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Application of Molecular Biology for Identification of Virus Resistance Gene in Melon

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Abstract

Source of resistance to an Indonesia isolate of *Cucumber mosaic virus* (CMV-B2) in melon cultivar Yamatouri has been reported. Moreover, *Creb-2*, a locus that confers resistance to CMV-B2 in Yamatouri has been determined as a single dominant gene. To elucidate the resistance mechanism conferred by *Creb-2* in more detail, it is necessary to clone the *Creb-2* gene and determine its molecular structure. One approach is by amplification and cloning of melon resistance gene analogs (MRGAs) based on degenerated PCR primers designed from conserved amino acids in the NBS-LRR motifs (P-loop, Kinase-2, and the GLPL) and Toll/ Interleukin-1 receptor-like region (TIR). This study was aimed to identify and characterize the resistance gene analogs from *Cucumis melo* L. cv. Yamatouri by employing polymerase chain reactions (PCR) as a molecular biology tools with degenerate primers based on conserved motifs of cloned *R* genes. The application of molecular biology such as DNA isolation, degenerate primers and PCR condition, cloning, sequencing, linkage analysis and mapping of resistance gene analogs to *Creb-2* gene in melon will be widely discussed in this paper.

Keywords: resistance gene analogs, *Cucumber mosaic virus*, *Cucumis melo* L.

Introduction

Plant disease resistance is often controlled by Mendelian genes and follows a gene for gene relationship in many plant species and their pathogens (Dixon *et al.*, 1996). According to this theory, there are many resistance (*R*) genes in a plant species against each of its pathogens and there is corresponding a virulence gene in the pathogen population for every *R* gene in the host plant. This theory has been well demonstrated in cases where plant resistance is associated with hypersensitivity. However, a clear cut resistance phenotype like hypersensitivity does not always exist in many other cases and plant resistance often shows both qualitative and quantitative components. The qualitative resistance in many plant-pathogen relationships is hypersensitive, race specific, and governed by interactions between avirulence genes in pathogens and resistance genes in hosts, while the quantitative resistance is non-hypersensitive, presumably non-race specific, and controlled polygenes (Mindrinos *et al.*, 1994).

Recent advances in DNA marker technology and genomic research have provided powerful tools for addressing many questions about genetics of interaction between plants and their pathogens. Large numbers of *R* genes in several plant species have been accurately mapped to their corresponding genomic locations (Meyers *et al.*, 1999). To date, at least 18 *R* genes including 17 dominant and 1 recessive one in nine plant species have been cloned (Bent *et al.*, 1994). A general model of plant defense responses to pathogen invasion involving complex biochemical pathways has been proposed, explaining the molecular basis of the gene for gene theory (Dangl, 1998).

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The presence of conserved domains permitted grouping of *R* genes into at least four classes and to propose their possible function in the defense response as part of signal transduction pathways (Backer *et al*., 1997). The first class of *R* genes contains *Pto*, a serine-threonine protein kinase, which confers resistance to bacterial speck disease in tomato (Backer *et al*., 1997). The second class includes the *Cf* family of tomato *R* genes, which are effective against leaf mold and encode putative trans-membrane receptors with extra cellularleucine-rich repeats (LRR) domains (Dixon *et al*., 1996; Jones *et al*., 1994; Timmerman-Vaughan *et al*., 2000). A kinase group, associated with a receptor domain results in a receptor-like kinase structure, characterizes the third class of genes, including *Xa21*, which confers resistance to rice bacterial blight (Shen *et al*., 1998). A nucleotide binding site (NBS) and a stretch of LRRs that characterize most of the functionally described *R* genes make up the fourth class, examples of which are *N* of tobacco, *L6* of flax, and *RPP5* and *RPS2* of *Arabidopsis* spp. (Bent *et al*., 1994; Lander *et al*., 1987; Leister *et al*., 1998; Michelmore & Meyer, 1998; Pan *et al*., 2000b; Thomphson *et al*., 1994). Lopez *et al*. (2003) reported that they also have been an NBS upstream domain, which is either a region contained coiled coils (CC) or a Toll/Interleukin-1 receptor-like region (TIR; so named because of its homology with the cytoplasmic domain of the corresponding proteins in *Drosophilla* spp. and mammals).

Based on sequence similarity between *R* genes, a method using degenerate primers to target the conserved motifs has been successfully employed to isolate resistance gene analogs (RGAs) from potato (Lawrence *et al*., 1995), soybean (Joyeuk *et al*., 1999; Witham *et al*., 1994), rice (Leister *et al*., 1996), *Arabidopsis* spp. (Aatrs et al., 1998), lettuce (Saitou & Nei, 1987), common bean (Geffroy *et al*., 1999; Leister *et al*., 1998; Pitrat & Lecoq, 1980), melon (Brotman *et al*., 1998), and other monocotyledonous and dicotyledonous.

Source of resistance to CMV-B2 in melon cultivar Yamatouri has been reported (Daryono & Natsuaki, 2002). Moreover, *Creb-2*, a locus that confers resistance to CMV-B2 in Yamatouri has been determined as a single dominant gene (Daryono *et al*., 2003). To elucidate the resistance mechanism conferred by *Creb-2* in more detail, it is necessary to clone the *Creb-2* gene and determine its molecular structure. One approach is by amplification and cloning of melon resistance gene analogs (MRGAs) based on degenerated PCR primers designed from conserved amino acids in the NBS-LRR motifs (P-loop, Kinase-2, and the GLPL) and Toll/Interleukin-1 receptor-like region (TIR).

