

Research Report

Developmental Changes of Recessive Genes-mediated *Cucumber mosaic virus* (CMV) Resistance in Peppers (*Capsicum annuum* L.)

Woong-Ki Min^{*}, Jae-Hwang Ryu, and Su-Hyeon Ahn

Breeding Research Station, DongbuFarm Hannong Co., Ltd., Anseong 456-933, Korea

Abstract: *Cucumber mosaic virus* (CMV) is one of the most important viral diseases in pepper (*Capsicum annuum* L.), and several genes for resistance were reported in *Capsicum* spp. In Korea, a single dominant gene that is resistant to CMV_{Fny} and CMV_{P0} has been used for breeding. Recently, a new strain (CMV_{P1}) was reported that could infect cultivars resistant to both CMV_{Fny} and CMV_{P0}. Therefore, breeding of more robust CMV-resistant cultivars is required. In this study, we surveyed the inheritance of CMV_{P1} resistance and analyzed the location of the resistance loci. After CMV_{P1} inoculation of various germplasms and breeding lines, one accession (ICPN18-8) showed no visual symptoms at 15 dpi (days post inoculation) but was susceptible after 45 dpi, and one resistant line (I7339) showed resistance until at 45 dpi. The latter line was used for tests of resistance inheritance. A total of 189 F₂ plants were examined, with 42 individuals showing resistance at 15 dpi and a phenotype segregation ratio close to 1:3 (resistant:susceptible plants). In a lateral ELISA test at 45 dpi, 11 plants showed resistance, and the segregation ratio was changed to 1:15. These results indicate that resistance in *C. annuum* 'I7339' is controlled by two different recessive genes; we named these resistance genes '*cmr3E*' and '*cmr3L*', respectively. To locate these two resistant loci in the pepper linkage map, various RAPD, SSR, and STS markers were screened; only nine markers were grouped into one linkage group (LG). Only one RAPD primer (OPAT16) was distantly linked with *cmr3E* (22.3 cM) and *cmr3L* (20.7 cM). To develop more accurate markers for marker-assisted breeding, enriching for molecular markers spanning two loci will be required.

Additional key words: linkage group, RAPD

Introduction

Various plant viruses infect peppers (*Capsicum annuum* L.) cause inhibition of growth, decrease of fruit quality and death. *Cucumber mosaic virus* (CMV) is one of the important viral diseases in Korea and worldwide, infecting many different crop species including peppers (Choi et al., 2005; Palukaitis et al., 1992). Besides its importance, no complete resistances were found capable of restriction of CMV replication or long-distance movement in pepper. In some cucurbits, resistance to CMV was controlled by one recessive gene (Kang et al., 2005), tomato and *Arabidopsis* have

single dominant gene for resistance (Stamova and Chetelat, 2000; Takahashi et al., 2001, 2002). Several poly-genes for resistance were reported in *Capsicum* species including 'Perennial' (Caranta et al., 1997; Chaim et al., 2001; Grube et al., 2000; Lapidot et al., 1997), 'Vania' (Caranta et al., 2002), *C. baccatum* 'PI439381-1-3' (Suzuki et al., 2003) and *C. frutescens* 'BG2814-6' (Grube et al., 2000). In *Arabidopsis* and tomato, single dominant gene *RCY* and *Cmr* were suggested for resistance (Stamova and Chetelat, 2000; Takahashi et al., 2001, 2002). Up to date, three different steps of inhibition of the virus cycles were reported in distinct resistant *Capsicum* accessions: restriction of host-cell infection (Caranta

*Corresponding author: shakespeare@dongbu.com

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et al., 1997), suppression of virus multiplication in the whole plants (Nono-Wondim et al., 1993) and restraint of long-distance movement (Dufour et al., 1989).

In Korea, CMV was reported all province cultivating peppers (Choi et al., 2005). Some commercial cultivars, such as 'Bukang' have been used for the resistance sources to CMV in seed companies. From progeny tests, it was revealed that 'Bukang' have a single dominant gene *Cmr1* resistant to CMV_{Fny} and CMV_{P0} (Kang et al., 2010). For the single dominant resistant gene, three closely linked molecular markers were developed and applied for breeding. Recently, a new strain CMV_{P1} was reported, which could infect cultivars resistant to both CMV_{Fny} and CMV_{Korean} (Kang et al., 2010; Lee et al., 2006). Korean pepper breeders have been developed many cultivars resistant to both CMV and *Phytophthora* root rot for open field cultivation, however occurrence of CMV infected plants did not decreased and strength of resistance to CMV was different in many CMV resistant cultivars in the repeated annual survey. Therefore, breeding of more robust CMV resistant cultivars will be important goal of seed companies. In this study, we surveyed inheritance of CMV_{P1} resistance, and described the development of markers linked to the resistance loci.

