Theor Appl Genet (2010) 120:1587–1596
DOI 10.1007/s00122-010-1278-9

ORIGINAL PAPER

Molecular mapping and characterization of a single dominant gene controlling CMV resistance in peppers (*Capsicum annuum* L.)

Won-Hee Kang · Ngoc Huy Hoang · Hee-Bum Yang · Jin-Kyung Kwon · Sung-Hwan Jo · Jang-Kyun Seo · Kook-Hyung Kim · Doil Choi · Byoung-Cheorl Kang

Received: 2 November 2009 / Accepted: 19 January 2010 / Published online: 24 February 2010

Abstract *Cucumber mosaic virus* (CMV) is one of the most destructive viruses in the Solanaceae family. Simple inheritance of CMV resistance in peppers has not previously been documented; all previous studies have reported that resistance to this virus is mediated by several partially dominant and recessive genes. In this study, we showed that the *Capsicum annuum* cultivar 'Bukang' contains a single dominant resistance gene against CMV_{Korean} and CMV_{FNY} strains. We named this resistance gene *Cmr1* (*Cucumber mosaic resistance 1*). Analysis of the cellular localization of CMV using a CMV green fluorescent protein construct showed that in 'Bukang,' systemic movement of the virus from the epidermal cell layer to mesophyll cells is inhibited. Genetic mapping and FISH analysis revealed that the *Cmr1* gene is located at the centromeric region of LG2, a position syntenic to the ToMV resistance locus (*Tm-1*) in tomatoes. Three SNP markers were developed by comparative genetic mapping: one intron-based marker using a pepper homolog of *Tm-1*, and two SNP markers using tomato and pepper BAC sequences mapped near *Cmr1*. We expect that the SNP markers developed in this study will be useful for developing CMV-resistant cultivars and for fine mapping the *Cmr1* gene.

Communicated by I. Paran.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1278-9) contains supplementary material, which is available to authorized users.

W.-H. Kang · N. H. Hoang · H.-B. Yang · J.-K. Kwon · D. Choi · B.-C. Kang (✉)
Department of Plant Science,
Research Institute for Agriculture and Life Sciences,
Plant Genomics and Breeding Institute, Seoul National University,
599 Gwanak-ro, Gwanak-gu, Seoul 151-921, Korea
e-mail: bk54@snu.ac.kr

S.-H. Jo
Bioinformatics Research Center, KRIBB, Taejon, Korea

J.-K. Seo · K.-H. Kim
Department of Agricultural Biotechnology,
Seoul National University, Seoul 151-921, Korea

Introduction

Crop production is constantly challenged by biotic stresses such as fungi, bacteria, and viruses. Viral disease in particular is one of the major problems affecting pepper production. *Cucumber mosaic virus* (CMV) was first described in 1916 and is one of the earliest known plant diseases (Jagger 1916). CMV, a member of the *Cucumovirus* genus in the family Bromoviridae, has the broadest host range of any virus throughout the temperate regions of the world. More than 800 species of plants can be infected by CMV, and more than 60 aphid species are known to be carriers for this virus (Palukaitis et al. 1992). Despite the importance of CMV disease, single dominant resistance genes against CMV in plants have rarely been reported, and most reported resistance genes are partial or polygenic (Troutman and Fulton 1958; Caranta et al. 1997, 2002). The *RCY* gene in *Arabidopsis* (Takahashi et al. 2001, 2002) and the *Cmr* gene in tomato (Stamova and Chetelat 2000) are the only known single dominant resistance genes in plants. The RCY gene is an NBS-LRR-type resistance gene that causes a hypersensitive response upon CMV infection (Takahashi et al. 2001). CMV resistance in cucurbits is mostly recessive (Kang et al. 2005b). In *Arabidopsis*, two recessive resistance genes identified by screening an EMS mutant population were reported to affect the cell-to-cell and long-distance movement of CMV (Yoshii et al. 2004).

In *Capsicum* spp., various sources of resistance to CMV have been identified. These include *C. annuum* 'Perennial' (Caranta et al. 1997; Lapidot et al. 1997; Grube et al. 2000a; Chaim et al. 2001), 'Vania' (Caranta et al. 2002), 'Sapporo-oonaga' and 'Nanbu-oonaga' (Suzuki et al. 2003); *C. frutescens* 'BG2814-6' (Grube et al. 2000a); 'LS 1839-2-4' (Suzuki et al. 2003); and *C. baccatum* 'PI 439381-1-3' (Suzuki et al. 2003). Most of these sources display partial resistance controlled by multiple genes, while a few cultivars show very good resistance (Grube et al. 2000a; Suzuki et al. 2003). Various resistance mechanisms have been documented, ranging from inhibition of replication to inhibition of cell-to-cell or long-distance movement. *C. annuum* 'Perennial' is an Indian hot pepper, first reported by an Indian group in 1977 (Pochard 1977) that has been extensively studied for CMV resistance. Several different resistance mechanisms have been suggested for 'Perennial': partial resistance to initial virus infection (Caranta et al. 1997), the inhibition of virus multiplication (Nono-Womdim et al. 1993a), and inhibition of long-distance movement of the virus (Nono-Womdim et al. 1993b). Caranta et al. (1997) showed that long-distance movement of CMV was impaired in 'Vania.' More recently, Grube et al. (2000a) showed that the resistance in *C. frutescence* 'BG2814-6' was controlled at the level of replication and cell-to-cell movement.