This study was aimed to identify and characterize the resistance gene analogs from *Cucumis melo* L. cv. Yamatouri by employing polymerase chain reaction (PCR) as a molecular biology tools with degenerate primers based on conserved motifs of cloned *R* genes.

Materials and Methods

Plant materials

Two melon cultivars, the resistant Yamatouri and the susceptible Vakharman were used for providing template DNA for PCR amplification of melon resistant gene analysis (MRGA). An F_2 population consisting of 154 individual plants derived from a cross between Yamatouri and Vakharman was used for linkage analysis and mapping of MRGAs.

Degenerate primers and PCR condition

Primers (Table 1) were designed from the conservative motifs P-loop and GLPLAL of the NBS-LRR domain according to Leister *et al*. (1995) and Brotman *et al*. (1998). TIR was used by a set of primers TIR1 and TIR5 according to Lopez *et al*. (2003).

DNA was extracted and purified from leaf tissues according to the protocol of Nucleon Phytopure (Amersham International). PCR reactions were performed for both cultivars in a total volume 50 µl reaction volumes in 600 µl tubes. Each reaction mixture containing

Table 1. Degenerate primers used to isolate resistance gene analogs in melon

Protein region	Primer	Sequence 5′ → 3′	Peptide encoded
Kinase-1 (*p-loop*)	S2	GGI GGR RTA GGI AAR ACI AC	GGVGKTT
	S2N	GGI GGI GTI GGI AAI ACI AC	GGVGKTT
C-terminal conserved domains	AS1	AR IGC IAR IGG IAR ICC	GLPLAL
	AS1N	CAA CGC TAG TGG CAA TCC	GLPLAL
	AS3	IAG IGC IAG IGG IAG ICC	GLPLAL
Kinase-3	AS2	YCT AGT TGT RAY DAT DAY YYT RC	(S/C)(R/K)(I/V) (I/M) (I/F) TTR
Kinase-2	310	CCA IAC RTC RTC NAR NAC	VLDDVW
Toll/Interleukin-1 receptor	TIR1	GAI GTN TTY TTI TCI TTY AGI GG	(D/E)VFLSF (R/ S)G
Toll/Interleukin-1 receptor	TIR5	IGG GTC IAC GTC GTA GAA IAC IGG	PVFYDVDP

Abbreviations: I = Inosine; R= A/G; N= A/C/G/T; Y=C/T; D= A/G/T

5µl of 10X ExTaq buffer, 4 µl of dNTPs , 0.5µl of ampliTaq™ (Takara, Japan), 25 picomoles of each primer and 10 ng of genomic DNA. Amplification was performed on a DNA Thermal Cycler (PTM-100™ Programmable Thermal Controller, MJ Research Inc.) with cycling conditions were initial denaturation at 95°C for 5 minutes followed by 35 cycles at 95°C for 1 min, 42-45° C for 1 min, and 72° C for 1.5 min and final extension step at 72°C for 10 min before holding at 4°C.

Cloning the PCR products

PCR products were separated by electrophoresis in a 1% agarose gel. Each expected band was eluted and purified with by the QIA Quick Gel Extraction Kit (QIAGEN, USA). PCR purified products were cloned into pGEM-T vector system (Promega Corp.) and transformed in *Escherichia coli* DH5α cells by electroporation, following GIBCO-BRL instruction. Approximately, 10-20 clones derived from each expected band of parents were randomly picked. Plasmids were extracted using LaboPass™ Mini Plasmid DNA Purification Kit (LaboPass).

Sequence analysis

Clones from each clone were sequenced using the Dye Terminator Cycle Sequencing Kit and an Applied Biosystem Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems). Homology searchers were performed using the Genbank, EMBL, and DDBJ databases with the BLAST algorithm. Nucleotide and amino acid sequences were analyzed using MacVector 6.5 software (Oxford Molecular Ltd., USA). Comparisons between sequences were initially aligned using CLUSTAL W (Thomphson *et al.,* 1994) with default parameters. Pair-wise sequence comparisons and alignments were conducted using multiple alignment parameters with a gap open penalty of 10 and a gap extension penalty of 5 for nucleotide analysis, and a gap open penalty of 10 and a gap extension penalty of 0.1 for amino acid analysis. Comparative sequences of members of the *R* genes were obtained from Genbank under the following accession numbers: the tobacco *N* gene (U51605); flax *L6* (U27081); rice *Pib* (AB013448); *Arabidopsis* RPS2 (U12860) and RPS5 (AF180942), tomato *Prf* (U65391), and cowpea *Cry* (AB020487). To make consistent sequence comparisons, only the region between P-loop and GLPLAL motifs was used. The regions corresponding to the primers S2, S2N, AS1, AS2, AS3, 310, TIR1, and TRI5 were not included.

Using the neighbor-joining (NJ) algorithm according to Saitou and Nei (1987), phylogenetic analysis of the MRGAs based

on NBS and TIR was performed to determine the relationship of these MRGAs with other members of the *R* genes. The strength of the internal branches of the resulting tree was statistically tested by bootstrap analysis from 1,000 bootstrap replications.

