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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Investigation of Virus Resistance Spectrum and Fine Mapping of the Potyvirus Resistance Gene Pvr7 in Capsicum annuum

고추에서 포티바이러스 저항성 유전자인 Pvr7의 바이러스 저항성 범위 조사 및 미세 유전자 지도작성

FEBRUARY, 2015

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MAJOR IN HORTICULTURAL SCIENCE
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY
Investigation of Virus Resistance Spectrum and Fine Mapping of the Potyvirus Resistance Gene

_Pvr7_ in _Capsicum annuum_

UNDER THE DIRECTION OF DR. BYOUNG-CHEORL KANG
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
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Investigation of Virus Resistance Spectrum and Fine Mapping of the Potyvirus Resistance Gene *Pvr7* in *Capsicum annuum*

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ABSTRACTS

*Pepper mottle virus* (PepMoV) is a frequently occurring virus in pepper field. PepMoV infected plants show symptoms including mosaic leaf, distortion of foliage and fruit deformation. Previous research revealed that *Pvr7* is located on chromosome 10 and linked to the dominant potyvirus resistance gene *Pvr4* and
*Tomato spotted wilt virus* (TSWV) resistance gene *Tsw*. Virus resistance test showed that *Capsicum annuum* ‘9093’ harboring *Pvr7* was resistant to PepMoV but susceptible to TSWV, *Tobacco etch virus* (TEV), and *Chilli veinal mottle virus* (ChiVMV). To develop a high resolution map of the *Pvr7* locus, an intraspecific F2 mapping population was constructed by crossing *C. annuum* ‘9093’ (PepMoV resistant) and *C. annuum* ‘Jeju’ (PepMoV susceptible).

A total of 916 of F2 plants were screened with green fluorescent protein (GFP) tagged PepMoV. For genotyping, a forty-four of randomly distributed single nucleotide polymorphism (SNP) markers located in chromosome 10 were tested to conform polymorphism among ‘9093’, ‘Jeju’ and ‘9093 x Jeju’ F1. In sequence, 4 SNP markers were selected and tested to F2 mapping population.

To define target region of *Pvr7*, *C. annuum* ‘CM334’ whole genome sequence (WGS) was used to develop SNP markers. Consequently, a four of SNP markers were developed additionally. Comparing phenotype and genotype data, eight recombinants were selected. A three of SNP markers were co-segregated with *Pvr7* exactly. These results indicated that the target region of *Pvr7* gene is located within 258kb. Using ‘CM334’ reference sequence, a nine of NBARC type genes were predicted in this region. We are expecting that 0 cM of *Pvr7* SNP markers can be used to breeding PepMoV resistant cultivars and fine mapping of *Pvr7*.

Keywords: potyvirus, *Pvr7*, *Pepper mottle virus* (PepMoV), dominant
resistance, marker development, single nucleotide polymorphism (SNP)

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<td>BC</td>
<td>Backcrossing</td>
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<tr>
<td>CAPS</td>
<td>Cleaved amplified polymorphic sequences</td>
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<tr>
<td>CP</td>
<td>Coat protein</td>
</tr>
<tr>
<td>ELSIA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>NB-LRR</td>
<td>Nucleotide binding-leucine rich repeat</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>WGS</td>
<td>Whole genome sequence</td>
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INTRODUCTION

PepMoV infects pepper, and is belonged to Potyvirus genus (Abdalla et al., 1991; Dogimont et al., 1996). Potyviruses are the largest group of viruses infecting plants (Valkonen et al., 1996) and transmitted by aphids. PepMoV infected plants show symptoms such as mosaic and dark green vein banding crinkled leaves, and plants become stunted (Kim et al., 2010). PepMoV is prevalent in Northern America and causes serious economical losses in Korea as well (Moury et al., 2005, Kim et al., 2009).

In Korea, there are four major pepper viruses causing the significant economical losses including Cucumber mosaic virus (CMV), Broad bean wilt virus (BBWV), Pepper mild mottle virus (PMMoV) and Pepper mottle virus (PepMoV) (Kim et al., 2010). The incidence of CMV, BBWV, PMMoV and PepMoV was 29.4%, 25.6%, 14.3% and 13.6% respectively (Cho et al., 2007). In the case of PepMoV, yield of infected peppers were decreased upto 64.4% (Kim et al., 2010).

In pepper, seven potyvirus resistance genes have been reported (Caranta et al., 1996; Lee et al., 2013; Grube et al., 2000; Kyle and Palloix 1997) and several resistance germplasms controlling different potyviruses have been found in Capsicum. The pvr1 renders resistance to TEV-HAT, Potato virus Y (PVY(0)), PepMoV and this resistace gene is originated from C.chinense ‘PI 159236’ and ‘PI152225’ (Kyle and palloix 1997; Murphy et al., 1998; Yeam et al., 2005). The
$pvr2$ ($pvr2^l = pvr1^l$) gives resistance to PVY and was found in *C. annuum* Yolo Y (Kang et al., 2005; Ruffel et al., 2002; Yeam et al., 2005). The $pvr3$ is resistant to PepMoV and this gene was reported in *C. annuum* ‘Avelar’ (Guerini and Murphy 1999; Murphy and Kyle 1995; Parrella et al., 2002). The $Pvr4$ is resistant to PepMoV and originated from *C. annuum* ‘Criollo de Morelos 334’ (CM334) (Caranta et al., 1999; Grube et al., 2000). The $pvr6$ is resistant to ChiVMV and this gene was reported *C. annuum* ‘Perennial’ (Caranta et al., 1996; Ruffel et al., 2006). The $pvr7$ is resistant to PepMoV and originated in ‘PI 159236’ and *C. annuum* ‘9093’ (Grube et al., 2000).