Materials and Methods

Plant Materials and DNA Extraction

A cayenne-pepper inbred line 'I7339 (I)' was used as a CMV_{P1} resistant parent and another inbred line 'E1338 (E)' was used as a susceptible parent, both were obtained from DongbuFarm Hannong (Anseong, Korea). A CMV_{Fny} resistant cultivar 'Bu-hong' (DongbuFarm Hannong, Korea) and one CMV_{P1} resistant accession ICPN-18-8 (provided from Dr. Myung-Cheorl Cho, National Institute of Horticultural & Herbal Science, Korea) were included. Yolo Wonder was used as susceptible controls in each inoculation. The F₁ hybrid (E1338 × I7339) was self-pollinated to produce 189 F₂ plants, and they were analyzed for both CMV resistance and molecular markers. Total genomic DNA was isolated using the protocol described by Kim and Kim (2005).

Virus Materials and Inoculation

CMV_{P1} was provided by Dr. Byoung-Cheorl Kang (Seoul National University, Seoul, Korea). The seedlings were inoculated with CMV_{P1} at two leaves stage. The inoculums were prepared from the infected leaves of *Nicotiana benthamiana*. One gram of infected leaves was ground with 10 mL of 0.1 M phosphate buffer pH 7.0 and carborundum #400

(Junsei Pure Chemical Ind., Japan) and inoculated by rubbing the virus 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 and symptoms were observed two times, 15 days and 45 days post inoculation (dpi).

Detection of the Presence of CMV by ELISA

To test systemic infection, ELISA was used to detect CMV_{P1} according to the manufacturer's protocol (Agdia, USA). Samples for ELISA test were collected and the upper leaves from inoculated cotyledons at 15 dpi and upmost leaves from top branches at 45 dpi, two times. Samples were considered to be positive for the presence of CMV_{P1} when the absorbance value (405 nm) of each sample was greater than the mean of two healthy control 'Yolo Wonder' plants.

RAPD Analysis and Linkage Group Construction

RAPD analyses were performed on each of DNA from resistant and susceptible bulks and 189 individual plants of F₂ population. A total of 1,200 random primers from OPA to OPBH primer sets (Operon Technologies, Alameda, CA) and 800 primers from UBC001 to 800 primer sets (University of British Columbia, Vancouver, BC, Canada) were screened. RAPD reactions were performed as described by Zhang et al. (2000). The genomic DNA of the parent cultivars were screened with simple sequence repeat (SSR) and sequence tagged sites (STS) markers (Lee et al., 2009; Mimura et al., 2012; Minamiyama et al., 2006). RAPD and other marker genotyping data were analyzed using CarthaGene software (Schiex and Gaspin., 1997).

Results and Discussion

Resistance Responses to CMV_{P1} of *C. annuum*

Cucumber mosaic virus is a major viral disease in Korea, their sequence variations, virulence and survey of resistant germplasm were rarely studied. A new strain CMV_{P1} infecting resistant cultivars to CMV_{Fny} and CMV_{P0} was reported, and belonged to subgroup IB different to CMV_{Fny} (subgroup IA) from movement protein (MP) and coat protein (CP) sequence analysis (Lee et al., 2006).

From CMV_{P1} inoculation, all of inoculated 'Yolo Wonder' plants were susceptible to CMV_{P1} strain, indicated that the CMV_{P1} screening method is reliable and consistent. Both vein clearing mosaic and leaf distortion symptoms were observed at 15 dpi and expanded to systemically.

One accession of CMV _{P1} resistant accession ICPN18-8 showed no visual symptoms at 15 dpi, only detected by ELISA test, finally showed delayed symptoms after 45 dpi. All 'Bu-hong' plants were susceptible to the CMV _{P1} strain, indicated that the CMV _{P1} strain can overcome the CMV resistance in 'Bu-hong' plants. CMV _{P1} symptoms were appeared on the leaves of 'E1338' plants at 15 dpi, but no visible symptoms were observed in 'I7339' plants. The result of ELISA test, CMV was detected only in the upper leaves of 'E1338' plants, not in the upper leaves of 'I7339' plants at 15 dpi (Table 1).

Times for virus resistance test of plant breeding materials will be different for each virus types, but usually started at cotyledon stages or one pair of true leaves completed 15 to 20 dpi.