Restriction fragment length polymorphism (RFLP) analysis

DNA samples from Yamatouri and Vakharman will be digested with five restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Bam*HI, and *Xba*I) according to the manufacture's instruction (Amersham Pharmacia Biotech). Ten µg of the digested DNA is separated on 1% agarose gel. DNA fragments are blotted on a Hybond N^+ membrane (Amersham Pharmacia Biotech) according to the manufacture's instruction. Digoxygenin-labeled probes are prepare using a PCR DIG Labeling Mix (Boehringer Mannheim, Germany) and use for hybridization to melon DNA according to the manufacture's instruction. After overnight hybridization at 65°C, the membrane is washed once in a solution of 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 10 minutes, at 65°C, then in 1X SSC and 0.1% SDS for 20 minutes at 65°C. The hybridization signal will be detected by exposure to X-ray films according to the manufacturer's instruction.

Linkage analysis

RFLP segregation will be evaluated in the F_2 population consisting of 154 individual plants derived from a cross between Yamatouri and Vakharman. Developed MRGA markers will be used for linkage mapping in 154 F_2 individual plants. Linkage analysis is performed using the MAPMAKER/EXP 3.0 software originally described by Lander *et al.* (1987).

Results

Cloning and sequence analysis of melon resistance gene analogs

To isolate melon resistance gene analogs (MRGAs), six combinations of degenerate primers (Table 2), were used on genomic DNA of cultivar Yamatouri. Although some amplification showed multiple band patterns, only fragments of the expected size (Table 2) were cloned. However, sequencing from the combination VI (MRGAs 12 to 15) revealed that they were not homologous with *R* genes and not used for further study.

Using primer combination I, several PCR products were obtained and ranging from 510 to 2300 bp (Figure 1). Primer combination II generated a single major product approximately 300 bp(Figure 1). Using primer combination III, a single major product of about 250 bp was detected (Figure1). Primer combination IV generated several PCR products ranging from 350 to 800 bp, and using primer combination V, three PCR products were obtained and ranging from 300 to 1200 bp (Figure 1). The 510 bp (I-510 and IV-510), 300 bp, and 250 bp products which had the mobility expected from the NBS sequences in *N*, *L6*, *Prf*, *Pib*, *Cry*, and *RPS2* protein, were cloned and approximately 50-70 clones were sequenced. Clones from combination I and IV were groped in three classes of MRGAs (MRGA1, MRGA2, and MRGA3), while from

Table 2. Primer combinations used in this study to isolate resistance gene analogs in melon

Combination	Primers	Tm	Expected size (bp)	Product clone
I	S2 + AS1	45	500-600	MRGAs 1- 2
II	S2 + AS2	45	300	MRGAs 4 - 7
III	S2 + 310	42	230-270	MRGAs 8 - 9
IV	S2N + AS3	45	500	MRGAs 3
V	TIR1 + TIR5	42	300	MRGAs 10 - 11
VI	S2N + AS1N	45	500-600	MRGAs 12-15*

* Not homologues to other *R*-genes

combination II were grouped in four classes of MRGAs (MRGA4, MRGA5, MRGA6, and MRGA7), and from combination III were grouped in two classes of MRGAs (MRGA8, and MRGA9; Table 2). On the other hand, clones from combination V were grouped in two classes of MRGAs (MRGA10 and MRGA11 (Table 2).

Different MRGAs were classified by pair-wise comparisons of their deduced amino acid sequences and those of the NBS of known *R* genes (Table 3). Identities between classes were equivalent to those between different *R* genes, although this was not the case for pairs MRGA4-MRGA5 and MRGA6-MRGA7, which were very similar but not identical (Table 3).

The melon NBS sequences exhibit high sequences divergent and the most closely related sequences are MRGA4, MRGA5, MRGA6, and MRGA7 (47-59% deduced amino acid identity), while homology between other MRGAs is low e.g. MRGA1 and MRGA9 (6% identity); MRGA2 and MRGA6 (14% identity). Furthermore, amino acid sequence comparison with NBS of known *R* genes revealed that MRGAs had 9 to 27% identities with the tobacco *N* gene, 10 to 28% identities with the flax *L6* gene, 10 to 17% identities with the tomato *Prf* gene, 8 to 18 % identities with rice Pib, 11 to 31% identities with cowpea *Cry*, and 10 to 14% identities with *RPS2* of *Arabidopsis* spp. (Table 3).

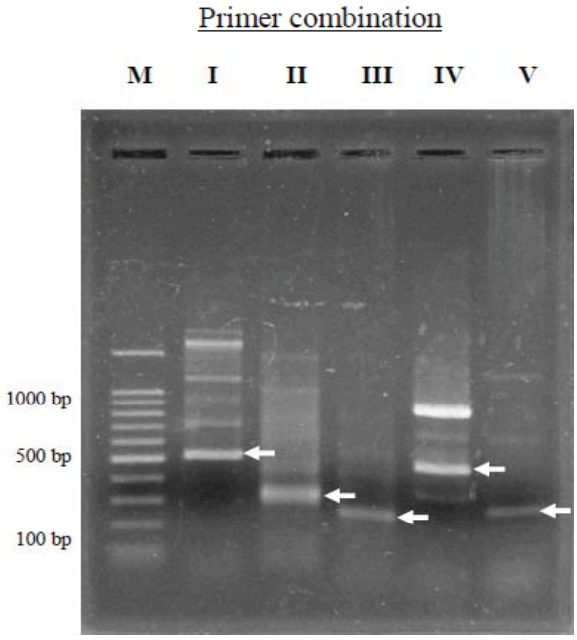

Figure1. Amplification of nucleotides-binding site and leucine-rich repeats (NBS-LRR) and Toll/Interleukin-1 receptor (TIR) sequences of melon. I:S2 and AS1; II: S2 and AS2; III: S2 and 310; IV:S2N and AS3; V: TIR1 and TIR5. M: 100 bp DNA ladder (Promega). Arrows indicate DNA bands showing expected mobility.

