVAKAVYNCI AYRF..EGCS FLPNIKEKCN FSRDDELTKL
NBS1        ....GGVGKT TIAKAVFKSV AREF..HGSC ILENVKKTLK NVR..GLVSL
NBS47-3     ....GGRGKT TIAQVVFDCI LSKF..DDCC FLTLPGGDSK QS....LVSL
NBS26-2     .....GVGKT TIAKVCYQRI RDEF..EAHC FLSDVRE.NY FRTSGDLPYL
NBS5        ....GGVGKT TLAKVLYNKI AYQF..EGCC FLQDVRREAS K...HGLVEL
NBS39-2     ....GGVGKT TLAKAVCQEL KPKFGYNSHC FL........ RVHTEKLVTV
NBS17-5     ....GGLGKT TLAKMVFNHE DIKGHSDQMI .WVCVSKPFN VMKILEE.IF
NBS42-12    ....GGWGKT TLAKTIFNHE EIRGHFDETI .WICVSEPFL INKILGA.IL
NBS45-8     ..WDSGGGET ALAKFLYNDT EVSKHFDIKM .WVWVSEQFH VKILVEKMII
NRT-A4      ....WGVGET TLALKFCHDK EVKDIFQEKI FFVAVSRKPD LKLILKDIIE
NBS37-4     LILKGMNGKS SMSY*..WKI L..VIFLPY* IRPNLFEFLM RKKK*SRVE*

                                                  Kinase-2
NBS2        QRELLHEI.L VDDSIKV.SN LPRGVTIIRN RLY.KKISLL ILDDVDTREQL
NBS7        HEKLLL*DFN G*LY*S*.QR S*RNYHRKGS TML.KKDSFN SYDDIDMSEQS
NBS23-2     QEKLLSEI.F KDNNLKV.DN VHKGMNIMKD RLC.SRKVLI VLDDVW.....
NBS46-7     QESLLYEI.L .TIYLKV.VN FDRGINIIRN RLC.SKKVLI VLDDVW.....
NBS3        QESLLQDILL IKMHRSI.SF SDEGSNVLRH RLR.NKKVLI VLDDVW.....
NBS1        QEKLLSD.TL MRGKVQI.KD .GEGVEMIKK NLG.NRKVFV VLDDVW.....
NBS47-3     QREMLSQI.F HKEDFRI.WH ENHGVEMIKN RLS.GRKVLI VFDDVW.....
NBS26-2     QTKLLSRMFS FKNN.HI.LD VEEGIAMINK AIF.RKKTLL VLDDVW.....
NBS5        QKTLLNDIL. .KEDLKV.VS RDRRI.IIRS RLF.KESSYS PRRRM......
NBS39-2     QKKLLLR.FG GKLDIVI.KN EDHGAKLIKK CLR.NIKVL. ..........
NBS17-5     Q...SMTDTC SGLKSKE.A. LLRRLQTEMQ DKKYFLVFSY VLDDVW.....
NBS42-12    Q...MIKGVS SGLDNKE.A. ....LLRELQ KVMRGKRYFL VFDDVW.....
NBS45-8     S...ATSDRN PNVHLMD.S. ...LQRELQK VIQ.GKKYLL VLDDVW.....
NRT-A4      S...LRGIQL PDLQSDE.RA FCYLEMWLKQ TSV.NRPVLM VLDD.......
NBS37-4     ING*GTMLQL *RN..KK*RA YV.LNLNDSP I*L.DVKTIL VLDDVW.....

NBS2        QALVGGHDWF GHGSKVIATT RNKQLLVTHG FDKMQSVVGL DYDEALELFS
NBS7        QVLVGG.T*L VWTWK*GRRD NKKHLLAIHG FNILQNVIRL NDDEALELFS

                                    HD
NBS2        WHCFRNSHPL NDYLELSKRA VDYCKGLPLAIK
NBS7        SMLLEIVTPQ MII*NFQNEL SLL*RPPP
```

B.