A higher frequency of recessive genes have been observed in resistance genes in *Capsicum* spp. In contrast, $Pvr4$ and $Pvr7$ genes show a dominant inheritance pattern. These two genes are located very closely on the lower arm of chromosome 10 but they are not allelic (Grube et al., 2000). Another virus resistance gene $Tsw$ giving resistance to *Tomato spotted wilt virus* (TSWV) is also located around the loci (Grube et al., 2000). The $Pvr7$ in ‘9093’ and was derived from three times backcrossing of a bell type *C. annuum* line with *C.chinense* ‘PI 159236’. Although the previous research showed that $Pvr7$ is located on chromosome 10, the precise location of $Pvr7$ in the pepper genome and the gene encoding $Pvr7$ have not been identified. The isolation of the $Pvr7$ gene promises to broaden our understanding of the molecular factors that have dominant inheritance mechanism and breed PepMoV resistant varieties more quickly.

In this study, the $Pvr7$ gene was characterized and an attempt was made to
A fine map $Pvr7$ and a candidate genes for $Pvr7$ were identified. Towards this end, virus the resistance spectrum of $Pvr7$ was investigated using three other viruses, TSWV, TEV, ChiVMV. In order to identify the target region of $Pvr7$, single nucleotide polymorphism (SNP) markers were developed and a phisical map around the $Pvr7$ locus was constructed.
LITERATURE REVIEW

1. The pathogen resistances in plant

Contrary to mammals, both mobile defender cells and somatic adaptive immune systems do not exist in plants. Instead, they defend on the innate immunity in each cell and resistant processes are progressed by detect systemic signals from infection sites (Ausubel 2005; Chisholm et al., 2006).

Innate immunity is an ability to defend plant against molecules derived from pathogen when plants are confront with infection. There is a two-way innate immune system. First is that uses one of the primitive part of the immune system which called pattern recognition receptors in transmembrane (PRRs, Parker 2003). These PRRs can recognize the pathogen-associated molecular patterns (PAMPs), such as flagellin (Gomez-Gomez and Boller 2000; Zipfel and Felix 2005) or avirulence factors (Liu et al., 2002). When PAMPs are recognized by PRRs, resistance mechanisms are activated at infected area, this called PAMP-triggered immunity (PTI). In case of pathogen, to survive against from PTI, release effectors that assist to pathogen virulence. Because effectors can interrupt to PTI, pathogens carrying effective effectors can break the plant resistance. This called effector-triggered susceptibility (ETS; Lapin and Van den Ackerveken 2013).

The second is that acts broadly inside the cell, using the NB-LRR protein
which is named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains and these products encoded by most resistance (R) genes (Dangl and Jones 2001). These NB-LRR proteins have ability to recognize molecules derived from pathogen. Counter mechanisms of ETS, some plants have evolved R genes for detecting effectors from pathogen. These R genes can either indirect, or through direct NB-LRR recognition of an effector (Jones and Dangl 2006). This mechanism we called effector-triggered immunity (ETI).

ETI is advanced disease resistance in plant kingdom and accompanied by a hypersensitive response (HR) at the infected site generally, such as cell death (Morel and Dangl 1997). Because NBLRR-mediated disease resistance is effective against pathogens, identifying NB-LRR genes and characterization of resistance mechanisms related to NB-LRRs are essential.

1.1 NB-LRR genes in plants

In plants, at least five distinct classes of R genes are reported (Ooijen et al., 2007). One of the most typical R-gene is the members of the gene family that code for proteins containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs; Dangl and Jones 2001). These NBS-LRR genes are one of the most numerous and ancient gene families in plants (Marone et al., 2013) and NB-LRR proteins are also called NLR, NBS-LRR or NB-ARC-LRR, and classified into the TIR or non-TIR class, rely on the sequences that prior to the NB domain, as well as
motifs within this domain (Meyers et al., 2005).

The TIR group of plant NB-LRR proteins (TNLs) are consist of a Toll, interleukin-1 receptor, R protein homology (TIR). This TIR protein is interacting with the NB-LRR domain and localize in front of the domain (Andolfo et al., 2014). In case of non-TIR group (CNLs) is less defined compare with TNLs, but some members of this group consist of helical coiled-coil–like (CC) sequences in front of the domain (Heath 2000).

Interestingly, most plant species have more CNLs than TNLs (Jacob et al., 2013). Because of mechanisms like gene duplication, gene conversion, ectopic recombination according to unequal crossing over and diversifying selections in NB-LRR genes, a number of NB-LRR gene clusters have emerged and resistance specificities are also formed (Michelmore and Meyers 1998; Young 2000; Sun et al., 2001). These results indicate that CNLs are more ancient type compare with TNLs in evolutionary perspectives.

Plant breeders rely on R genes in order to breed varieties for disease resistance. To date, several NB-LRR type R genes have been cloned from tomato, potato and pepper, and are used in current breeding programs (Andolfo et al., 2014). Mostly, NB-LRR genes are clustered and many cases have shown co-localized. This allows the identification of resistance genes and the development of molecular markers linked to resistance genes more easy (Marone et al., 2013).
2. Potyvirus resistance genes in pepper

The relationships among potyvirus resistance genes and the resistance spectrum of each gene have been abstruse to characterize because potyvirus resistance is co-segregate with two or more related potyviruses, such as *Tobacco etch virus* (TEV), *Pepper mottle virus* (PepMoV) and *Potato virus Y* (PVY) in pepper. The resistance germplasm and symbols are also poorly characterized and genetic background insufficient (Caranta et al., 1997; Deom et al., 1997; Murphy et al., 1998). To unify the resistance symbols, Kyle and Palloix re-investigate the resistance alleles linked to germplasm and virus (Kyle and Palloix 1997). Consequently, they construct new symbols to either $pvr$ or $Pvr$ toward 5 potyvirus resistance genes. Afterward, three different resistance gene, $pvr5$, $pvr6$ and $Pvr7$ are reported (Caranta et al., 1996; Grube et al., 2000; Parrella et al., 2002; Ruffel et al., 2006).