Various resistance inheritance patterns for 'Perennial' have been described: single recessive, partial dominant, or polygenic inheritance (Pochard and Daubeze 1989; Nono-Womdim et al. 1991; Nono-Womdim et al. 1993b; Dogimont et al. 1994; Caranta et al. 1997; Lapidot et al. 1997; Grube et al. 2000a). These inconsistent results may be ascribed to different viral isolates, environmental conditions, and inoculation methods (Grube et al. 2000a). Analyses have been conducted to identify genomic regions controlling resistance in 'Perennial,' quantitative trait locus (QTL). Caranta et al. (1997) identified two QTLs while Chaim et al. (2001) identified four QTLs that control resistance in 'Perennial.' QTL analysis of the resistance in 'Vania' revealed that one major QTL and several minor QTLs govern CMV resistance in this cultivar (Caranta et al. 2002). Quantitative trait loci detected in these different studies could not be aligned, with the exception of QTLs in LG11 due to the lack of common markers.

Pathogen-derived transgenes have been widely used to engineer resistance to plant viruses (Prins et al. 2008). Among pathogen-derived transgenes, coat protein (CP) genes have been most successfully utilized, and transgenic papaya tolerant to *Papaya ring spot virus* and transgenic squash resistant to *Watermelon mosaic virus* have been commercialized (James 2008). Recently, Lee et al. (2009) reported that transgenic peppers expressing CMV-CP were tolerant to two strains of CMV.

Approximately three decades ago, a Korean breeder identified a CMV resistance source from a Chinese open-pollinated variety called 'Likeumjo.' Since then, Korean seed companies have used this variety to develop commercial pepper that are resistant to CMV. Despite the practical use of the CMV resistance gene by seed companies, there have been no scientific reports on the spectrum and inheritance of the resistance gene. In this study, we describe the spectrum and inheritance of CMV resistance using a commercial F_1 hybrid variety 'Bukang' and describe the development of markers linked to the resistance locus.

Materials and methods

Plant materials and DNA extraction

The three mapping populations used in this study were a *C. annuum* 'Bukang' F_2 population, a backcross population from a cross between *C. annuum* 'Bukang' and *C. annuum* 'Jeju,' and an AC 99 F_2 population (Livingstone et al. 1999).

Two varieties of peppers, *C. annuum* 'Bukang' and *C. annuum* 'Jeju,' were used as parental lines. The 'Bukang' variety, which is a commercial variety known to contain a resistance gene against CMV, was obtained from Monsanto Korea (Chochiwon, Korea). A total of 94 breeding lines were also provided by Monsanto Korea and used for linkage analysis. 'Jeju' is a local variety susceptible to CMV and was obtained from Monsanto Korea. To study the inheritance pattern of the resistance gene to CMV, the F_1 'Bukang' plants were self-pollinated to obtain an F_2 population, and a BC_1 population of 'Bukang' was obtained by crossing 'Bukang' with 'Jeju.' A total of 94 breeding lines were provided by Jaehyung Yoo (Monsanto Korea, Chochiwon, Korea). These materials were used to confirm the linkage of SNP markers with the resistance phenotype.

Total DNA was extracted with a hexadecyltrimethylammonium bromide (CTAB) procedure (Hwang et al. 2009).

Genomic DNA samples from an F_2 AC 99 population (Livingstone et al. 1999) derived by crossing *C. chinense* 'PI 159234' (CA4) and *C. annuum* 'NuMex RNaky' (RNaky) were used to map *Cmr1* using SSR and CAPS markers.

Virus materials and inoculation

CMV_{Korean} was provided by Dr. KyungHee Paek (Korea University, Seoul, Korea), CMV_{FNY} was provided by

Dr. Ki-Hyun Ruy (Seoul Women's University, Seoul, Korea), and CMV_{P1} was provided by Nam-Han Hur (Nong Woo Bio Ltd., Korea).

The seedlings were inoculated with CMV strains when two cotyledons were fully expanded and two true leaves began to appear. The inoculums of the CMV_{Korean} and CMV_{FNY} strains were prepared from infected leaves of *Nicotiana benthamiana* or *Cucumis sativus*. One gram of infected leaves was ground in 10 ml of 0.1 M phosphate buffer pH 7.0. The plants were dusted with carborundum #400 (Hayashi Pure Chemical Ind., Japan) and inoculated by rubbing the viruses onto the two cotyledons. Control plants were mock-inoculated with 0.1 M phosphate buffer pH 7.0. After inoculation, the inoculated plants were kept in a growth chamber at 25°C. CMV symptoms were first observed 1 week after inoculation, and the development of symptoms was monitored continuously until the experiment was completed.

Detection of the presence of CMV by ELISA

We performed ELISA to test systemic infection at 7 dpi, and we checked the symptoms once more at 14 dpi to avoid contamination. ELISA was used to detect CMV according to the manufacturer's protocol (Agdia, USA). Samples were considered positive for the presence of CMV when the absorbance value (405 nm) of each sample was greater than that of a healthy control plant.

Confocal laser scanning microscopy

CMV_{FNY}-Green fluorescent protein (GFP) was developed by Dr. Kook-Hyung Kim (Seoul National University, Seoul, Korea). *Agrobacterium tumefaciens* strain GV2260-containing CMV constructs were grown in 3 ml of YEB media containing kanamycin 50 μg/ml and rifampicin 50 μg/ml at 30°C. *Agrobacterium* cells were harvested by centrifugation at 13,000 rpm for 2 min and resuspended in 3 ml infiltration buffer containing 10 mM MES (pH 5.5), 10 mM $MgCl_2$, and 100 μM acetosyringone. Each *Agrobacterium* cell suspension was grown to an OD_{600} of 0.5 and mixed at 1:1:1 ratio of CMV_{FNY} RNA1:CMV_{FNY} RNA2-GFP:CMV_{FNY} RNA3. The cell suspension was incubated at room temperature for 3 h. Then, the cell suspension was infiltrated into *N. benthamiana* using a 1 ml syringe. At 5–7 dpi, systemically infected leaves of *N. benthamiana* were used to inoculate 'Bukang' and 'Jeju' cotyledons as described earlier. Two to six days after inoculation, GFP fluorescence in cotyledons was observed using a confocal laser-scanning microscope (Carl Zeiss-LSM510, NCIRF, Seoul National University). The optimal brightness and contrast of all images were enhanced using Adobe Photoshop.