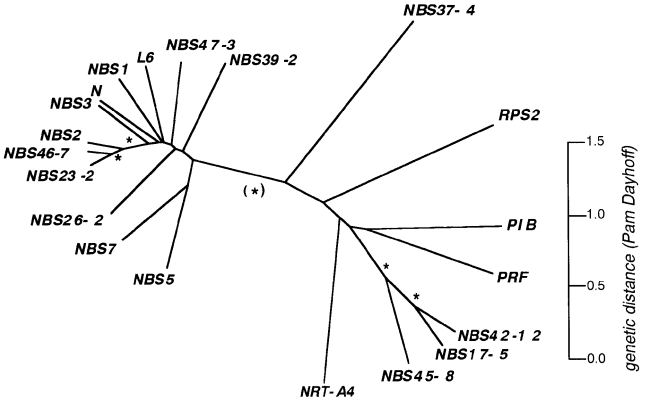

Fig. 2 **A** Alignment of deduced amino-acid sequences encoded by the 15 NBS-LRR homologous fragments from melon. Sequences encoded by the primers used for PCR amplification are *underlined*. Clones NBS2 and NBS7 were amplified by downstream primers corresponding to the Hydrophobic Domain and are longer than the other clones. **B** Dendrogram of NBS genomic fragments from melon and from representative *R*-genes of other plants. Cluster analysis was performed as in Fig. 2. The *node* marked by (*) is supported by 12 out of 25 Bootstrap replicates, those marked by * were supported by the majority of replicates. *Unmarked nodes* were not supported by the Bootstrap test. Non-melon sequences included the tobacco *N* gene, GenBank accession from U51605; *L6* from flax, accession U27081; rice *PIB*, AB013449; *Arabidopsis RPS2*, U12860; and tomato *PRF*, U65391

cluster, comprises five melon genes, *Arabidopsis Rps2*, rice *Pib* and tomato *Prf*. Such grouping probably reflects the well-documented phylogenetic dichotomy between Group I proteins, which contain a TIR domain, and Group II which lack the TIR element. The internal node that separates the two groups is the longest in the tree, and has been marked by (*) to indicate that it is supported by 12 out of 25 Bootstrap replicates.

Genomic organization and rates of polymorphism of NBS-like sequences

We applied Southern-blot analysis to investigate the genomic organization of melon RGH sequences. When the NBS fragments were hybridized at a stringent temperature (60 °C for short, 250-bp probes, and 65–68 °C for the longer ones), one or two strongly hybridizing fragments were detected using several restriction enzymes to digest the genomic DNA. In a few cases, additional faint bands were present as well. Only NBS5 yielded multiple strong bands at stringent temperatures, indicating that most of the RGH sequences that were cloned are probably represented by a single locus in the melon genome. At lower stringency conditions (50–55 °C), many additional bands were apparent, indicating that multiple, more distantly related sequences exist. The genomic organization of a representative gene is shown in Fig. 3

Since NBS-LRR sequences represent a highly variable gene family, apparently subjected to diversifying evolution (Parniske et al. 1997), we wished to know whether they would provide a class of particularly polymorphic RFLP markers. We therefore compared the levels of polymorphism detected between two diverged melon genotypes, cultivar Top Mark and PI 414723, using different sources of RFLP probes and four restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I). Out of 30 randomly chosen cucumber cDNA probes hybridized at high stringency, 20 (67%) were polymorphic by at least one restriction enzyme. This is similar to the proportion of polymorphic NBS probes (10 of 15, 67%). However, if we take into account the number of restriction enzymes that detected an RFLP with each probe, the ratio of polymorphic probe × enzyme combinations is 35% for NBS fragments, that often detected two or more polymorphisms per probe, compared to 23% for the random cDNAs. Moreover, since the cDNA probes were much larger (average size 1 kb) than the NBS probes (average 0.28 kbp), and larger probes have a higher probability of detecting an RFLP, it appears that NBS probes are significantly more polymorphic. A sample of 23 random genomic clones from melon was the least polymorphic category (44% of the probes, 16% of the probe × enzyme combinations were polymorphic), consistent with results of a previous study (Silberstein et al. 1999).