Up to date, seven potyvirus resistance loci have been reported in the pepper (Lee et al., 2013). Except for dominant resistance genes $Pvr4$ and $Pvr7$, recessive potyvirus resistance genes are related to interact with host-factor and viral genome-linked proteins. In general, these recessive resistances are known as translation elongation factors, such as eIF4E (Hwang et al., 2009; Kang et al., 2005).

2.1 The recessive potyvirus resistance genes
The \textit{pvr1} is located in pepper chromosome 3 and this gene was identified to be identical to the \textit{pvr2} locus (Caranta et al., 1996; Ruffel et al., 2002), which is resistant to TEV strains, PepMoV, PVY (Kang et al., 2005). This gene locus was described in ‘PI 152225’ and ‘PI 159236’ (Kyle and Palloix 1997). In case of \textit{pvr3}, resistance source is \textit{C. annuum} ‘Avelar’ and this gene is resistant to PepMoV but locus is not determined yet (Parrella et al., 2002). Although \textit{pvr3} is recessive, this gene is different allele to \textit{pvr1} (Murphy et al., 1998). The \textit{pvr5} is located in chromosome 3. According to former researches (Parrella et al., 2002; Yeam et al., 2005), \textit{pvr5} also inferred to be another allele of \textit{pvr1} and resistance source is not determined. Another recessive gene is \textit{pvr6}. This gene is located in chromosome 9. The \textit{pvr6} is resistant to ChiVMV which is another potyvirus. The ChiVMV-resistant germplasm is \textit{C. annuum} ‘Perennial’ and this germplasm has been known as carrying \textit{pvr6} (Caranta et al., 1996; Ruffel et al., 2006).

\section*{2.2 The dominant potyvirus resistance genes}

Most potyvirus resistance genes are recessive, whereas \textit{Pvr4} and \textit{Pvr7} have a single dominant resistance mechanism (Grube et al., 2000). The \textit{Pvr4} gene was identified in \textit{C. annuum} ‘CM334’ (Caranta et al., 1997). ‘CM334’ is a semi-domesticated hot pepper originating in Mexico (Sala et al., 2004). This pepper is broadly used for either research or breeding because it possesses amount of
pathogen resistance genes (Janzac et al., 2009). Such as resistance genes of *Phytophthora capsici* (Sala et al., 2004) and root-knot nematode (Djian-Caporalino et al., 2007). The *Pvr4* confers a complete resistance to the three pathotypes of PVY and to PepMoV and was located in pepper chromosome 10 (Grube et al., 2000; Janzac et al. 2009).

Grube et al. (2000) reported that the *Pvr7* was identified in ‘PI 159236’ and *C. annuum* ‘9093’. Because ‘PI 159236’ is carries *pvr1* and *Pvr7*, ‘9093’ is developed from backcrossing ‘PI159236’ with *C. annuum* recurrent parent which is bell type and selected from PepMoV-resistant BC3F3 line. The *Pvr7* confers resistance to PVY and PepMoV. In common with *Pvr4*, *Pvr7* also located in pepper chromosome 10.
MATERIALS AND METHODS

Plant materials and mapping population

A total of five Capsicum accessions were used in this study including C. chinense ‘PI 159236’, C. chinense ‘PI 152225’, C. annuum ‘ECW’, C. annuum ‘9093’, and C. annuum ‘Jeju’. ‘PI 159236’ and ‘PI 152225’ are known resistance sources for PepMoV (Grube et al., 2000) and ‘ECW’ is susceptible to PepMoV (Kim et al., 2011).

To perform a genetic analysis and fine mapping of the PepMoV resistance gene Pvr7, F1 and F2 populations were obtained by a cross between C. annuum ‘9093’ (resistant to PepMoV) and C. annuum ‘Jeju’ (susceptible to PepMoV). One thousand F2 seeds were obtained from 10 individuals of ‘9093 x Jeju’ F1.

In observation of PepMoV infection, ‘PI 159236’, ‘9093’ ‘9093 x Jeju’ F1, ‘Jeju’ and ‘ECW’ were used. To investigate virus resistance spectrum of Pvr7, ‘PI 159236’, ‘PI 152225’, ‘9093’, ‘Jeju’ and 9093 x Jeju F1 plants were used and inoculated with Tomato spotted wilt virus (TSWV), Tobacco etch virus (TEV) and Chilli veinal mottle virus (ChiVMV).

Each pepper seeds were soaked in a solution of 10% disodium phosphate and 2% Clorox for 20 min and 10 min respectively. The germinated seeds were sown in plastic trays (50 cells) and grown in the growth chamber.
Inoculum preparation and plant inoculation

To inoculate pepper plants, a total of 4 frozen virus stocks (GFP tagged PepMoV strain Vb1, TEV strain NW, ChiVMV and TSWV strain Pap) were used. These frozen stocks were stored at -80℃.

PepMoV, TEV and ChiVMV were maintained in *Nicotiana benthamiana* plants. TSWV was maintained in *N. rustica* plants. Infected tobacco tissue was ground in chilled 0.1 M potassium phosphate buffer, pH 7.0 (approximately 1g tissue: 5ml buffer), mixed with 400-grit carborundum, and rubbed manually on two youngest fully expanded tobacco leaves and washed after 20 minute with distilled water.

In the case of pepper plants, the first or second pair of leaves on 3- to 4-week-old plants were inoculated. After inoculation, plants were grown in the growth chamber conditions (16-h light, 21℃ night and 23℃ day with fluorescent lighting). Infected pepper plants were checked for viral symptoms up to 21 days post-inoculation (dpi). ELISA tests were performed for PepMoV, TSWV, TEV and ChiVMV.