Table 3. Comparison of nucleotide binding site-type melon resistance gene analogs (MRGAs) from melon with cloned resistance genes[a]

MRGA	MRGA													
	2	3	4	5	6	7	8	9	N	L6	Prf	Pib	Cry	RPS2
1	13	14	7	9	8	10	8	6	15	14	15	10	14	16
2		24	13	10	14	13	7	10	17	19	17	18	19	15
3			6	7	12	6	7	7	14	14	15	17	16	13
4				59	47	47	14	19	21	23	13	13	25	14
5					44	61	14	16	22	21	13	12	22	14
6						47	18	19	27	28	17	15	31	14
7							16	14	23	22	13	12	23	14
8								27	12	10	10	8	11	10
9									9	10	11	9	12	14
N										35	21	18	42	25
L6											19	19	37	20
Prf												27	20	19
Pib													25	17
Cry														25

[a]Values correspond to the percent identity of the deduced amino acid sequences.

Table 4. Comparison of Toll/Interleukin-1 receptor (TIR)-type resistance gene analogs (RGAs) from melon with cloned resistance genes[a]

MRGA	MRGA11	TIR-N	TIR-L6	TIR-RPS5
MRGA10	41	55	44	53
MRGA11		31	33	34
TIR-N			44	57
TIR-L6				38

[a]Values correspond to the percent identity of the deduced amino acid sequences.

The expected 300 bp band was cloned from combination V, which targeted the TIR domain of *R* genes. Deduced amino sequences of TIR-type MRGAs were compared and assembled in two classes (MRGA10 and MRGA11; Table 4). These MRGAs also showed an internal conserved motif (TIR-2; as reported by Meyer *et al*., 1999) of *R* genes (Figure 3). Identities between TIR-type MRGAs and the TIR domain of *R* genes are higher than in the case of the NBS (Table 4). MRGAs had 31 to 55% identities with the tobacco *TIR-N* gene; 33-44% identities with the flax *TIR-L6* gene; and 34-57% identities with *TIR-RPS5* of *Arabidopsis* spp.

According to the amino acid sequences predicted from MRGA nucleotide sequences, MRGAs 1 to 3 contained the kinase 2 and kinase 3 with C-terminal conserved domains which function in binding with phosphate groups of nucleotides and with bases or sugars, respectively. Whereas, MRGAs 4 to 7 encompassing a p-loop to kinase 3. On the other hand, MRGAs 8 and 9 contained a p-loop and the kinase 2 (Figure 2). Furthermore, MRGAs 10 and 11 encompassing a TIR-1 to TIR-5 and also showed an internal conserved motif (TIR2, Figure 3).

The dendrogram (Figure 4) divides the sequence into two broad subfamilies on NBS type: the first, rather-tight cluster includes five melon gene fragments (MRGA1, MRGA2, MRGA3, MRGA8, and MRGA9) as well as the *RPS2 Arabidopsis*, the rice *Pib*, andthe tomato *Prf*. The second much loser cluster comprises four melon gene fragments (MRGA4, MRGA5, MRGA6, and MRGA7) and three NBS (the tobacco *N* gene, flax *L6* gene, and the cowpea *Cry*; and *Arabidopsis* RPS2). Similarly, in TIR type (Figure 5), the dendrogram divides the sequence into two subfamilies on TIR type;

Table 5. Nucleotides sequence of MRGAs primers used in Yamatouri and Vakharman

Primer name	Sequence 5′ - 3′	TM	Size (bp)
MRGA-1F	TCGGCACAGCCGTTGGTGTCG	48	534
MRGA-1R	CGCTCCTGTAGTATCTACGAC		
MRGA-2F	CTTAACAATATCTAAAAATATAGG	50	507
MRGA-2R	GGCGAGGGGCAAGAAGGATGCGTG		
MRGA-3F	TGGCTACTCAAAAAGGCCTTGG	52	498
MRGA-3R	ATCGAGAGGTAGGAAGGATGCG		
MRGA-4F	ATTGCTGAAGCTTGTTACAAC	48	333
MRGA-4R	CCTGCTTCCGGGTGCAAACCA		
MRGA-7F	TGACTATACCCTTTCACGACC	50	333
MRGA-7R	TACTTCCGCGTCCGCGGGATT		
MRGA-8F	GGCAAAGACAATCTTCCATCACG	53	240
MRGA-8R	ATATCTTTTACCTCGCATCACCT		
MRGA-9F	GTTGAAGAAATTGCAAGATTGGC	53	240
MRGA-9R	CACCTTCTTCTCATCTCTAACCT		
MRGA-10F	GGGAGAGGATACTCGAGACAAG	53	294
MRGA-10R	AACTCTTTGTTTCTTCGATCTC		
MRGA-11F	CGATCTCATGACACTCTAATTA	53	294
MRGA-11R	GTTTCTTTGTCGTTCAGGGGAG		

Figure 2. Alignment of the putative amino acid sequences of nine melon resistance gene analogs (MRGA) with those of the resistance genes *N* (tobacco), *L6* (flax), *Prf* (tomato), *Pib* (rice), *Cry* (cowpea), and *RPS2* (*Arabidopsis* spp.) using CLUSTAL W. Kinase 2 and 3 domains are indicated in bold. Arrows indicate position and direction of PCR primers used for amplification of melon NBS-LRR sequences.