Comparative genome mapping among different species raises considerable interest from both evolutionary and applied perspectives (e.g. Grube et al. 2000). We therefore hybridized, under moderately stringent condi-

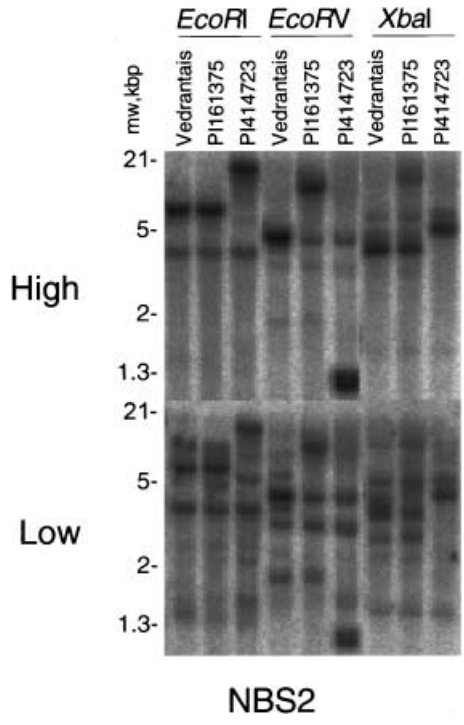

Fig. 3 Genomic organization of an NBS-LRR homologue. DNA samples (3 µg/lane) of three melon genotypes used as mapping parents in this study, namely the French cultivar Vedrantais, the Korean landrace PI 161375 and the Indian landrace PI 414723, were digested with three restriction endonucleases. The blot was hybridized with the melon NBS2 probe under two stringency regimes, differing in hybridization and washing ternperature: "High" represented 60 °C, "Low" was 50 °C. MW = molecular-weight standards

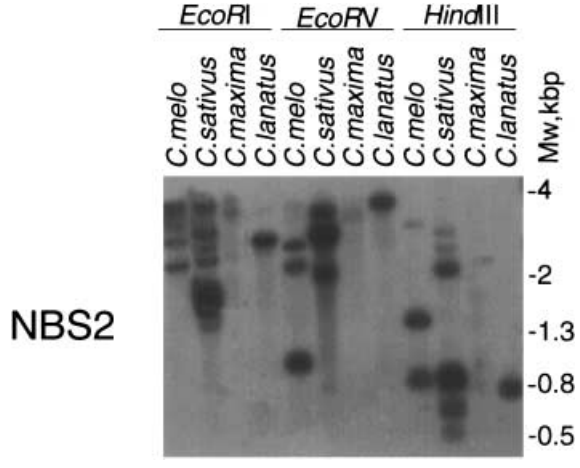

Fig. 4 Conservation of the NBS2 sequence among species and genera of the Cucurbitaceae. DNA from *C. melo*, the species from which the probes had been cloned, and three other cucurbit species, *C. sativus* (cucumber), *C. maxima* × *C. moschata* (a pumpkin hybrid) *C. lanatus* (watermelon), was digested with three different restriction endonucleases (3 µg per lane), blotted and hybridized at 60 °C. MW = molecular-weight standards

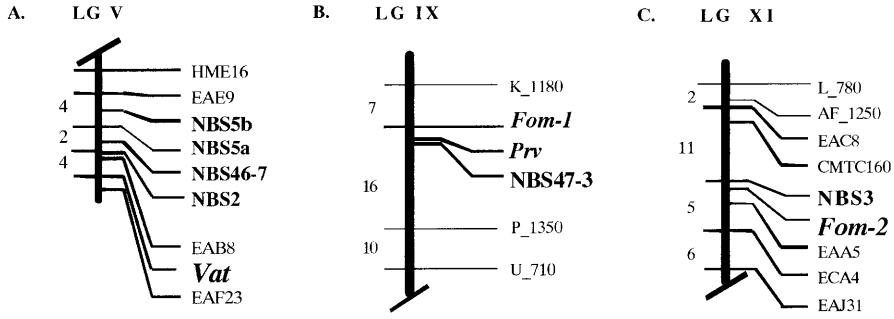

Fig. 5 Genetic maps of regions harboring NBS homologues that map to the proximity of agronomically interesting loci. Linkage group designation and molecular markers are according to Perin et al. (1998, 2000, and submitted manuscript). Unit distances between skeletal markers (those crossing the vertical bar) are shown in centi-Morgans. Pairwise distances between NBS markers and linked phenotypic traits are specified in the text. RGH markers are in *bold*, agronomic traits are in *bold-italic*. **A** Distal region of Linkage Group V, harboring the aphid resistance gene *Vat* and a Powdery mildew resistance gene, *Pm-w*. **B** Region of Linkage Group IX harboring resistance genes *Fom-1* and *Prv*, conferring resistance to *Fusarium* races 0 and 2, and to PRV, respectively. **C** Region of Linkage XI with resistance gene *Fom-2*, conferring resistance to *Fusarium* races 0 and 1

Table 3 Map position of RGH clones. Mapping populations used: 1 – RIL derived from Védrantatis × PI 161375, 2 – RIL derived from Védrantais × PI 414723. Linkage groups and markers are according to Perin et al. (2001, submitted manuscript)

Clone	Mapping population	Linkage group	Flanking markers