Testing CAPS markers

A total of 309 'Bukang' F_2 individuals were used to test the three previously reported CAPS markers (Kim et al. 2004). PCR was performed in 25 μl reaction volumes containing 2.5 μl of 10× PCR buffer (20 mM Tris–HCl (pH 8.0), 100 mM KCl and 2 mM $MgCl_2$), 2 μl of 10 mM dNTPs, 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer, 18.3 μl dH_2O, 0.2 μl of *Taq* DNA polymerase, and 1 μl of 50 ng/μl DNA template. The PCR profile comprised initial 4 min incubation at 94°C for denaturation, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, and a final extension step of 5 min at 72°C. The PCR reaction was performed in a thermocycler (My Cycler™, BioRad, USA).

PCR products were digested with the restriction enzymes *Xba*I and *Eco*RI (Roche, Germany). Restriction enzyme digestion reactions contained 2 μl of 10× PCR buffer, 0.4 μl of enzyme, 10 μl of PCR product, and 7.6 μl dH_2O in a total reaction volume of 20 μl. The reactions were incubated for 1 h at 37°C. The DNA bands of the digested products were separated by electrophoresis and observed on a UV trans-illuminator (Bio-Rad, USA).

Locating the dominant CMV resistance gene on a pepper linkage map

Genomic DNA from the two parent strains, *C. chinense* 'PI 159234' (CA4) and *C. annuum* 'Numex RNaky' (RNaky), and 91 individuals from the F_2 AC 99 population were used to locate the CAPS-A and -B markers in a pepper linkage map (Table 1). Then, the same marker analysis procedure as described earlier was performed.

The genomic DNA of the parent cultivars was amplified using 20 simple sequence repeat (SSR) markers (Supplementary Table) that are located on linkage group 2 of the SNU map (Yi et al. 2006) to detect polymorphisms. Polymorphic SSR markers were then used to analyze a total of 91 individual plants from the F_2 AC-99 population. The SSR procedure was performed as described by Yi et al. (2006). SSR and CAPS marker genotyping data were analyzed using CarthaGene software (Schiex and Gaspin 1997).

BAC screening and BAC FISH

The CAPS-A marker was used as a probe for BAC library screening. The BAC screening procedure was performed as described in Yoo et al. (2003). Pachytene preparations from the floral bud and FISH were carried out as described by Kwon and Kim (2009) with minor modifications. All images were improved for optimal brightness and contrast using Adobe Photoshop.

Table 1 Molecular markers linked to the *Cmr1* gene

Marker	Type	Primer sequences (5′–3′)	Product size (bp)	Mapping population[a]	Recombinants/total number of F_2 individuals
CMV	CAPS (*Xba*I)	GTAGTAGGGTACGGACTCATA	1,000	AC99	12/309
CAPS A		GTCCCGACGATAGCCCAAAAG		Bukang	
CMV	CAPS (*EcoR*I, *Xba*I)	GTAGTAGGGTACGGACTCATA	1,200	AC99	7/309
CAPS B		GGAGTTTCATCATATGAAGCC		Bukang	
CaTm-int1	CAPS (*Hinf*I)	TCAGCAAAGAAAGATTCACGAAC	496	AC99	
		ACGTACACTTGATGATGCCTTGT			
CaTm-int3HRM	SNP	TGGTGTTTTTATCAGCCTTAGC	158	Bukang	6/309
		GAAGGACAAGAATTCATGATATGG			
CaT1616BAC	SNP	AATTGGTTCTGGATCACTGCC	200	Bukang	6/309
		CACCTTGATCTGCTCCTTTCTG			
240H02sp6	SNP	TTGGTTGAGGCAAGTTTTCA	472	Bukang	6/309
		TCATTTTCCTATGTCATTCATGG			

[a] AC99, *C. chinense* 'PI 159234' (CA4) × *C. annuum* 'NuMex RNaky' F_2 population; 'Bukang,' 'Bukang' F_2 population

Development of *Cmr1*-linked markers

The *Tomato mosaic virus* (ToMV) resistance gene *Tm-1* was used to develop *Cmr1*-linked markers in peppers. The *Tm-1* gene sequence was aligned against the pepper EST database (http://www.210.218.199.240/SOL/), and one EST sequence (cacn2211) was obtained. Primers were designed based on the pepper EST sequence homologous to *Tm-1*. SGN Intron Finder software (http://www.sgn.cornell.edu) was used to design primers for intron amplification. PCR was performed in a total volume of 20 µl containing 1× PCR reaction buffer [20 mM Tris–HCl (pH 8.0), 100 mM KCl, and 2 mM $MgCl_2$], 0.1 mM dNTP, 0.2 U *Taq* DNA polymerase, 10 pmol of each primer, and 20 ng of genomic DNA. PCR conditions involved denaturing the DNA for 4 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 40 s at 72°C. PCR product sequencing was performed at NICEM (Seoul National University, Seoul, Korea). PCR products were digested using 2 U of *Hinf*I (Roche, Germany) for 2–3 h at 37°C. The digested products were separated on a 1% agarose gel. The third intron of *CaTm1* showed a single nucleotide polymorphism in the 'Bukang' F_2 population. Genotype screening was performed using a high-resolution melting (HRM) method. PCR was carried out in 20 µl reaction mixtures containing 60 mM KCl, 10 mM Tris–Cl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5 pmol of each primer, 1 unit *Taq* polymerase, 1.25 µM Syto9 (Invitrogen, USA), and 50 ng gDNA, using a Rotor-Gene™ 6000 thermocycler (Corbett, Australia). Cycling conditions were 95°C for 4 min, followed by 95°C for 20 s, 57°C for 20 s, and 72°C for 30 s for 40 cycles, and HRM was analyzed for every increment of 0.1°C between 70 and 90°C.