Changes in resistance through growth stages of host plant had been rarely reported. In *C. annuum*, Tobacco Mosaic Virus (TMV)-induced gene (*CaTin2*) encodes a mature 23 kDa protein that shares little sequence similarity with other cell wall proteins, fluorescently-tagged CaTin2 appeared to localize and in common with other cell wall proteins (Shin et al., 2003). *CaTin2* was usually expressed in leaves and roots, constitutive expression of *CaTin2* in transgenic tobacco resulted in some resistance against TMV or CMV, between 7 and 14 dpi after inoculation, but the resistance was lost one month after inoculation, the expression of the CaTin2 cell wall protein probably restricted virus movement and accumulation (Shin et al., 2003).

Inheritance of Resistance to CMV _{P1} Strain in *C. annuum* 'I7339'

In the F₂ population, a total of 189 plants were examined for CMV _{P1} resistance in two times, first at 15 dpi and

second at 45 dpi. Of these, 42 individuals showed resistance, 147 plants showed susceptible from ELISA test at the first test (15 dpi). The phenotype segregation ratio was closed to the 1:3 ratio (resistance : susceptible plants), indicating single recessive gene inheritance pattern ($\chi^2 = 0.002, P = 0.967$). In the lateral ELISA test at 45 dpi, 11 plants showed resistance, 178 plants showed susceptible, the phenotype segregation ratio was close to the 1:15 (resistance: susceptibility) ($\chi^2 = 0.060, P = 0.807$) (Table 1). A total of 31 resistant plants at 15 dpi became susceptible at 45 dpi. These results indicated that the resistance in *C. annuum* 'I7339' is controlled by two different genes with a recessive manner. We named these resistance genes '*cmr3E*' and '*cmr3L*' respectively.

Studies for the resistance to CMV in *C. annuum* have been reported many research groups, most of them were incomplete dominant and quantitatively inherited (Caranta et al., 1997, 2002; Grube et al., 2000), except for *Cmr1* (Kang et al., 2010). *Cmr1* on the LG2 is the only dominant resistance gene known in pepper, but resistance is broken down by CMV _{P1} strain, requiring more robust CMV resistant germplasm (Kang et al., 2010). Recently, single recessive gene '*cmr2*' resistant to CMV _{P1} was reported in pepper, located on chromosome 1 (Park et al., 2011). The resistance was controlled at the level of virus replication, different to '*Cmr1*' which involves inhibition of the systemic movement of CMV (Kang et al., 2010).

In this study, virus infection was tested two times, 15 dpi and 45 dpi. Firstly, 47 plants showed no visible symptoms and negative in ELISA test at 15 dpi, 36 individuals become susceptible at 45 dpi. These results indicated that two different mechanisms might be involved for the resistance to CMV _{P1} - one gene for suppression in cellular level of

Table 1. Resistance responses and segregation analysis of *C. annuum* to CMV _{P1} strain.

Population	Observed ratio (R:S)		χ^2 (P)		ELISA (Absorbance 405 nm)		
	15 dpi	45 dpi	15 dpi	45 dpi	Non-inoculated	Inoculated	
						15 dpi ^z	45 dpi
Yolo Wonder	0:20	0:20	-	-	0.299	1.625	1.636
ICPN-18-8	0:10	0:10	-	-	-	0.673	1.581
I7339	17:0	17:0	-	-	0.217	0.246	0.289
E1338	0:20	0:20	-	-	0.25	1.477	1.629
EI F ₁	0:15	0:15	-	-	-	0.636	1.623
EI F ₂	47:142	11:178	0.002 (0.967)	0.060 (0.807)	-	-	-

^zDays post inoculation.



Fig. 1. Phenotypic differences of CMV P_1 strain in susceptible plant ICPN-18-8 (A) and resistance plant '17339' (B) at 45 days after inoculation. Susceptible plant showed vein clearing mosaic and leaf distortion symptoms.

virus replication (*cmr3E*), the other for inhibition of long distance movement of CMV (*cmr3L*). Because CMV inoculated plants of $E_1 F_2$ population were transplanted to vinyl greenhouse at 30 dpi, gene for suppressing CMV replication (*cmr3E*) could be affected by environmental conditions such as high temperature.