NBS2	1 & 2	V	EAE9 – EAB8
NBS3	1 & 2	XI	CMTC160 – *Fom-2*
NBS5a	2	V	EAE9 – EAB8
NBS5b	2	V	EAE9 – EAB8
NBS7	2	IX	HMD61 – ECD55
NBS17-5	1 & 2	IV	EAG8a – N 950
NBS23-2	2	VI	EAJ33 – EAD15
NBS26-2	1	Unlinked	
NBS37-4	1	Unlinked	
NBS42-12	1	VII	ECF8 – S 1300
NBS46-7	2	V	EAE9 – EAB8
NBS47-3	2	IX	P 1350 – *Fom-1*

tions, melon RGH probes with comparative blots that included, in addition to *C. melo*, three other cucurbits: *C. sativus* (cucumber), *C. lanatus* (watermelon) and a *C. maxima* × *C. moschata* hybrid (pumpkin). Figure 4 depicts the results obtained with a representative RGH clone. The NBS-LRR homologue, NBS2, detected strong signals in *C. sativus* and in *Citrullus*, but not in the more distant genus *Cucurbita* where only very faint bands hybridized. Another fragment, NBS46-7, exhibited melon-specific hybridization and did not react with the other three species (data not shown). We conclude that RGH probes vary in their degree of phylogenetic conservation and some of them may be used efficiently across species, and even across some inter-generic boundaries, in the cucurbits.

Genetic mapping of RGH fragments

Detailed genetic maps of melons have been constructed in the last few years by Pitrat and co-workers using two different RIL (recombinant inbred lines) populations. The two maps have been merged by computational methods and, together, they include a total of six resistance gene traits, 15 horticultural traits and about 700 molecular and isozyme markers. Based on this reference map, the positions of the RGH clones were determined on the 12 linkage groups, representing the 12 melon chromosomes. We also tested for linkage between these clones and disease resistance loci that were phenotypically scored on the two populations. Population 1 was derived from an F2 progeny of the cross Vedrantais × PI 161735, followed by repeated selfing of single-seed descendants down to the F6 to F8 generations. Resistance traits scored in this population included *Fusarium oxysporum* race 1 (*Fom-2* gene), *F. oxysporum* race 2 (*Fom-1* gene), *Aphis gossypii* (*Vat* gene) and melon necrotic spot virus (*nsv* gene). Population 2 was derived from the cross Vedrantais × PI 414723 by a similar crossing scheme, and was scored for zucchini yellow mosaic virus resistance (*zym* gene), papaya ringspot virus (*prv*) as well as the *Vat*, *Fom-1* and *Fom-2* genes (Perin et al. 1998, 2000; Perin et al., submitted manuscript).