Double-antibody sandwich enzyme-linked immunosorbent (DAS-ELISA) assay

At 7, 8, 13 and 14 days after inoculation, the viral coat protein (CP) of TSWV,
TEV, PepMoV and ChiVMV were detected by an enzyme-linked immunosorbent assay (ELISA) respectively. ELISA was conducted in accordance with the manufacturer’s protocol (Agdia, Elkhart, IN, USA).

The samples were taken from inoculated and non-inoculated upper leaves. In each samples, were used for ELISA analysis. Each sample was measured at an absorbance value of 405 nm in an ELISA reader (Anthon zenith 340 micro plate reader, UK). The evaluations were repeated three times.

**Monitoring infection of PepMoV tagged with GFP**

PepMoV constructs expressing the green fluorescent protein (GFP) was used to monitor the systemic spread of PepMoV. GFP tagged PepMoV was provided by Prof. Choi (Horticulture Crop Genomics Lab., Seoul National University). In infected plants, GFP was visualized with a Multidisc-it Digital Imaging System using a SYBR Green filter (bandpass 515–570 nm; UVP) or a GFP filter (excitation 470/40 nm; emission 525/50 nm) (UVP, http://www.uvp.com). After 14 dpi, resistance and susceptibility could be distinguished in 916 plants of ‘9093 x Jeju’ F2 population using Multidisc-it Digital Imaging System.

**Genomic DNA extraction**

Total DNA was obtained from fully expanded cotyledon tissues of the each plant using modified cetyltrimethylammonium bromide (CTAB) method (Doyle and
Doyle 1987) in a 1.5ml micro tube.

After DNA extraction, each sample was moved to 96 well plates. DNA concentrations were measured by NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE, USA). DNA was diluted with 0.1 M TE buffer (PH 7.0) to final concentration of 10 ng/μl for SNP marker analysis. DNA samples were stored at -20°C prior to use.

**Pepper genome information and development of SNP markers**

Out of 44 SNP markers located randomly in chromosome 10, we obtained 4 polymorphic SNP markers (SNP-005, SNP-006, SNP-007 and SNP-008) co-segregating with the PepMoV resistance phenotype of F₂ plants and PCR primers were designed based on sequences around the four polymorphic markers. To obtain sequences the PCR products, amplicons were purified with a Zymoclean PCR purification Kit following the manufacturer’s protocol (Invitrogen Korea, Seoul, Korea). Sequences were determined by National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Korea), and primers were redesigned to develop SNP markers. To design the SNP markers, reference pepper genome sequence, *C. annuum* ‘CM334’ was used (Kim et al., 2014). Designed primer sets were tested polymorphism and four additional SNP
markers (SNP-001, SNP-002, SNP-003 and SNP-004) were developed (table 2). Selected polymorphic SNP markers were used to determine genotype of 916 F2 plants.

**Genotype analysis using high resolution melting (HRM) analysis**

Eight SNP markers (formerly developed four SNP markers and newly developed four SNP markers) were used for genotyping 916 F2 individuals. Linkage with marker and phenotype were analyzed by comparing marker genotype and resistance phenotype. Genotype was determined by HRM analysis using Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia).

Eight SNP markers linked to Pvr7 was used to identify a corresponding pepper genome scaffold sequence. Scaffold A were obtained from *C. annuum* 1.5 scaffold version of pepper genome database (http://cab.pepper.snu.ac.kr), by BLASTN search program.
RESULTS

PepMoV resistance in ‘9093’

To test resistance of ‘9093’, we inoculated PepMoV-Vb1 (GFP) on the first or second pair of true leaves of ‘PI 159236’, ‘9093’, ‘9093 X Jeju’ F₁ ‘Jeju’ and ‘ECW’ with. Ten individual plants were tested for each accession. Red arrows in the pictures indicate the inoculated leaves with PepMoV.

By 10 dpi, the susceptible control ‘Jeju’ and ‘ECW’ uniformly developed bright systemic mosaic symptoms characteristics of PepMoV on newly emerged uninoculated and inoculated leaves, and no symptoms were observed in the controls ‘PI 159236’, ‘9093’ and ‘9093 X Jeju’ F₁ (Fig. 1A).

At 13 dpi, systemic virus spreading of the GFP tagged PepMoV was also confirmed in ‘Jeju’ and ‘ECW’ whereas no GFP signals both inoculated and systemic uninoculated leaves in ‘PI 159236’, ‘9093’ and F₁ (Fig. 1A). This was confirmed in ELISA test, inoculated and uninoculated upper leaves of ‘Jeju’ and ‘ECW’, viral coat protein were highly detected (Fig. 1B) whereas viral coat protein was never detected in both inoculated and uninoculated tissue of ‘9093’, ‘PI 159236’ and ‘9093xJeju’ F₁ (Fig. 1B). These results confirmed that ‘9093’ and ‘9093xJeju’ F₁ are resistant to PepMoV.
**Fig. 1.** GFP signals in PepMoV infected plants and ELISA at 13 days post inoculation. Resistant plants are ‘PI 159236’, ‘9093’ and ‘9093xJeju’ F₁ and susceptible plants are Jeju and ECW. (A) Red arrows indicate inoculated leaves. Resistant plants did not show GFP signal in both inoculated and uninoculated upper leaves. Susceptible plants showed GFP signal in the both leaves. (B) In ELISA results, *error bars* represent standard deviation above the mean absorbance value. Each experiment was repeated 3 times.
Resistance spectrum of Pvr7 in ‘9093’

1.1 Resistance response to TSWV

In the previous research, it was reported that ‘PI 159236’ carries TSWV resistance gene Tsw (Grube et al., 2000). Because ‘9093’ was derived from ‘PI 159236’, we tested whether ‘9093’ carries the Tsw as well as Pvr7. Because Tsw and Pvr7 are tightly linked, we expected that ‘9093’ is also resistant to TSWV. ‘PI 159236’, ‘PI 152225’, ‘Jeju’ and ‘9093 x Jeju’ F1 were used to test TSWV resistance.