Three BAC clones (AC215399, AC215483, and AC215456), *Bs3*, and COS marker sequences located near *Tm-1* on tomato chromosome 2 were used for additional marker development. To obtain homologous sequences, pepper EST sequences were selected from a pepper EST database. The primers to amplify intron sequences were designed using Primer3 software (http://www.sgn.cornell.edu/tools/primer3/). Marker polymorphism was tested using the HRM method.

Results

Survey of resistance spectra of *C. annuum* 'Bukang' to CMV strains

Cucumber mosaic virus screening revealed that all of the inoculated 'Jeju' plants were completely susceptible to all three CMV strains, indicating that the CMV screening method that we used is reliable and consistent. Two major CMV symptoms, vein clearing mosaic and leaf distortion, started to develop at 2–3 days post inoculation (dpi) and continued to develop systemically. A large number of severely infected plants died, while the surviving plants developed severe mosaic symptom and leaf distortion. As seen in Table 2, 'Bukang' plants showed complete resistance to two CMV strains, CMV_{Korean} and CMV_{FNY}, but were susceptible to the CMV_{P1} strain. These results indicated that the CMV_{P1} strain can overcome the CMV resistance in 'Bukang' plants.

Resistance responses to CMV in *C. annuum* 'Bukang'

Cucumber mosaic virus symptoms were visible on the first true leaves of 'Jeju' at 1 week after inoculation with CMV_{Korean} and CMV_{FNY}, while no observable symptoms

Table 2 Resistance spectra and segregation analysis of *C. annuum* 'Bukang,' *C. annuum* 'Jeju,' F_2 population and BC_1 to *Cucumber mosaic virus* (CMV)

Populations	CMV strains			Expected ratio (R:S)	χ^{2b}	P[c]	ELISA (Absorbance 405 nm)		
	CMV_{Korean} (R:S)[a]	CMV_{FNY} (R:S)	CMV_{P1} (R:S)				Non-inoculated	Inoculated	
								Cotyledon	Upper leaves
Jeju	0:10	0:20	0:15	–	–	–	0.44[d]	4.0	3.9
Bukang	17:0	20:0	0:15	–	–	–	0.3	3.8	0.452
BC_1	7:10	–	–	1:1	0.52	0.4708	–	–	–
Bukang F_2	236:73	–	–	3:1	0.1167	0.7326	–	–	–

[a] Resistant plants (R) versus susceptible plants (S)

[b] Chi-square test

[c] Probability value

[d] Mean of values in triplicate

appeared on the first true leaves of 'Bukang.' To investigate resistance responses in 'Bukang,' accumulation of coat protein (CMV CP) was examined by ELISA. As can be seen in Table 2, accumulation of CMV CP was observed in both inoculated cotyledons and non-inoculated upper leaves of 'Jeju'. Interestingly, CMV CP was detected in inoculated cotyledons of 'Bukang' plants. However, no CMV accumulation was detected in the non-inoculated leaves of these plants. These results demonstrate that resistance in 'Bukang' involves inhibition of the systemic movement of CMV.

Observation of virus movement at the cellular level

To determine in more detail the resistance mechanism of 'Bukang' to CMV, CMV_{FNY}-GFP (abbreviated as CMV-GFP) was inoculated onto 'Bukang' and 'Jeju' cotyledons. At 2, 4, and 6 dpi, GFP fluorescence was observed in inoculated leaves by confocal laser scanning microscopy (Fig. 1). At 2 and 4 dpi, green fluorescence was detected in the epidermal cells of inoculated 'Bukang' and 'Jeju' leaves (Fig. 1a, b, g, h). These two pepper cultivars appeared to have a similar quantity of CMV-GFP at the infection site. At 6 dpi, CMV-GFP movement to epidermal cells of infected sites was observed in 'Bukang' (Fig. 1c, f), whereas in 'Jeju' at the same time point, CMV-GFP moved into epidermal and mesophyll cells (Fig. 1i, l). Therefore, movement of CMV-GFP to mesophyll cells appears to be restricted in 'Bukang' (Fig. 1f, l).

Inheritance of resistance to CMV in *C. annuum* 'Bukang'

To analyze the inheritance pattern of resistance to CMV in 'Bukang' plants, we observed the segregation ratio of disease responses of both F_2 and BC_1 populations against CMV_{Korean} strain. Results of the segregation analysis of the CMV resistance gene are presented in Table 2. The segregation analysis of resistance and susceptibility in the BC_1 population and F_2 'Bukang' population were fitted to the expected 1:1 and 3:1 Mendelian segregation models (Table 2). These results are consistent with a dominant inheritance pattern and strongly demonstrate that resistance in *C. annuum* 'Bukang' is controlled by a single dominant resistance gene. We named this resistance gene '*Cmr1.*'

Locating the *Cmr1* gene using linkage analysis

Previously, Kim et al. (2004) reported three CAPS markers linked to the *Cmr1* gene; however, the map location of *Cmr1* has been not reported. We confirmed that CAPS markers linked with the *Cmr1* gene and determined that they are located about 3 cM away from the gene in the 'Bukang' F_2 population. To locate the *Cmr1* gene in a pepper linkage map, the CAPS-A and CAPS-B markers were mapped using the AC 99 F_2 population. Figure 3 shows that the CAPS-A marker is located at the centromeric region of LG2 near TG31A. This result suggests that the *Cmr1* gene is located near this position.