Eleven of the NBS clones were scored as RFLPs in either one or both mapping populations; the other probes did not detect an RFLP between the mapping parents. Table 3 summarizes the linkage data obtained, placing each RGH on the reference map, in an interval between two previously mapped markers in a linkage group, except for two NBS clones that remained unlinked. We observe that RGH sequences are widely distributed among eight out of the 12 melon linkage groups. A significant proportion of the mapped NBS homologues maps to the vicinity of three resistance loci: NBS2 maps at a distance of 4.75 cM (calculated as the pair-wise distance between the two loci, with nine recombinants out of 95 RILs assayed) from the *Vat* locus that controls resistance to the aphid pest *A. gossypii* and resistance to virus transmission by this aphid (Kishaba et al. 1976; Pitrat and Lecoq 1980). NBS 46-7 is linked to *Vat* at 7.5 cM. A third NBS fragment, NBS5, detects two RFLP loci that map at distances of 10 and 11 cM, respectively, from *Vat* (Fig. 5A). Another cluster of resistance genes harbors the *Fom-1*

and *prv* genes, which specify resistance to *Fusarium* races 0 and 2 and the potyvirus PRV, respectively. NBS47-3 maps at an approximate distance of 2.6 cM from *Fom-1*, with two RILs showing recombination between the two genes (Fig. 5B), and 1.2 cM from *prv* (a single recombinant). A third resistance gene, *Fom-2*, controlling resistance to races 0 and 1 of the same pathogen, is tightly linked to NBS3 (0.7 cM, two recombinants out of 114 individuals; Fig. 5C).

Discussion

Resistance gene homologues in melon: diversity versus conservation

We have isolated a family of 15 genomic fragments homologous to NBS-LRR genes. The PCR-based approach taken in this study thus allowed for good sampling of this well-studied *R*-gene family.

Genome-wide sequence data reveal that the NBS-LRR class of *R*-genes may represent as much as 1% of the *Arabidopsis* genome (Meyers et al. 1999). Large repertoires of distantly related resistance genes with diverse recognitional specificities are found within a single plant species; this may reflect an on-going "arms race" between pathogens and plants that causes rapid evolution of such sequences (Parniske et al. 1997). The LRR domain, and sometimes the TIR domain, have a proven role in determination of specificity (reviewed by Ellis et al. 2000). Using the few conserved motifs to construct degenerate PCR primers, it has been possible to clone *R*-gene homologues (RGH) from many plant species, e.g. rice and barley (Leister et al. 1998), potato (Leister et al. 1996), tomato (Pan et al. 2000b), pepper (Pflieger et al. 1999), soybean (Kanazin et al. 1996; Yu et al. 1996), common bean (Rivkin et al. 1999), *Arabidopsis* (Speulman et al. 1998), lettuce (Shen et al. 1998), *Brassica* (Joyeux et al. 1999) and pea (Timmerman-Vaughan et al. 2000).

Cloning of melon NBS-LRR homologues proved to be a laborious task, because many cloned fragments harbored unrelated sequences. Longer primers, with fewer inosine bases, such as primers 464 and 310 (Table 1), improved our rate of success. Most of the clones were generated using primers based on the 'P-loop' and 'Kinase-2' motifs and only two were produced by a primer based on the 'Hydrophobic Domain'. Further variation of primer sequence should result in the cloning of a larger repertoire of RGH fragments in this species. Meyers et al. (1999) and Pan et al. (2000a) divided NBS-LRR genes into two groups. Type I contains the TIR element (Toll-interleukin-1 receptor-like domain) and is only found among dicots, while group II, which is more diverse, lacks the TIR domain and is found in both dicots and monocots. The authors of both studies showed that the partial sequence of the NBS portion is sufficient to assign a given gene to either Group I or Group II. Thus, both groups seem to be represented in our collection: ten of the melon RGH fragments are tightly clustered with typical Group-I genes such as tobacco *N* and flax *L6*, while five fragments clustered with rice *Pib*, *Arubidopsis Rps2* and tomato *Prf*, that belong to Group II (Fig. 2B). The high rate of diversification within the NBS-LRR family was also reflected by the long terminal branches, the poorly resolved inner nodes of the tree and by the high rates of polymorphism detected with NBS probes, as compared to random cDNA or genomic probes.

In view of the difficulty in cloning a large number of such fragments, the conservation of NBS genes among genera in the *Cucurbitaceae* and the direct application of RGH clones from *C. melo* to *C. sativus* (cucumber) and *C. lanatus* (watermelon) is of practical value. *Cucurbita* appears to be more distantly related. Grube et al. (2000) used a similar approach and compared, by DNA gel-blot hybridization with heterologous probes, the genomic organization of *R*-genes and *R*-gene homologues across genera in the *Solanaceae*. Their study indicated that such genes map to syntenic positions in pepper, potato and tomato, but only in a few cases did the syntenous loci specify resistance to the same pathogen in the different genera. It thus appeared that recognition specificity of such genes is rapidly evolving and only the general function (i.e. pathogen recognition) remains conserved.