By 5 dpi, the resistant line ‘PI 159236’ and ‘PI 152225’ showed hypersensitive response (HR) on the inoculated leaves (data not shown), whereas no HR response was observed in inoculated leaves of other lines. At 8 dpi, except for resistant plants, 9093, Jeju and 9093xJeju F1 showed typical TSWV symptoms in uninoculated upper leaves (Fig. 2A). ELISA results (Fig. 2B) also confirmed that viral coat protein (CP) did not accumulate in resistant plants, but CP was highly accumulated in susceptible plants. Altogether, it was concluded that ‘9093’ derived from ‘PI 159236’ only carries Tsw and not Pvr7.

1.2 Resistance response to TEV

_Tobacco etch virus_ (TEV) is belonged to the potyvirus genus and _C. chinense_
'PI 159236' and ‘PI 152225’ are well known resistance sources for TEV. These accessions carry the recessive resistance gene \textit{pvr1} located on chromosome 3. Because ‘9093’ was derived from ‘PI 159236’, ‘9093’ may also carry \textit{pvr1}.

To investigate whether ‘9093’ is carrying the \textit{pvr1}, ‘9093’ and control plants were inoculated with TEV as the same manner as TSWV inoculation (Fig. 2). For each accession, 10 plants were inoculated. By 5 dpi, the resistant line ‘PI 159236’ and ‘PI 152225’ shown to any virus symptoms in inoculated and upper leaves (Fig. 2A) and by 7 dpi, other plants were not shown to any HR or abscission in the inoculated leaves. At 7 dpi, except for resistant plants, ‘9093’, ‘9093xJeju’ F\textsubscript{1} and ‘Jeju’ showed complete TEV symptoms in uninoculated upper leaves (Fig. 2A). ELISA results (Fig. 2B) also indicated that CP was not accumulated both inoculated and uninoculated upper leaves in resistant plants, whereas susceptible plants, CP was highly accumulated. These results demonstrate that ‘9093’ is susceptible to TEV and does not carry \textit{pvr1}. 
1.3 Resistance response to ChiVMV

*Chilli veinal mottle virus* (ChiVMV) is also belonged to the potyvirus genus. Because *Pvr7* is a potyvirus resistance gene, we investigated the resistance response to ChiVMV. By 14 dpi, no HR response was observed in 9093 and other control plants including ‘PI 159236’, ‘PI 152225’, ‘Jeju’ and ‘9093 x Jeju’ F\textsubscript{1} (Fig. 2A). At this stage, all the tested plants showed typical ChiVMV symptoms in both inoculated and uninoculated upper leaves (Fig. 2A). ELISA results (Fig. 2B) also indicated that inoculated plants accumulated viral CP. These results demonstrate that ‘9093’ carrying *Pvr7* is susceptible to ChiVMV.

In summary, ‘9093’ susceptible to all three tested viruses and only carries *Pvr7* which is effective only to PepMoV (Table 1).
A

TSWV
PI 159236
PI 152225
9093
Jeju
9093 x Jeju F₁
8 dpi

TEV

ChiVMV

14 dpi
Fig. 2. Symptoms and virus accumulation of TSWV, TEV and ChiVMV at 8, 7 and 14 days post inoculation. *Capsicum* spp including ‘PI 159236’, ‘PI 152225’, ‘9093’, ‘Jeju’, ‘9093 x Jeju’ F₁ were inoculated on the first two true leaves. (A) Symptoms of three viruses in plants. In case of TSWV and TEV, ‘PI 159236’ and ‘PI 152225’ showed resistant phenotype. Susceptible plants showed typical virus symptoms in uninoculated upper leaves. In contrast to TSWV and TEV infection, ‘PI 159236’ and ‘PI 152225’ showed typical CVMV symptoms in uninoculated upper leaves. (B) ELISA results at 8, 7 and 14 dpi respectively. PC=positive control, NC=negative control
Table 1. Summary of resistance response of 9093 to three viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Pepper accession</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>‘PI 159236’</td>
<td>‘PI 152225’</td>
<td>‘9093’</td>
<td>‘Jeju’</td>
</tr>
<tr>
<td>TSWV</td>
<td>R (10/10)*</td>
<td>R (10/10)</td>
<td>S (0/10)</td>
<td>S (0/10)</td>
</tr>
<tr>
<td>TEV</td>
<td>R (10/10)</td>
<td>R (10/10)</td>
<td>S (0/10)</td>
<td>S (0/10)</td>
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<tr>
<td>ChiVMV</td>
<td>S (0/10)</td>
<td>S (0/10)</td>
<td>S (0/10)</td>
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</tr>
</tbody>
</table>

*Resistant plants/total number of plants
Resistance screening of F2 mapping population

In order to determine resistance phenotype of F2 plants, a total of 1,000 F2 seeds were sown and 916 plants germinated. After sampling the one cotyledon per each line for DNA extraction, we screened 916 F2 individuals with PepMoV together with control plants. ‘PI 159236’ and ‘9093’ is shown to any virus symptoms in inoculated and upper leaves. Except for ‘9093 x Jeju’ 10 plants per each control were inoculated. After 13 dpi, resistance phenotypes of 916 F2 individuals were evaluated by Multidisc-it digital imaging system. In case of ambiguous phenotypes, ELISA test was performed at 13 dpi. After scoring the resistance phenotype, it turned out that 688 of F2 individuals were resistant and 288 of F2 individuals were susceptible to PepMoV (Table 2). This segregation ratio of resistance confirmed that the \( P_{vr7} \) gene in ‘9093’ is controlled by a single dominant gene (Table 2).
<table>
<thead>
<tr>
<th>Parent lines and population</th>
<th>Number of plants</th>
<th>Expected ratio (R:S)</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Total</td>
<td>Resistant (R)</td>
<td>Susceptible (S)</td>
<td></td>
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<tr>
<td>‘Jeju’</td>
<td>10</td>
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<td>0:1</td>
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<td>10</td>
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<td>10</td>
<td>0:1</td>
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<td>‘PI 159236’</td>
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<td>0</td>
<td>1:0</td>
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<td>‘9093’</td>
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<td>10</td>
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<td>1:0</td>
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<td>‘9093 x Jeju’ ($F_1$)</td>
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<td>5</td>
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<tr>
<td>‘9093 x Jeju’ ($F_2$)</td>
<td>916</td>
<td>688</td>
<td>228</td>
<td>3:1</td>
</tr>
</tbody>
</table>