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Consensus  EVFLSFRG-DTR-F.SHL...LRGK-I.TF-DD-L.RG.-I---L.KAI .---ES.-ISI      60
MRGA10     EVFLSFRGED TRDKFISHL VVALRQKGVN FFIDDK-LDR GHQISKSLLK SIE-ESRISI  60
MRGA11     EVFLSFRSHD TLIIFISHL VQTPRGRCII LGENND---R NPRLLYRFEK GFRNLVTPISL  60
TIR-RPS5   DVFPSFSGVDVRKTFLSHL LKALDGKSIN TFIDHG-IER SRTIAPELIS AIR--EARISI  60
TIR-N      DVFLSFRGED TRKTFTSHL YEVLNGKGIK TFQDDKRLEY GATIPGELCK AIE--ESQFAI  60
TIR-L6     EVFLSFRGPD TREQFTDFL YQSLRRYKIH TFRDDDELLK GKEIGPNLLR AID--SKIYV  60
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TIR-1

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Consensus  VIFS.NYA.S TWCL-EL-EI.-.-----.Q-VLPVFYDVD P    101
MRGA10     IIFSQNYASS TWCLDELGEN NLSVMRSKKQ RVLPVFYDVD P  101
MRGA11     VIYEEIYALS TWCHDKIDEI VSSILSPERQ RNLPVFYDVD P  101
TIR-RPS5   VIFSKNYASS TWCLNELVEI HKCFNDLG-Q MVIPVFYDVD P  101
TIR-N      VVFSENYATS RWCLNELVKI MECKTRFK-Q TVIPIFYDVD P  101
TIR-L6     PIISSGYADS KWCLMELAEI VRRQEEDPRR IILPIFYMVD P  101
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Figure 3. Alignment of the putative amino acid sequences of the Toll/Interleukin-1 receptor (TIR)-type resistance gene analogs (RGAs) with the TIR domain of *N* (tobacco), *L6* (flax), and RPS5 (*Arabidopsis* spp.). Amino acids in the conserved motifs TIR-1 and TIR-5 are shown in the bold, while TIR-2 is shown in blue color.

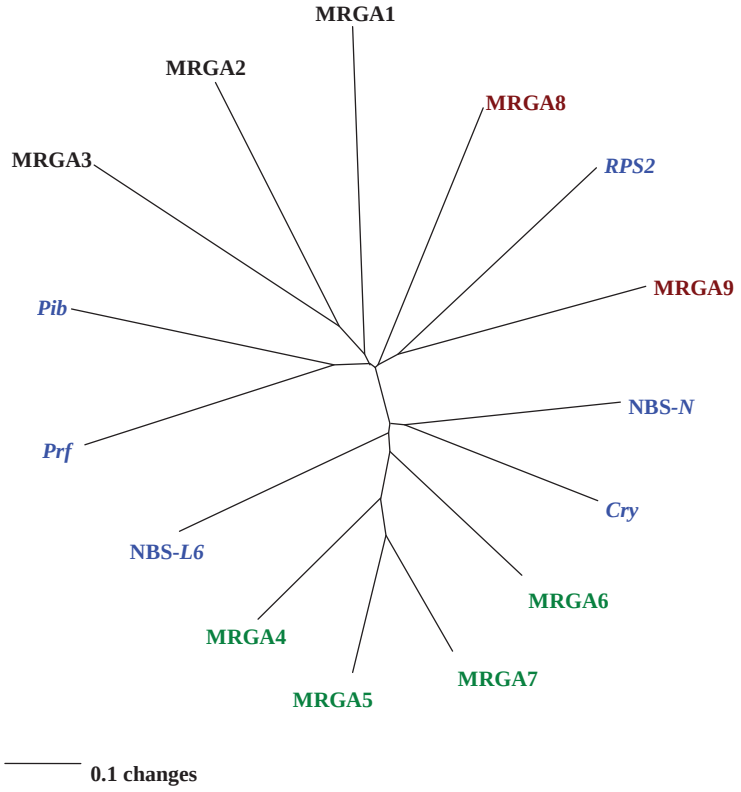

Figure 4.Dendrogram of nucleotide binding sequences (NBS) genomic fragments from melon and from representative *R*genes of other plants. Non melon sequence included the tobacco *N* gene (U51605); flax *L6* (U27081); rice *Pib* (AB013448); *Arabidopsis* RPS2 (U12860), tomato *Prf* (U65391), and cowpea *Cry* (AB020487).

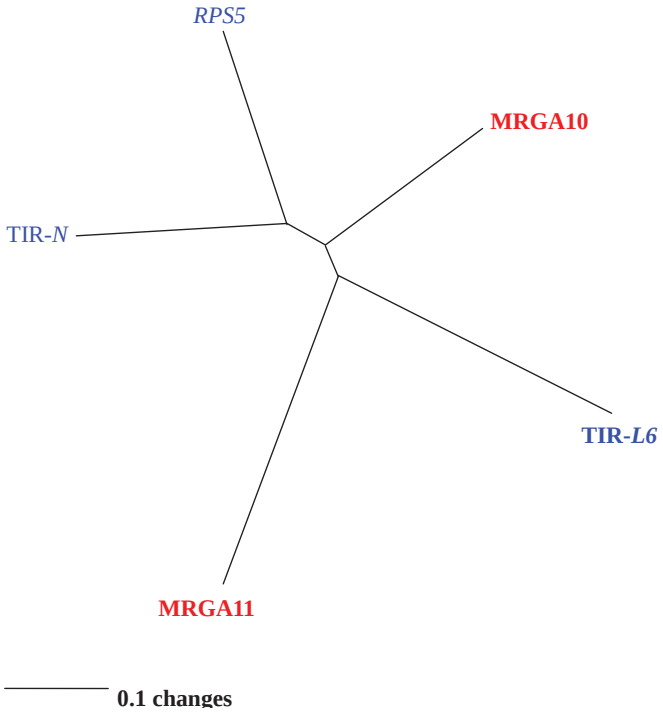

Figure 5. Dendrogram of the Toll/Interleukin-1 receptor (TIR) genomic fragments from melon and from representative *R*genes of other plants. Non melon sequence included the tobacco *N* gene (U51605); flax *L6* (U27081); tomato *Prf* (U65391); and *Arabidopsis* RPS5 (AF180942).