Several RGH clones map to the vicinity of disease and pest resistance genes in melon

Mapping studies in various species proved that RGH sequences are often organized as gene clusters, consistent with the genetic observation that disease resistance genes often occur in clusters (Shen et al. 1998). Interestingly, when RGH gene-fragments were placed as RFLP markers on genetic maps of the respective species, they often mapped to disease resistance loci. For example, one gene cluster in soybean (Kanazin et al. 1996) contained two phenotypically defined resistances, against *Phytophtora* and powdery mildew, and five RGH clones. Such organization may be of evolutionary importance, as clusters of *R*-genes and their homologues may undergo unequal cross-over and generate new alleles. Genomic analysis of clusters with multiple LRR gene-members has been carried out in detail (Parniske et al. 1997; Meyers et al. 1999; Ellis et al. 2000). The structure of such clusters may be rather complex, including both *R*-genes and unrelated sequences.

In melon, positional cloning of genes of agronomic interest has not been achieved yet, and saturating the melon map with many RGH genes may render the future identification of functional resistance genes much easier. Out of 11 NBS-LRR homologues that we mapped, five were linked to *R*-genes involved in responses to an insect pest, as well as viral and fungal pathogens. The *Vat* locus appears to be part of a cluster that comprises the aphid resistance gene, NBS2, NBS46-7 and NBS5. An additional *R*-gene, conferring powdery mildew resistance, also maps to the same region (Pitrat 1991). Mapping of

Vat with respect to NBS2 and NBS5 has been reported using a different mapping population (Klingler et al. 2001). The cluster also includes a lipoxygenase homologous sequence (Klingler et al. 2001), reminiscent of the lipoxygenase gene fragments that are part of the *Cf-4/9*) cluster (Parniske et al. 1997). Interestingly, two of the NBS sequences linked to *Vat*, NBS2 and NBS46-7, are also tightly clustered on the phylogenetic tree, while NBS23-2, which also shares high homology with these two, is unlinked. Examples of closely related sequences residing in the same cluster have been recorded, but in other cases clusters contained very diverse sequences (Meyers et al. 1999). Breeding for aphid resistance is economically important, both to reduce direct damage by the pest and to reduce transmission of viruses such as ZYMV by this vector; markers for this locus could be useful to assist breeders in selecting for this trait.

The *Fom-1* gene, which controls resistance to races 0 and 2 of *F. oxysporum fs.melonis* also appears to be part of such a cluster, with an RGH sequence, NBS47-3, mapping to its vicinity, as well as a second *R*-gene for papaya ringspot virus resistance (Pitrat 1991). The same region also harbors a QTL for CMV resistance (Dogimont et al. 2000). No markers for this locus have been available to breeders. The fusarium resistance trait is difficult to select phenotypically, since up to 50% of genetically susceptible plants may escape wilting following standard inoculation (R. Cohen and Y. Burger, personal communication). Molecular markers greately enhance selection for this trait in breeding programs. Another marker, NBS3, mapped close to the *Fom-2* gene which controls resistance to races 0 and 1 of the pathogen. Tightly linked RAPD, AFLP, CAPS and RFLP markers have been published for *Fom-2* (Zheng et al. 1999; Wang et al. 2000; Zheng and Wolff 2000) but an NBS sequence at only 0.7 cM from the gene probably suggests that the *R*-gene residing in this locus is a member of an NBS-LRR cluster.

In conclusion, the RGH approach proved an efficient strategy to obtain melon markers that are close to *R*-genes. None of the probes that we have mapped actually co-segregates with an *R*-gene, but additional linked markers and eventually candidate *R*-genes may be obtained, e.g. by cloning more RGH sequences related to members of a given cluster. Saturation of a locus of interest with additional RGH markers or with other molecular markers may set the stage for positional cloning of the locus. Our results will also enable comparative mapping of RGH sequences in other cucurbit species and the development of RGH-derived markers to assist selection.