Table 2. Inheritance of PepMoV resistance in ‘9093 x Jeju’ $F_2$ population
Development of HRM markers based on genomic information of CM334

To delimit the Pvr7 region, we randomly select 44 SNP markers located on pepper chromosome 10. Four of 44 markers showed polymorphisms in HRM melting curves between two parental lines distinguishing resistance and susceptibility. HRM analysis revealed that four selected markers, SNP-005, SNP-006, SNP-007 and SNP-008, were linked to the Pvr7 gene. These 4 SNP markers are co-dominant markers.

We then tried to add more markers around the Pvr7 locus using genomic information of Capsicum. We designed 10 primer sets around the Pvr7 region in chromosome 10 based on the CM334 genome sequence (Kim et al., 2014). Of the 10 primer sets tested for 9093, Jeju and 9093xJeju F1, four sets of primers SNP-001, SNP-002, SNP-003 and SNP-004, showed clear polymorphic HRM curves (Fig. 3). Consequently, four additional polymorphic co-dominant markers were developed (Fig. 3). These polymorphic markers were used for genotype scoring for the F2 individual plants (Table 3, 4). Among the eight SNP markers, three markers (SNP-004, SNP-005 and SNP-006) perfectly co-segregated with phenotype data (Fig. 4, Table 3). Of 916 of ‘9093 x Jeju’ F2 plants the number of homozygous resistant type (RR), heterozygous resistant type (Rr) and homozygous susceptible type (rr) is 215, 473 and 228 respectively (Table 4). This genotype result was accordance with
the phenotype data in this research.
Fig. 3. Normalized fluorescence curves of the 8 SNP markers. (A) SNP-001, (B) SNP-002, (C) SNP-003, (D) SNP-004, (E) SNP-005, (F) SNP-006, (G) SNP-007, (H) SNP-008. SNP-001 to 004 are newly developed 4 polymorphic SNP markers and SNP-005 to 008 are former developed 4 polymorphic SNP markers. In each graph, red curves indicate homozygous resistance to PepMoV, blue curves indicate susceptible to PepMoV and green curves indicate hetero genotype. Above 8 SNP markers are co-dominant.
**Fig. 4.** Co-segregation analysis of genotype with phenotype data. A total of 8 SNP markers were tested to F$_2$ population. Three SNP markers (SNP-004, SNP-005 and SNP-006) showed perfect co-segregation with phenotypes. Green shade and yellow shade indicate homozygous resistant (R) and homozygous susceptible (S) type respectively. Numbers in figure indicate individual numbers in F$_2$ population. Individual plants showing recombination are presented.

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Phenotype</th>
<th>31</th>
<th>96</th>
<th>101</th>
<th>167</th>
<th>322</th>
<th>568</th>
<th>695</th>
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<td>S</td>
<td>H</td>
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<td>S</td>
<td>S</td>
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<td>SNP-002</td>
<td></td>
<td>S</td>
<td>S</td>
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<td>S</td>
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<td>S</td>
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<tr>
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<td>S</td>
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<tr>
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<tr>
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<td>S</td>
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<td>H</td>
</tr>
<tr>
<td>SNP-008</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>H</td>
<td>H</td>
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</table>
Table 3. The number of recombinants in accordance with 8 SNP markers

<table>
<thead>
<tr>
<th>Population</th>
<th>Marker name</th>
<th>SNP-001</th>
<th>SNP-002</th>
<th>SNP-003</th>
<th>SNP-004</th>
<th>SNP-005</th>
<th>SNP-006</th>
<th>SNP-007</th>
<th>SNP-008</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>‘9093 x Jeju’ (F₂)</td>
<td></td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>8</td>
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Table 4. Genetic analysis of *Pyr7* using co-dominant SNP marker

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of plants</th>
<th>Expected ratio</th>
<th>$\chi^2$</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Resistant (RR)</td>
<td>Hetero type (Rr)</td>
<td>Susceptible (rr)</td>
</tr>
<tr>
<td>'9093 x Jeju' (F$_2$)</td>
<td>916</td>
<td>215</td>
<td>473</td>
<td>228</td>
</tr>
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</table>


Physical map of *Pvr7* and candidate gene identification

The above-mentioned 8 SNP markers (SNP-001, 002, 003, 004, 005, 006, 007 and 008) were used for construction of a genetic map (Fig. 5). A total of 916 F$_2$ plants were used to obtain more detailed information of the position of *Pvr7* in pepper genome. Using the reference pepper genome sequence of *C. annuum* ‘CM334’ (Kim et al., 2014), we obtained scaffold A corresponding to marker sequences by BLAST. The size of scaffold A is 1,791,376 base pair (bp).

The plants of the population were selected for recombination between two SNP markers, SNP-001 and SNP-008, which are located 0.0076 cM and 0.0011 cM away from *Pvr7* respectively (Figure 5A). The genotype analysis of 916 F$_2$ plants using the eight flanking SNP markers showed recombinants 7, 5, 3, 0, 0, 0, 1 and 1, respectively (Table 3). These recombinant plants were susceptible to PepMoV.