first cluster includes MRGA10, MRGA11, and the flax *TIR-L6* gene, while the second cluster includes the tobacco *TIR-N* gene and the *TIR-RPS5* gene of *Arabidopsis* spp.

Application of MRGAs primers in Yamatouri and Vakharman

Based on nucleotides sequence results of MRGAs, some MRGAs primers were designed to detect polymorphism of resistance gene analogues (RGAs) in Yamatouri and Vakharman (Tabel 5). Among nine MRGAs primers, only MRGA-1 primers showed RGA polymorphism between Yamatouri and Vakharman in 534 bp (Figure 6), while other MRGAs primers failed obtained RGA polymorphism.

Southern-blot analysis

The 534 bp amplified by MRGA1 primers was then used as probe in Southern-blot analysis. To investigate the genomic distribution of MRGAs in melon, genomic Southern-blot analysis was applied. MRGAs-1 clone was selected and used as a probe against DNAs of the resistant Yamatouri and susceptible Vakharman digested with five restriction endonucleases (*Eco*RI, *Eco*RV, *Hind*III, *Xba*I, and *Bam*III). A polymorphism between Yamatouri and Vakharman was detected when MRGAs1 was used as a probe; a single major band was present in lanes of Yamatouri DNA digested with *Eco*RI, *Eco*RV, and *Xba*I, whereas weak hybridization signals with different mobility were detected in corresponding lanes of Vakharman DNA (Figure7). In contrast, MRGA-1 showed copious hybridizing bands both in Yamatouri and Vakharman digested with *Hind*III and *Bam*III respectively (Figure 7).

Linkage analysis and mapping of MRGA with the Creb-2 locus

The linkage of MRGA1 with the *Creb-2* resistance locus was investigated. RFLP marker MRGA1 was converted into sequenced-

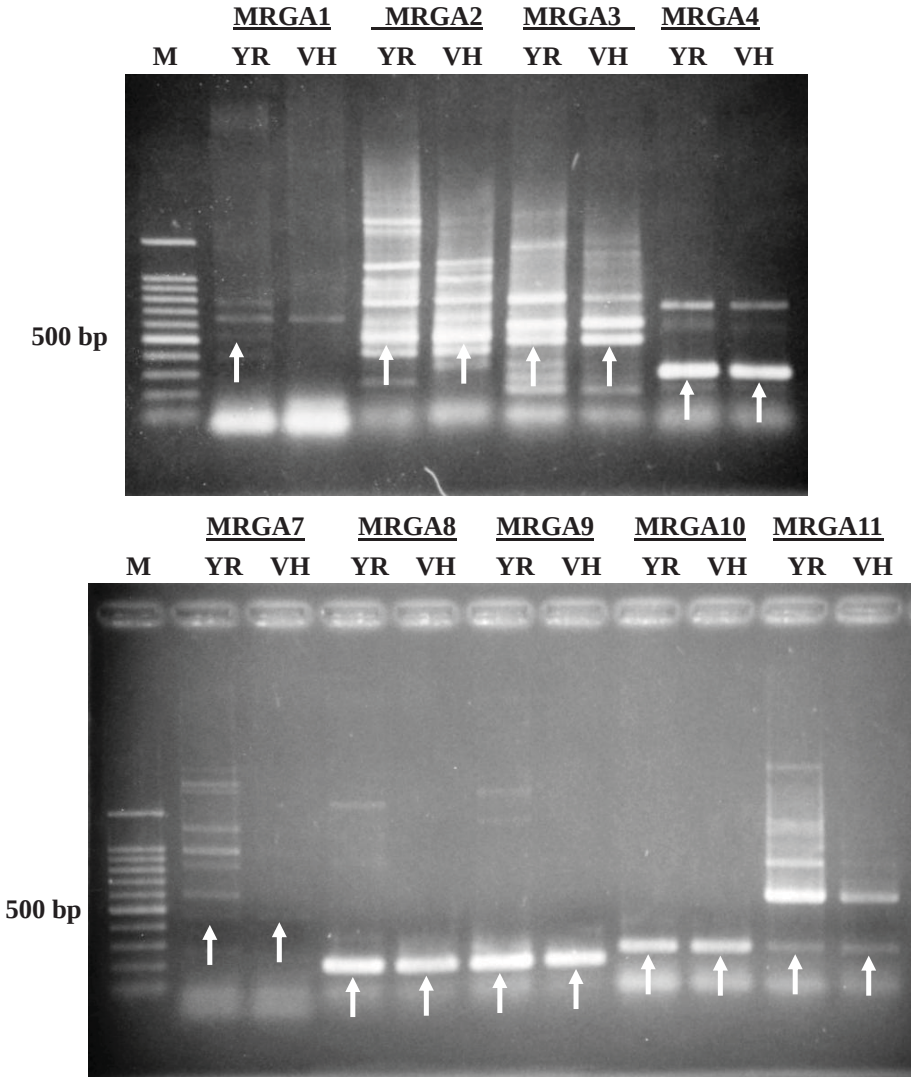

Figure 6. PCR products amplified by SCAR-MRGAs primers in cultivars Yamatouri (YR) and Vakharman (VH). MRGAs 1-4 and MRGAs 7-9 consist nucleotides-binding site and leucine-rich repeats (NBS-LRR), while MRGAs 10-11 contain Toll/Interleukin-1 receptor (TIR) sequences of melon. M: 100 bp DNA ladder (Promega). Arrows indicate DNA bands showing expected mobility.