Acknowledgements The authors thank Prof. Robert Fluhr and Dr. Qilin Pan of the Israeli Plant Genome Centre for providing oligonucleotide primer for this study. This research was supported by grant No. IS-2708-96 from the Us–Israel Binational Agricultural and Development Fund, and by Grant No. 1140-1-99 from the France–Israel Association for Scientific Research and Technology (AFIRST).

References

Anagnostou K, Jahn M, Perl-Treves R (2000) Inheritance and linkage of ZYMV and WMV resistance in *Cucumis melo*. Euphytica 116:265–270

Baudracco-Arnas S (1995) A simple and inexpensive method for DNA extraction from *Cucumis melo* L. Cucurbit Genet Coop Reports 18:50–51

Baudracco-Arnas S, Pitrat M (1996) A genetic map of melon (*Cucumis melo* L.) with RFLP, RAPD, isozyme, disease resistance and morphological markers. Theor Appl Genet 93:57–64

Bent AF (1996) Function meets structure in the study of plant disease resistance genes. Plant Cell 8:1757–1771

Buschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, vanDaelen R, vanderLee T, Diergaarde P, Groenendijk J, Topsch S, Vos P, Salamini F, SchulzeLefert P (1997) The barley *mlo* gene: a novel control element of plant pathogen resistance. Cell 88:695–705

Chisholm ST, Mahajan SK, Whitham SA, et al. (2000) Cloning of the Arabidopsis *RTM1* gene, which controls restriction of long-distance movement of tobacco etch virus. Proc Natl Acad Sci USA 97:489–494

Danin-Poleg Y, Reis N, Baudracco-Arnas S, Pitrat M, Staub JE, Oliver M, Arus P, deVicente CM, Katzir N (2000) Simple sequence repeats in *Cucumis* mapping and map merging. Genome 43:963–974

Dogimont C, Lecomte L, Perin C, Thabuis A, Hagen L, Lecoq H, Pitrat M (2000) Identification of QTLs contributing to resistance to different strains of cucumber mosaic cucumovirus in melon. In: Proc Eucarpia Cucurbitaceae 2000 Congress, 19–23 March 2000, Ma'aleh Hahamisha, Israel. Acta Hort 510: pp 391–398

Ellis J, Dodds P, Pryor T (2000) Structure, function and evolution of plant disease resistance genes. Curr Opin Plant Biol 3:278–284

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Grube RC, Radwanski ER, Jahn M (2000) Comparative genetics of disease resistance within the Solanaceae. Genetics 155: 873–887

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575–607

Johal GS, Briggs SP (1992) Reductase-activity encoded by the *hm1* disease resistance gene in maize. Science 258:985–987

Joyeux A, Fortin MG, Mayerhofer R, Good AG (1999) Genetic mapping of plant disease resistance gene homologues using a minimal *Brassica napus* L. population. Genome 42:735–743

Kahana A, Silberstein L, Kessler N, Goldstein RS, Perl-Treves R (1999) Expression of cucumber ACC oxidases differs among sex genotypes and sex phases. Plant Mol Biol 41:517–528

Kanazin V, Fredrick ML, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750

Kishaba AN, Bohn GW, Toba HH (1976) Genetic aspects of antibiosis to *Aphis gossypii* in *Cucumis melo* from India. J Am Soc Hort Sci 101:557–561

Klingler J, Kovalski I, Silberstein L, Thompson GA, Perl-Treves R (2001) Mapping of cotton-melon aphid resistance in melon. J Am Soc Hort Sci 126:56–63

Lander ES, Green P, Abrahamson J, Barlow A, Daley MI, Lincoln SE, Newberg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genet 14:412–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Shulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Martin GB (1999) Functional analysis of plant disease resistance genes and their downstream effectors. Curr Opin Plant Biol 2:273–279

McCreight JD, Kishaba AN, Bohn GW (1984) AR Hale's Best Jumbo, AR 5, and AR Topmark: melon aphid-resistant muskmelon breeding lines. HortScience 19:309–310