Because the CAPS markers are not completely linked to the *Cmr1* gene, we attempted to develop additional markers using SSR markers already mapped to LG2. Of 20 SSR markers, 13 markers were mapped onto the AC 99 map (Fig. 3). Of these 13 markers, three, HpmsE003, HpmsE066, and HpmsE141, were found to be linked to the *Cmr1* locus. These SSR markers were then used to evaluate the segregation of CMV resistance in the 'Bukang' F_2 population, but all of the markers in this population were monomorphic.

Development of molecular markers linked to *Cmr1* using comparative analysis between peppers and tomatoes

The *Tm-1* gene, a *Tomato mosaic virus* resistant gene, is located in the centromeric region of tomato chromosome 2

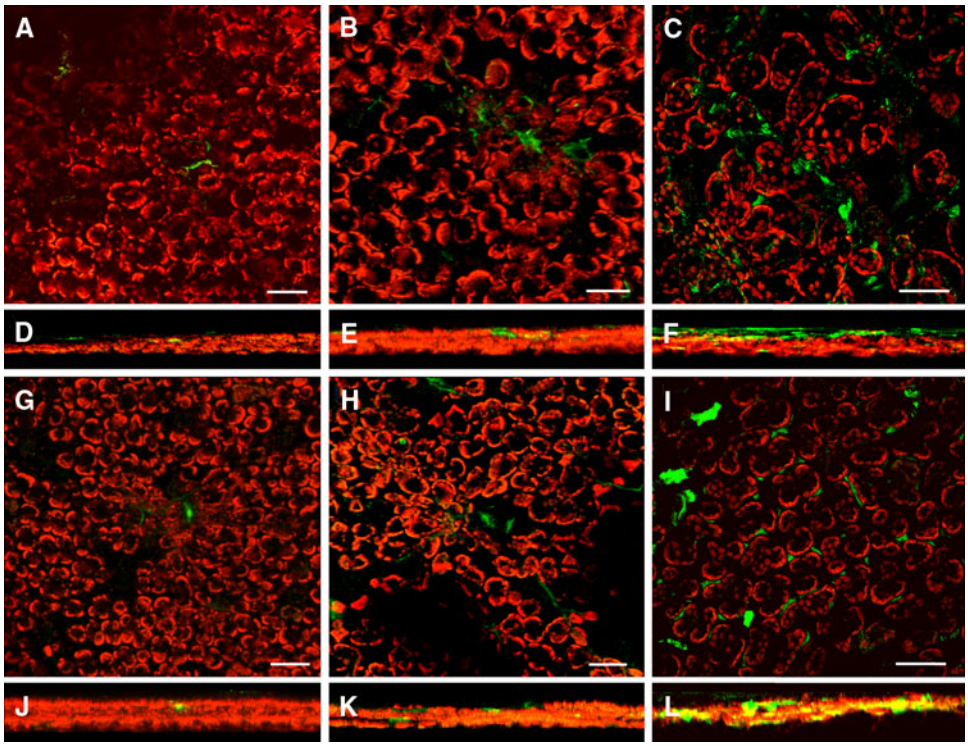

Fig. 1 Localization of CMV-GFP in *C. annuum* 'Bukang' and 'Jeju.' 'Bukang' (**a**–**f**) and 'Jeju' (**g**–**l**) leaves were inoculated with CMV-GFP. GFP fluorescence was visualized by confocal laser scanning microscopy at 2 days (**a**, **d**, **g**, **j**), 4 days (**b**, **e**, **h**, **k**), and 6 days (**c**, **f**, **i**, **l**) after inoculation. All images are "Z projections" of 15–20 slices, 20–30 μm thick. Panels **d**–**f** and **j**–**l** are three-dimensional images of **a**–**c** and **g**–**i**, respectively. *Three-dimensional images* indicate the sides of leaves. *Green* indicates GFP signal, and *red* indicates autofluorescence. *Scale bars* 50 μm

(Ishibashi et al. 2007). We found that the *Cmr1* gene is located in a region syntenic to the *Tm-1* gene. Therefore, we used the *Tm-1* gene sequence as a candidate sequence for *Cmr1*. One pepper EST (Cacn2211), which has highest nucleotide similarity with *Tm-1*, was used for marker development. A total of five introns were predicted in this EST sequence using the Intron Finder program of the SGN project (http://www.sgn.cornell.edu). PCR products were obtained from the first, third, and fourth predicted introns with sizes of 500 bp, 540 bp, and 1 kb, respectively. No PCR products were obtained for the second and fifth introns. Amplicons from the two parental genotypes of AC99 were of the same size. Sequence analysis of the first intron revealed a polymorphic endonuclease recognition site, *Hinf*I, and this was used to develop the CAPS marker CaTm-int1 (Fig. 2). CaTm-int1 mapped near TG31 on chromosome 2 as expected (Fig. 3). However, CaTm-int1 did not show polymorphism between the 'Bukang' parents. Further analysis showed that the third intron sequence was polymorphic between the two 'Bukang' parents. This sequence polymorphism was detected by HRM analysis, and the marker was named 'CaTm-int3HRM' (Fig. 2). Co-segregation analysis of CaTm-int3HRM and the *Cmr1* gene revealed six recombinants out of 309 individuals.

Bs3 maps between the RFLP markers TG31 and TG33 (Pierre et al. 2000) where *Cmr1* is predicted to be located. A total of nine markers (H17-6, H17-52, H6-4, Y65-2, B103T7, B3T7, S1464, S2245, and Y110-2) from both sides of the *Bs3* gene (Pierre et al. 2000; Jordan et al. 2006) were used in this study. However, two markers flanking *Bs3* (Y65-2 and S2245) did not map to the predicted region between TG31 and TG33 (Fig. 3). Therefore, the *Bs3* gene mapping did not provide any additional information that could aid in the development of closely linked markers.