tagged sites (STSs), Monna *et al*. (1994). Primer sequenced used for STS were MRGA1-F: 5'-TCGGCACAGCCGTTGGTGTCG-3' and MRGA1-R: 5'-CGCTCCTGTAGTATCTAC GAC-3') and used in the genetic analysis of F_2 mapping population. Among 154 F_2 individual plants, only 2 recombinants were detected between *Creb-2* and MRGA1, while in the remaining 152 F_2 individual plants, MRGA1 co-segregated with *Creb-2* (Table 6). A map distance between MRGA1 and *Creb-2* was estimated to be 1.7 cM (Figure 8).

MRGA1 in diverse melon

Some melon cultivars such as Yamatouri, Mawatauri, Miyamauri, Shinjong, and Sanuki-shirouri were reported resistance to CMV-B2 with no visible symptoms in upper leaves (Daryono *et al.,* 2003). Furthermore, cultivars Kohimeuri, Mi Tang Ting, and PI 161375 were also reported resistance to others CMV isolates (Hirai & Amemiya, 1989; Pflieger *et al.;* 1999; Speulman *et al.,* 1998). These cultivars were tested by MRGA1 primer whether or not they maintained

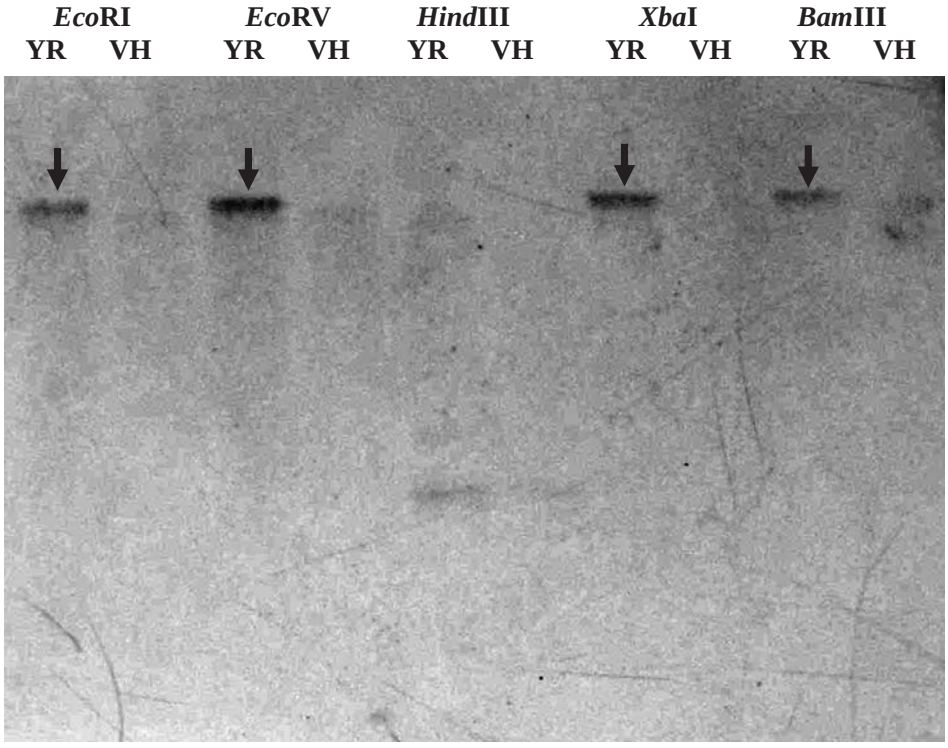

Figure 7. Southern hybridization with DNA fragments cloned from the MRGA1. The restriction endonucleases used are indicated above the lanes.

Table 6. Phenotypic frequencies of 154 F_2 individual plants

Loci		Phenotypes			
		(Resistant F_2 Individuals)		(Susceptible F_2 Individuals)	
A	B	r/+[1]	r/-[1]	s/+[1]	s/-[1]
Creb-2	SCOPE14542[2]	111	8	11	24
Creb-2	SCAPB051046[2]	113	6	12	13
Creb-2	MRGA1	117	2	0	35

[1]: Resistant and susceptible were judged by SCAR markers; [2]: Daryono *et al*. (2005,2010).

resistance gene analogous linked to *Creb-2*. Result showed that MRGA1 primer was amplified in cultivars Yamatouri, Mawatauri, Sanuki-shirouri, Kohimeuri, and PI 161375, except cultivars Miyamauri, Shinjong, and Mi Tang Ting. This result suggested that these cultivars did not have *Creb-2* and showed to be susceptible against CMV-B2.