Eight selected F$_2$ recombinants were planned to self-pollinate for construction of a detailed linkage map around *Pvr7* (Fig. 5). The genotypes between SNP-008 and SNP-007 showed the same marker genotypes and marker genotypes corresponded to phenotype data. Therefore, the *Pvr7* gene is likely to be located within 258kb.

To perform candidate gene approach, gene prediction also performed in this target region. In the target region, nine NBARC-LRR (nucleotide binding domain shared by Apaf-1, certain R gene products, and CED-4 fused to C-terminal leucine-
rich repeats) type genes were predicted (Fig. 5B). These 9 NBARC-LRR types (NBARC a to i) were belong to resistance gene family.

In former research (Grube et al., 2000) reported that linkage map of region containing \textit{Pvr7} is located in chromosome 10 and linked 1.6 cM around \textit{Pvr4} (Fig. 6A). To construct fine map of \textit{Pvr7}, eight molecular markers of SNP-001, SNP-002, SNP-003, SNP-004, SNP-005, SNP-006, SNP-007 and SNP-008 were used and these markers showed 7, 5, 3, 0, 0, 0 and 1 recombinants respectively (Table 3). Markers SNP-006, about 0 cM above the \textit{Pvr7} locus, and SNP-004, about 0 cM below the \textit{Pvr7} locus, were the closest markers to \textit{Pvr7} (Fig. 6B). Taken together, I was able to construct more high-resolution of linkage map around the \textit{Pvr7} locus using five markers: SNP-003, SNP-004, SNP-005, SNP-006 and SNP-007 (Fig. 6B).
**Fig. 5.** Physical and genetic map around the *Pvr7* gene. (A) Target region of *Pvr7*. Red letters indicate the number of screened 9093 x Jeju F$_2$ plants and recombinants. (B) Gene prediction of the *Pvr7* target region using CM334 reference sequence. NBARC a to i indicate NBARC-LRR (nucleotide binding domain shared by Apaf-1, certain R gene products, and CED-4 fused to C-terminal leucine-rich repeats) type.
Fig. 6. Linkage map of the region containing the potyvirus resistance gene Pvr7. (A) Previous report on the Pvr7 locus (Grube et al., 2000, Jahn et al., 2000) (B) The linkage map of the region containing the Pvr7 locus based on ‘9093 x Jeju’ F₂ population (total 916 individuals). The Pvr7 locus was flanked by 3 SNP markers (SNP-004, SNP-005, and SNP-006). Numbers in parenthesis indicate centi Morgan (cM).
DISCUSSION

Two dominant potyvirus genes, Pvr4 and Pvr7, are known in Capsicum. Pvr4 and Pvr7 are tightly linked each other and they were originated from different sources, C. annuum ‘CM334’ and C.annuum ‘9093’, respectively. It was reported that although Pvr4 and Pvr7 are tightly linked and have the same inheritance mechanism, they are not allelic (Grube et al. 2000).

It was reported that C. annuum ‘9093’ was derived from a cross between C. chinense ‘PI 159236’ with a recurrent parent C. annuum and subsequent three times backcrossing with the recurrent parent (Grube et al., 2000). Because the recurrent parent of ‘9093’ is C. annuum, ‘9093’ is considered to have a C. annuum genetic background. This was confirmed by a recent research that showed ‘9093’ is belonged to C. annuum species (Kang et al., 2014). In this research, we tested whether ‘9093’ is resistant to PepMoV by inoculating GFP-tagged PepMoV to monitor virus accumulation (Fig. 1). At 13 dpi, resistant plants did not show any virus symptoms and GFP signals. No hypersensitive response was observed in ‘PI 159236’, ‘9093’, and ‘9093 x Jeju’ F1. It is commonly observed that resistance response is accompanied by HR in dominant resistance genes. For example, dominant resistance gene Tsw was shown apparent HR to inoculated leaves (Fig. 2). Previous study also demonstrated that inoculated leaves on ‘PI 159236’ showed HR
and abscission (Hoang et al., 2013). However, we were not able to observe any HR or abscission in this research indicating that Pvr7 has a different resistance mechanism compared to other dominant resistance genes although Pvr7 in ’9093’ is a single dominant resistance gene (Table 1, Table 4).

Since ‘PI 159236’ is known to carry both Pvr7 and Tsw (Kyle and Palloix 1997; Lee et al., 2013; Parrella et al., 2002), there is a high chance that ‘9093’ may also carry Tsw because Pvr7 and Tsw are tightly linked. To test whether ‘9093’ also carry other virus resistance genes, we inoculated ChiVMV, TSWV and TEV and observed resistance responses of ’9093’ together with control plants, ‘PI 159236’. ‘9093’ was susceptible to all three viruses indicating that ‘9093’ only carries the PepMoV resistance gene Pvr7. However, we need to confirm that ‘9093’ is derived from ‘PI 159236’ actually or not. According to some research (Boiteux et al., 1996), ‘PI 159236’ is carrying only recessive resistance gene. Another research (Huh et al., unpublished data) also demonstrate that F1 plants crossed with ‘PI 159236’ and susceptible annuum parent were susceptible to potyvirus. For these reasons, existence of Pvr7 is suspicious.

In this study we attempted to clone Pvr7 by a map-based cloning approach. In case of first successful effort in pepper (Tai et al., 1999). They isolates bacterial leaf spot resistance gene Bs2. Using near isogenic line (NIL) they applied to random amplified polymorphic DNA (RAPD) primer and amplified fragment length polymorphism (AFLP) primer then develop two markers which tightly linked to the Bs2 gene. Eventually, a yeast artificial chromosome (YAC) approach
was conducted (Tai and Staskawicz 2000) and they cloned Bs2 gene, but this method take a long time and spend a lot of money. Compare to previous method, however, my research have an advantage of time and money. Because mapping population is F₂ (that is take an only 2 generation) markers are SNP marker, so this study provide possibility of map based cloning easy.