Linkage Analysis of the CMV P_1 Resistance Loci

To locate *cmr3E* and *cmr3L* loci in pepper linkage map, total 2,000 RAPD primers, 115 SSR markers and 37 STS markers were screened using two parental lines, then polymorphic markers were test in the resistant and susceptible bulks of $E_1 F_2$ population, only 9 primers were showed polymorphisms between bulked plants and mapped (3.6%). Because two parents of $E_1 F_2$ population were breeding lines they shared low polymorphisms compared to wild types. One linkage group was constructed containing 9 markers (Fig. 2). It shared one common marker (CAMS352-2) with LG4 in the map of Minamiyama et al. (2006), other SSR markers were monomorphic between parents used int this study. Only one RAPD primer OPAT16 (5'-CTCTCCGTAG-3') was distantly linked with *cmr3E* (22.3 cM) and *cmr3L* (20.7 cM). Two loci for resistance to CMV P_1 were located on the LG4 in this study, different to the *cmr2* on chromosome 1 in previous study (Park et al., 2011), due to differ in genetic resources. Therefore, it is supposed that the gene required for inhibition of the systemic movement of CMV are absent in 36 plants, resulting in susceptibility. In the RNA3 sequence analysis for P_0 resistant-breaking isolate (Ca-P1-CMV), the same CMV isolate used in this study, revealed Ca-P1-CMV belongs to CMV subgroup IB whereas

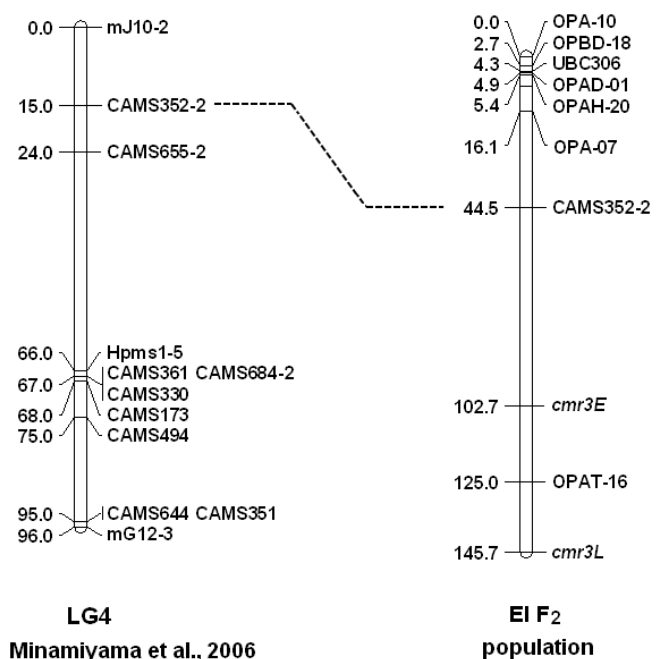


Fig. 2. The linkage map showing genetic distances of the pepper CMV P_1 resistance loci and molecular markers from $E_1 F_2$ population compared with LG4 in Minamiyama et al. (2006). Markers on the LG4 are partially shown here. One common marker CAMS352-2 was mapped in $E_1 F_2$ map. The marker names and distances (Kosambi, cM) are presented on the right and left sides of the central bar, dotted line indicate common marker.

the ordinary strain (Fny-CMV) group to subgroup IA from movement protein (MP) and coat protein (CP) comparison (Lee et al., 2006). They also suggested the possibility of

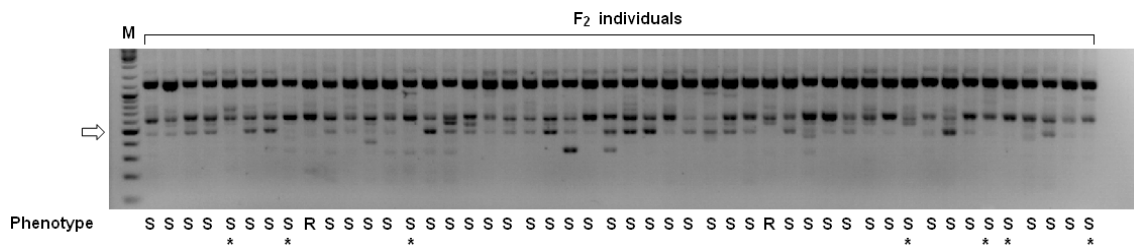


Fig. 3. Amplification pattern of part of individuals from EI F₂ population with OPAT-16. M 1kb plus ladder. Arrow indicates susceptible plant specific band, star-marks denote individuals showed inconsistent results when compared phenotype and marker result.

changes in the MP and CP sequences resulting in different responses between pepper and virus. Similar response was found in the 'Bu-hong' cultivar (resistant to CMV_{Fny}), it showed susceptible symptoms after inoculated with CMV_{P1}. Further research remains for the comparison of genes for resistance to different CMV isolates in different pepper germplasms.

One distantly linked marker OPAT16 is insufficient for marker assisted selection of breeding lines or germplasms, requiring more closely linked markers. To narrow down and delimit loci, AFLP analysis and diverse approaches using pepper genome sequences being performed, further experiments will be needed to clarify genetic and inheritance of genes for resistance to diverse CMV strains including CMV_{P1}.

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