Discussion

Two families of 11 melon genomic fragments homologous to NBS and TIR genes were isolated using PCR-based approach with degenerate primers that targeted the conserved motifs of the NBS and TIR domain from different plant *R* genes. The primers used in this study were designed according to Leister *et al.* (1996) and Brotman *et al.* (1998). Another primer combination (Leister *et al*., 1998) that had not been used for this purpose in melon and which matches the TIR domain was used to isolate another type of putative resistance genes in melon. As the results, two families include 11 MRGAs have been isolated. The first family of NBS includes 9 MRGAs, while the second family of TIR contains two MRGAs. The PCR based approach taken in this study allowed for good sampling of these *R* genes families.

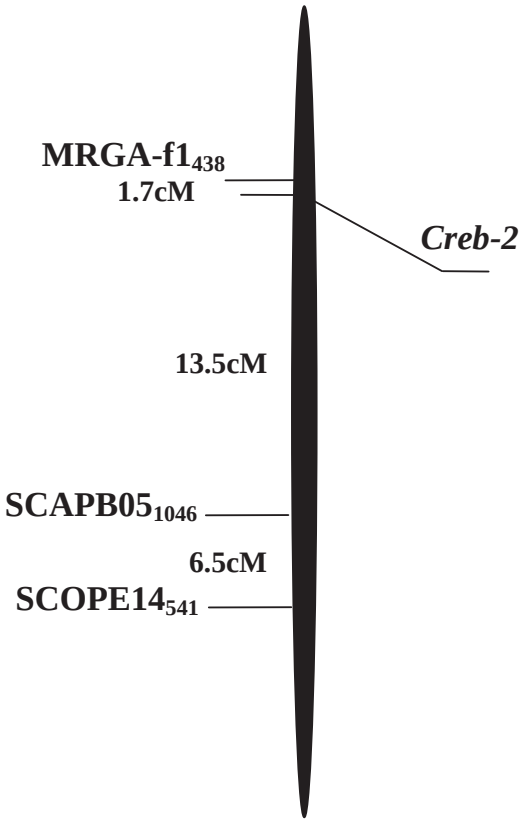

Figure 8.Linkage analysis of MRGA-f1 and SCAR markerswiththe *Creb-2* locus

Furthermore, using the few conserved motifs to construct degenerate PCR primers, it has been possible to clone R-gene analogs (RGAs) from many plant species, e.g. rice and barley (Leister *et al.,* 1996), potato (Lawrence *et al.,* 1995), tomato (Pan *et al.,* 2000), pepper (Parker *et al.,* 1997), soybean (Joyeuk *et al.,* 1999; Witham *et al.,* 1994), common bean (Leister *et al.,* 1998; Pitrat & Lecoq, 1980), Arabidopsis (Song *et al.,* 1995), lettuce (Saitou & Nei, 1987), Brassica (Li *et al.,* 1999), pea (Takada, 1979), and melon (Brotman *et al.,* 1998, Garcia-Mas *et al.,* 2001).

Cloning of melon NBS homologues proved to be a laborious task, because many cloned fragments harbored unrelated sequences, e.g. in combination V (MRGAs 12 to 15). Longer primers with fewer inosine bases, such as primer AS1 and 310 (Table 1) improved the rate of homologous. Most of clones were degenerated using primers based on the 'P-loop' and GLPL which also called Hydrophobic domain (Brotman *et al.,* 1998), and Kinase 2. However, only two were produced by a primer based on 310 or Kinase-3.

The analysis of the NBS domains of different *R* genes and RGAs of NBS type suggest classification into either TIR or non-TIR linked sequence (Martin *et al.,* 1993; Pan *et al.,* 2003). The first are characterized by the presence of an aspartic acid residue (D) at the final portion of the kinase-2 motifs. Examples of these genes are the genes *N* and *L6*. In contrast, the non-TIR sequences have a tryptophan residue (W) in the same position and included *RPS2*, *RPM1*, and *12C-2* (Leister *et al.,* 1998). Based on this criterion, MRGA3 is predicted to be non-TIR containing sequences (Figure2).

MRGAs from the NBS type in this study showed 6 to 59% amino acid identities to cloned *R* genes and between them. This level of similarityis equivalent to that shown between cloned *R* genes. In fact, low levels of identities are obtained when NBS sequences of the TIR type are compared with those of non-TIR type.

Using the primers designed from the converted TIR domain of the genes *N*, *L6*, and *RPS5*, a new type of RGAs from melon (MRGA10 and MRGA11) was obtained indicating that the TIR containing *R* genes are specific to dicotyledonous (Martin *et al.,* 1993).

Among nine NBS homologous tested, one (MRGA1)was shown in resistant Yamatouri and absent in susceptible Vakharman. Thus, MRGA1 is to be linked to *R* genes involved in response to CMV-B2 and the *Creb-2* locus appeared to be part of *R* genesin MRGA1.

Moreover, several hypotheses in regard to *R* genes can be summarized; **First**, *R* genes in a plant species are distributed in cluster in many locations of its genome (Crute & Pink, 1996; Botella *et al.,* 1997). **Second**, *R* genes from different species appear to share sequence similarities in certain domains of their gene products such as leucine rich

repeats (LRR) and nucleotide binding sites (NBS; 3). **Third**, different *R* genes from a particular plant species against one of its pathogens may not show high homology in DNA sequence. **Fourth,** quantitative trait loci (QTL) for partial resistance tend to map at genomic regions in the vicinity of clustered *R*-genes (Li *et al*., 1999; Wang *et al*., 1994).

In conclusion, positional cloning of genes of agronomic interest has not been achieved yet in melon, and saturating the melon map with some MRGA genes may render the future identification of functional resistance genes much easier.

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