Because tomato genome sequence information is available, we attempted to add more linked markers around the *Cmr1* locus using tomato sequence information around the *Cmr1* region. Three BAC clones were anchored to a tomato COS marker (T1616) located on the short arm of tomato chromosome 2. Several pepper EST sequences homologous to the tomato BACs were identified by Blast analysis. One EST (cacn11444) showed polymorphisms in the *Cmr1* segregating and AC99 populations (CaT1616BAC marker). The CaT1616BAC marker showed six recombinants in the 309 'Bukang' F_2 individuals evaluated; these were the same recombinants detected with CaTm-int3HRM (Fig. 3).

To confirm linkage, a total of 94 pepper breeding lines with a known CMV resistance phenotype were subjected to marker analysis using CaTm-int3HRM. This marker identified 60 resistant lines and 33 susceptible lines. Marker genotype and phenotype comparisons showed that 92 out of 93 genotypes corresponded to disease resistance phenotypes (Table 3).

Development of BAC end markers and FISH analysis

Ten positive BAC clones (235B02, 240H02, 291M13, 352C19, 367G19, 410E04, 416D09, 564I19, 770O18, and

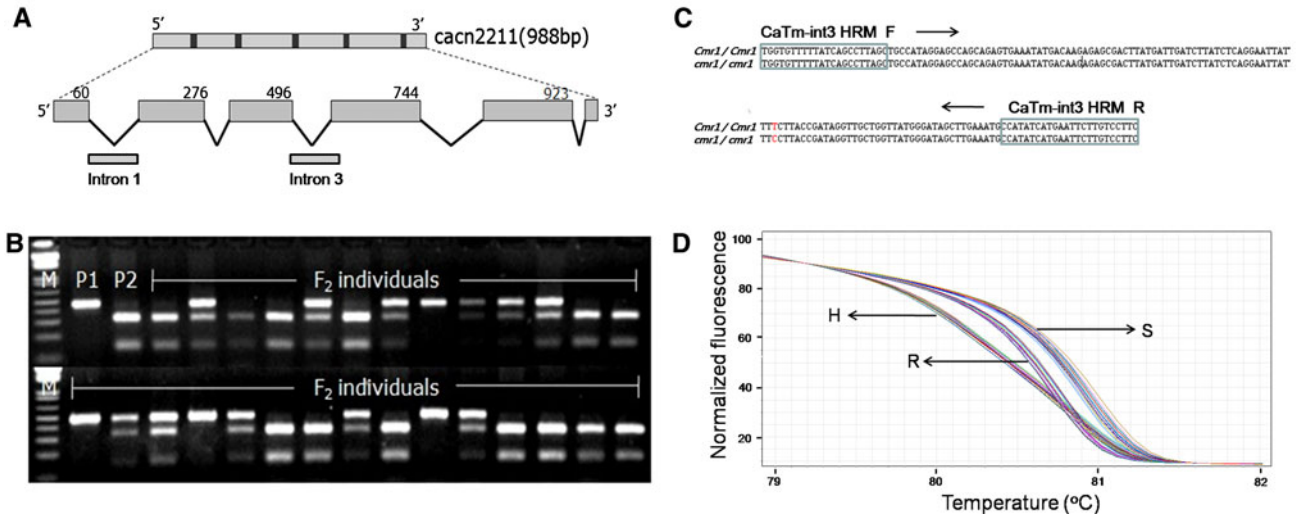

Fig. 2 **a** Intron prediction in *Tm-1* homologous ESTs and the positions of SNP markers. Five introns were predicted in the pepper EST sequence using the Intron Finder program. *Gray shaded boxes* and *black lines* indicated the six exons and five introns, respectively. The *numbers* on the *gray boxes* indicated the locations (bp) of the introns in the pepper EST sequence. The *Intron1* and *Intron3 boxes* indicate the positions of the CaTm-int1 and CaTm-int3HRM markers, respectively. **b** Analysis of the CaTm-int1 CAPS marker in the AC99 F_2 population. Parents P1 (RNaky) and P2 (CA4) of the AC99 population (*lanes 2* and *3* from the *left*) were polymorphic after digestion with *Hinf*I. *M* 1 kb plus ladder. **c**, **d** HRM analysis of the CaTm-int3HRM marker in the 'Bukang' F_2 population. One SNP existed between the *Cmr1/Cmr1* and *cmr1/cmr1* genotypes (**c**). Melting curve analysis of the CaTm-int3 HRM marker (**d**). Three different melting curve types were identified (*R* homozygous resistance genotype, *S* homozygous susceptible genotype, *H* heterozygous genotype)