Several markers around Pvr7 were developed in the previous research (Grube et al., 2000) but the genetic region was too wide covering 4.5 cM in chromosome 10. To narrow down the target region and develop more closely linked markers, we screened a large F₂ population consisting of 916 F₂ plants. The position of the Pvr7 gene was delimited within 258kb region between SNP-003 and SNP-007 (Fig. 5, Fig. 6) and a total of three 0 cM SNP markers were developed (Table 3). Using the delimited target region, we performed gene prediction to identify candidate genes encoding Pvr7. Nine of NBARC type genes were predicted. These NBARC genes are typical dominant resistance genes and related either directly or indirectly to pathogen resistance mechanism (Eitas and Dangl 2010; Mucyn et al., 2006).

We assumed that the Pvr7 gene will correspond to one of the 9 predicted genes. To identify the Pvr7 gene, we are planned to perform transient overexpression in N. benthamiana. Transient overexpression (TOE) is co-infiltration with resistance gene and a virus avirulence (Tran et al., 2014). If any of NBARC gene is corresponded to Pvr7, N. benthamiana co-infiltration with PepMoV will show HR or will not infected.
In addition, these results will contribute to marker-assisted breeding for PepMoV resistance pepper and provide genomic information to clone \( Pvr7 \). Tightly linked marker will be used to screen the resistant individuals quickly in the segregating population.
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alleles at the pvr1 locus encoding eIF4E in *Capsicum. Theoretical and Applied Genetics, 112*(1), 178-186.


고추반점무늬바이러스(PepMoV)는 고추가 재배되는 지역에서 빈번하게 발생하는 바이러스이다. PepMoV에 감염된 식물체는 잎에 모자이크 증상, 잎의 뒤틀림, 기형으로 형성되는 과실의 모양이 병징이다. 기존에 연구되었던 결과는 포티바이러스 저항성 유전자인 Pvr7가 고추 염색체 10번에 위치하고 있으며 이 유전자는 포티바이러스의 다른 우성 저항성 유전자인 Pvr4와 토마토반점위조바이러스(TSWV)의 우성 저항성 유전자인 Tsw와 연관되었다고 알려졌다. 우성 저항성 유전자인 Pvr7은 고추 annuum속에 속하는 ‘9093’이라는 식물체가 보유하고 있으며 이 저항성 유전자에 의해 포티바이러스의 하나인 고추반점무늬바이러스에 저항성이다. 이 ‘9093’고추는 고추 chinense속에 속하는 ‘PI 159236’으로부터 유래하였다. ‘PI 159236’고추가 Tsw 및 포티바이러스 열성 저항성 유전자인 pvr1도 보유하고 있기 때문에, 우리는 ‘9093’고추에 TSWV와 담배식각바이러스(TEV)를 접종하여 ‘9093’에 Pvr7 이외의 다른 저항성 유전자가 있는지를 확인하였다. 또한 포티바이러스에 속하는 고추잎맥반점무늬바이러스(ChiVMV)도 ‘9093’에 접종하여 이 식물체에 다른 포티바이러스 저항성 유전자가 있는지를 확인하고자 하였다.
이러한 일련의 실험 결과에 따라 ‘9093’의 저항성 범위는 오직 고추 반점 무늬 바이러스에만 저항성만 갖고 있는 것으로 밝혀졌다.

\( Pvr7 \) 유전자를 동정하기 위해 \( Pvr7 \)과 아주 근접한 마커를 개발하는 것은 필수적이다. 그래서 우리는 마커를 개발하기 위해 ‘9093’과 고추 반점 무늬 바이러스에 이병성인 ‘제주재래’를 교배하여 세대를 진전시킨 \( F_2 \)집단을 구축하였다. 총 916개체의 \( F_2 \)집단이 녹색형광단백질 (GFP)로 부착된 고추 반점 무늬 바이러스에 접종되어 표현형이 조사되었고, 한편으로 각 개체 별 DNA도 추출하여 유전형 분석에 이용되었다.

정교한 유전자 지도를 만들기 위해, 고추 염색체 10번에 위치하는 44개의 단일 염기 다형성 (SNP) 마커를 임의로 선발하여 ‘9093’, ‘제주재래’, ‘9093x제주’ \( F_1 \)의 DNA를 통해 4개의 다형성이 발견된 마커를 선발하였다. 그 후 선발된 4개의 마커는 916개의 \( F_2 \) DNA로 유전형을 분석하였으며, \( Pvr7 \)의 목표 위치를 설정하기 위해 \( C. annuum \) ‘CM334’의 전체 유전체 서열 (WGS)을 참고하여 4개의 추가적인 SNP 마커를 개발하였다. 이들 마커 8개의 유전형과 표현형을 비교분석하고 8개체 재조합 개체가 선발되었고, 총 3개의 SNP 마커가 \( Pvr7 \)과 완벽하게 공분리되는 결과를 얻게 되었다. 이러한 결과는 \( Pvr7 \)의 목표 위치가 258kb안에 위치하는 것을 밝혀준다. 또한 ‘CM334’ 전체 유전체 서열을 이용하여 이 영역 안에 총 9개의 후보 저항성 유전자들을 예측하였다. 따라서 우
리는 이러한 0cM의 근접한 $Pvr7$ SNP 마커를 통해 고추반점무늬바이러스 저항성 고추를 육종할 때에 유용하게 쓰일 것으로 기대하며, $Pvr7$ 유전자를 동정할 것으로 기대한다.

주요어: 포티바이러스, $Pvr7$, 고추 반점 무늬 바이러스(PepMoV), 우성 저항성, 마커 개발, 단일 염기 다형성(SNP)

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