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20: 317–332

Oliver M, Garcia-Mas J, Cardus M, Pueyo N, Lopez-Sese A, Arroyo M, Gomez-Paniagua H, Arus P, de Vincente MC (2001) Construction of a reference linkage map for melon. GENOME 44 (5):836–845

Pan QL, Wendel J, Fluhr R (2000a) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol 50:203–213

Pan QL, Liu YS, Budai-Hadrian O, Sela M, Carmel-Goren L, Zamir D, Fluhr R (2000b) Comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes of two dicotyledons: tomato and *Arabidopsis*. Genetics 155:309–322

Parniske M, HammondKosack KE, Golstein C, Thomas CM, Jones DA, Harrison K, Wulff BBH, Jones JDG (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91:821–832

Perin C, Hagen L, Dogimont C, DeConto V, Pitrat M (1998) Construction of a genetic map of melon with molecular markers and horticultural traits. In: Cucurbitaceae '98 – evaluation and enhancement of cucurbit germplasm, Proc Cucurbitaceae Conference, Pacific Grove, California, 30 Nov.–4 Dec. 1998, pp 370–376

Perin C, Hagen L, Dogimont C, DeConto V, Lecomte L, Pitrat M (2000) Construction of a reference genetic map of melon. In: Proc Eucarpia Cucurbitaceae 2000 Congress, 19–23 March 2000, Ma'aleh Hahamisha, Israel. Acta Hort 510:367–374

Pflieger S, Lefebvre V, Caranta C, Blattes A, Goffinet B, Palloix A (1999) Disease resistance gene analogs as candidates for QTLs involved in pepper–pathogen interactions. Genome 42:1100–1110

Pitrat M (1991) Linkage groups in *Cucumis melo* L. J Hered 82:406–411

Pitrat M, Leqoc H (1980) Inheritance of resistance to cucumber mosaic virus transmission by *Aphis gossypii* in *Cucumis melo*. Phytopathology 70:958–961

Rivkin MI, Vallejos CE, McClean PE (1999) Disease-resistance related sequences in common bean. Genome 42:41–47

Shen KA, Meyers BC, Islam-Faridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant–Microbe Interac 11:815–823

Silberstein L, Kovalski I, Huang R, Anagnostou K, Jahn MM, Perl- Treves R (1999) Molecular variation in *Cucumis melo* as revealed by RFLP and RAPD markers. Scien Hort 79:101–111

Speulman E, Bouchez D, Holub EB, Beynon JL (1998) Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana*. Plant J 14:467–474

Timmerman-Vaughan GM, Frew TJ, Weeden NF (2000) Characterization and linkage mapping of *R*-gene analogous DNA sequences in pea (*Pisum sativum* L.). Theor Appl Genet 101:241–247

Traut TW (1994) The functions and consensus motifs of nine types of peptide segments that form different types of nucleotide-binding sites. Eur J Biochem 222:9–19

Wang Y, Thomas CE, Dean RA (1997) A genetic map of melon (*Cucumis melo* L.) based on amplified fragment length polymorphism (AFLP) markers. Theor Appl Genet 95:791–798

Wang YH, Thomas CE, Dean RA (2000) Genetic mapping of a fusarium wilt resistance gene (*Fom-2*) in melon (*Cucumis melo* L.). Mol Breed 6:379–389

Whitham SA, Anderberg RJ, Chisholm ST, et al. (2000) The *Arabidopsis RTM2* gene is necessary for specific restriction of tobacco etch virus and encodes an unusual small heat shock-like protein. Plant Cell 12:569–582

Yu YG, Buss GR, Maroof MAS (1996) Isolation of a superfamily of candidate disease resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11,751–11,756

Zheng XY, Wolff DW (2000) Randomly amplified polymorphic DNA markers linked to fusarium wilt resistance in diverse melons. Hortscience 35:716–721

Zheng XY, Wolff DW, Baudracco-Arnas S, Pitrat M (1999) Development and utility of cleaved amplified polymorphic sequences (CAPS) and restriction fragment length polymorphisms (RFLPs) linked to the *Fom-2* fusarium wilt resistance gene in melon (*Cucumis melo* L.). Theor Appl Genet 99:453–463