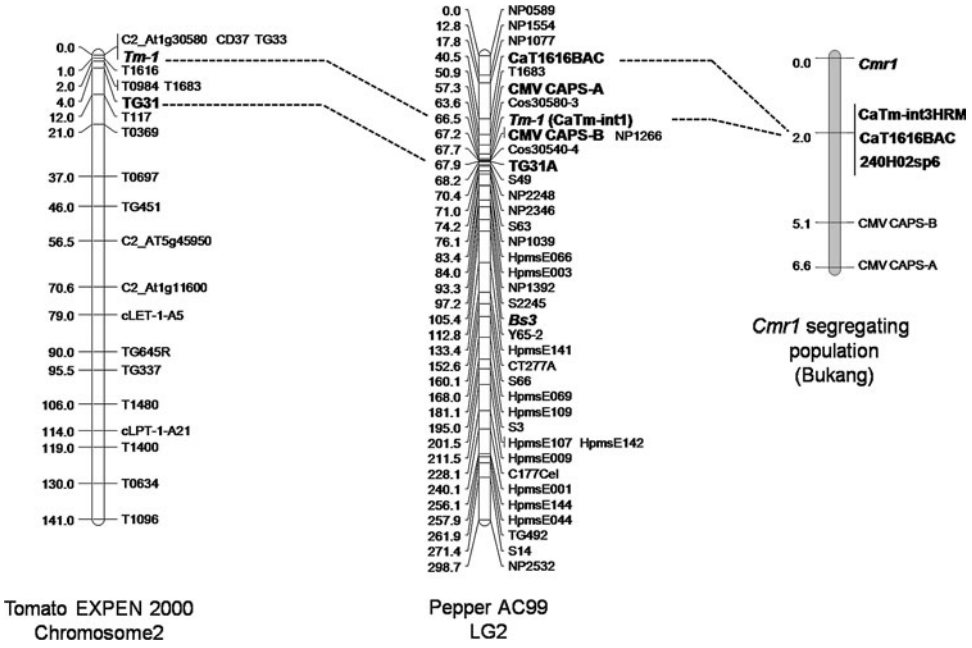

Fig. 3 The linkage map of the *Cmr1* region in peppers and tomatoes using comparative analysis. Markers on the tomato EXPEN 2000 chromosome 2 are only partially shown here. The locations of the *Tm-1* gene and CD37 marker are based on Tanksley et al. (1992) and the tomato EXPEN 1992 map, respectively. Two CAPS markers (CMV CAPS-A and CAPS-B) and two SNP markers (CaTm-int1 and CaT1616BAC) were mapped in AC99. Three SNP markers (CaTm-int3HRM, CaT1616BAC, and 240H02sp6) were linked to the *Cmr1* locus in a *Cmr1* segregating population. *Numbers to the left* indicate genetic distances (cM). *Dotted lines* indicate common markers of the *Cmr1* region in peppers and tomatoes

806K23) were obtained by hybridization of CAPS-A. To test which of the BAC clones were potentially linked to *Cmr1*, SNP markers were developed from the BAC end sequences and were mapped onto the AC99 population. One SNP marker derived from 240H02 mapped near the end of LG2 near the *Cmr1* region. Furthermore, 240H02-sp6

Table 3 Test of CMV resistance in pepper breeding lines using CaTm-int3HRM marker

Phenotype of breeding lines	Number of lines	Maker genotype		
		R	H	S
R	60	58	2	0
S	34	0	1	33

R homozygous resistance genotype, *S* homozygous susceptible genotype, *H* heterozygous genotype

co-segregated with other *Cmr1*-linked markers in the 'Bukang' F_2 population (Fig. 3).

After confirming the genetic location of 240H02 BAC, the physical location of this clone was determined using FISH analysis. FISH was performed on meiotic pachytene from *C. annuum* 'Bukang.' 240H02 clone DNA as well as 25S rDNA were used as FISH probes. 240H02 co-localized on the 45S rDNA region with high intensity at the end of the pachytene (Fig. 4).

Discussion

This was the first study to demonstrate that CMV resistance in *C. annuum* can be controlled by a single, dominant gene. All prior studies have shown that resistance to CMV in *C. annuum* is incompletely dominant and quantitatively inherited (Lapidot et al. 1997; Caranta et al. 1997; Grube et al. 2000a; Chaim et al. 2001). We named this single dominant CMV resistance gene '*Cmr1*' (*Cucumber mosaic resistance 1*). Genetic mapping and FISH analysis demonstrated that the *Cmr1* gene is located at a position syntenic to the ToMV resistance locus (*Tm-1*) in tomatoes. Using a comparative genetic mapping study, we were able to develop several SNP markers linked to *Cmr1*.

Despite the fact that several studies have examined resistance sources in *Capsicum* spp., none of the previous studies have reported a complete dominant resistant gene. Because of this, all previous studies of CMV resistance have focused on identifying QTLs associated with resistance (Caranta et al. 1997, 2002; Chaim et al. 2001). In this present study, we determined that *Cmr1* is located on LG2; however, none of the QTLs were detected by previous studies correspond to *Cmr1,* which demonstrates that the resistance mechanisms of *Cmr1* are totally different from those previously reported. Because *Cmr1* was isolated and marketed by a private company, the resistance source of this cultivar has never been open to the scientific community.

Natural virus resistance can be classified according to the viral life cycle stage at which resistance is observed (Kang et al. 2005a, b): resistance at the cellular level, cell-to-cell movement and long-distance movement. Various resistance responses to CMV have been observed in plants, with inhibition of long-distance movement seen as a common CMV resistance mechanism (Dufour et al. 1989; Valkonen and Watanabe 1999; Stamova and Chetelat 2000; Canto and Palukaitis 2001; Caranta et al. 2002). In this

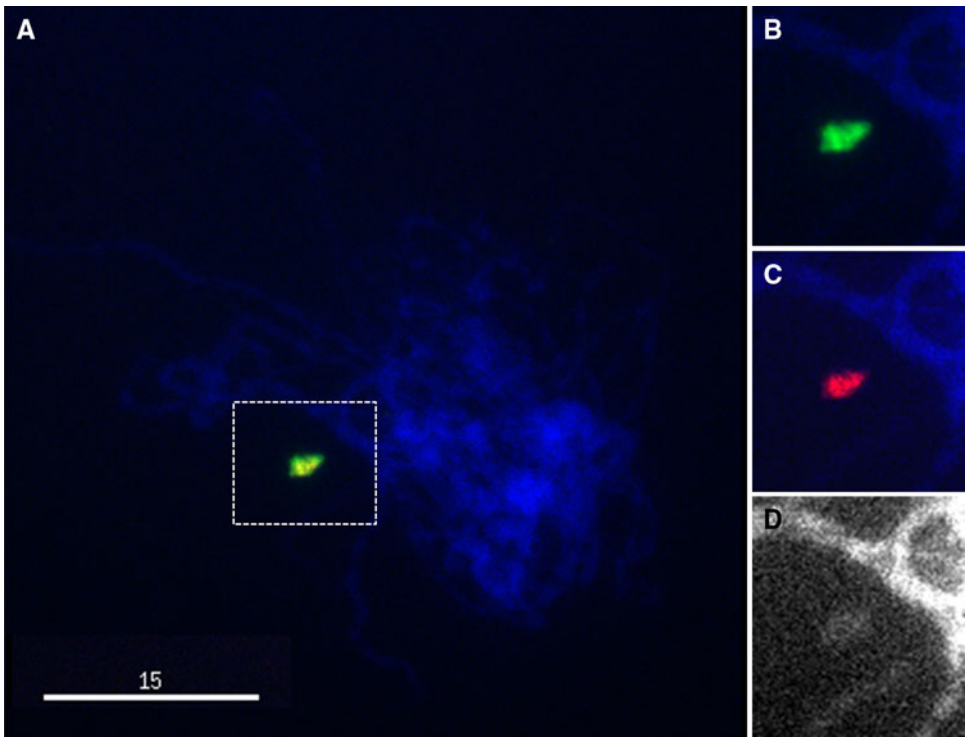

Fig. 4 FISH with BAC240H02 and 25S rDNA on meiotic pachytene from *C. annuum* 'Bukang.' DNA was counter-stained with DAPI in *blue*. BAC240H02 is shown in *red* and 25S rDNA is shown in *green*. Panel **a** shows the merged green and red signals. Panels **b**, **c**, **d** show enlarged views (×1.5) of the *boxed area* on the end of the pachytene. *Scale bar* 15 μm

study, we observed that the CMV CP accumulated in the inoculated leaves but not in the systemic leaves of 'Bukang,' indicating that *Cmr1* inhibits the systemic movement of CMV. There have been extensive studies of CMV movement in plants (reviewed by Palukaitis and Garcia-Arenal 2003). However, cellular observations of virus movement in relation to resistance are very rare. When we visualized the movement of CMV_{FNY}-GFP in the inoculated leaves of *Cmr1* plants, we found that CMV spread preferentially between epidermal layers but less efficiently through the mesophyll. Thus, *Cmr1* seems to render resistance against CMV by preventing the movement of CMV from the epidermal cell layers to the deeper cell layers. Our results are consistent with the previous observation that CMV moves radially from primary inoculated cells through the epidermal tissue before moving to deeper cell layers (Soards et al. 2002).

R genes and resistance gene analogs (RGA) are located at syntenic positions in *Solanaceae* crops (Grube et al. 2000b). This result indicates that the locations of R genes are broadly conserved in genomes of plants in the same genera. Based on this observation, the *Phythophthora* resistance gene, *R3a*, was successfully isolated in potatoes (Huang et al. 2005). Similarly, we used *Bs3* and *Tm-1* as candidate loci and found that the *Cmr1* gene is syntenic to the *Tm-1* gene. We were able to develop a *Cmr1*-linked marker using *Tm-1*; however, the *Tm-1*-derived marker did not co-segregate with the *Cmr1* gene (Fig. 3). This indicates that *Tm-1* and *Cmr1* do not have the same structure and function. Unlike other R genes, *Tm-1* does not encode a nucleotide binding site (NBS) or a leucine-rich repeat (LRR) gene; rather, the *Tm-1* gene is a member of an as yet uncharacterized protein family and contains a TIM-barrel domain (Ishibashi et al. 2007). The *Tm-1* gene inhibits the replication of wild type ToMV RNA by binding to replication proteins, while the *Cmr1* gene inhibits CMV systemic movement. Colocalization of *Tm-1* and *Cmr1* may therefore be coincidental.

In this paper, we developed SNP markers linked to the *Cmr1* locus using a F_2 population derived from *C. annuum* 'Bukang.' We found that the closest marker, CaTm-int3HRM, estimated to be located about 2 cM away from *Cmr1*, predicted the phenotypes of breeding lines fairly accurately. The discrepancy between using a segregation population and breeding lines seems to be due to scoring errors during virus screening using the F_2 population. Therefore, the actual distance between CaTm-int3HRM and *Cmr1* appears be closer than that estimated in the F_2 population.

All developed markers were located on one side of the *Cmr1* locus in a segregating population. Markers on the opposite side and more closely linked markers are needed in order to clone *Cmr1*. However, finding more closely linked markers may be very challenging, because *Cmr1* is located on the short arm of pepper chromosome 2, for which little genomic information is available as it is a heterochromatic region (Gill et al. 2008). We confirmed the heterochromatic nature of this region by FISH analysis. The pepper BAC clone 240H02 was located at the end of the NOR region of the pepper pachytene chromosome. This result indicates that *Cmr1*-linked markers are also linked to 45S rDNA in pepper. Previous studies have reported clustering of pepper and tomato 45S rDNA at corresponding regions of chromosome 2 (Tanksley et al. 1992; Brasileiro-Vidal et al. 2009; Kwon and Kim 2009; Wu et al. 2009). In summary, the lower recombination rate and lower gene density in the chromosomal region to which *Cmr1* maps will make development of more closely linked markers challenging (Gill et al. 2008).

Acknowledgments This work was supported by a grant (Code 20070401034028) from the BioGreen21 program, Rural Development Administration, Republic of Korea and by a grant (GC1132-1) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program, funded by the Ministry of Education, Science and Technology of the Republic of Korea.

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