A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Fine Mapping and Identification of Candidate Genes of the Root-Knot Nematode (*Meloidogyne incognita*) Resistance Locus *Me7* in Pepper (*Capsicum annuum*)

고추(*Capsicum annuum*)의 뿌리혹 선충 (*Meloidogyne incognita*) 저항성유전자 *Me7* 의 정밀지도 작성 및 후보유전자 동정

AUGUST, 2019

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ABSTRACT

The root-knot nematode (RKN) *Meloidogyne incognita* severely reduces yields of pepper (*Capsicum annuum*) worldwide. A single dominant locus, *Me7* conferring RKN resistance was previously mapped on the long arm of pepper chromosome P9. In the present study, the *Me7* locus was fine mapped using an F_2 population of 714 plants derived from a cross between the RKN-susceptible parent *C. annuum* ECW30R and the RKN-resistant parent *C. annuum* CM334. CM334 exhibits suppressed RKN juvenile movement, suppressed feeding site enlargement and significant reduction in gall formation compared with ECW30R. RKN resistance screening in the F_2 population identified 558 resistant and 156 susceptible plants, which fit a 3:1 ratio confirming that RKN resistance is controlled by a single dominant gene. Using the *C. annuum* CM334 reference genome and BAC library sequencing, fine mapping of *Me7* markers was performed. The *Me7* locus was delimited between two markers G21U3 and G43U3 covering a physical interval of approximately 394.7 kb on the CM334 chromosome P9 corresponding to the ~198 kb region on

Dempsey scaffold 10. Nine markers co-segregated with the *Me7* gene. A total of 42 genes were predicted in the ~394.7 kb CM334 *Me7* region, while in the corresponding Dempsey region of ~198 kb detected only 30 genes. Bulked segregant analysis (BSA) using Resistance gene enrichment Sequencing (RenSeq) sequence data enabled detection of SNPs with significant Δ(SNP-index) value <-0.3 linked to the *Me7* locus. A total of 492 SNPs/InDels were detected in 224.92 – 270.94 Mb on P9 at the significance level of 99%. Among the variants, 104 SNPs/InDels were located within the 16 candidate genes. The sizes of candidate genes ranged 0.192 kb to 2.835 kb. Among the 16 candidate genes, two Coiled coil-Nucleotide binding site-Leucine rich region (CNL) gene types (*1640.1* and *1640.7*) showed presence of missense, nonsense and InDels mutations, whereas three non-CNL genes (*1640.6*, *1640.15* and *1640.21*) had only missense mutation. RT-PCR analysis showed expression difference between resistant and susceptible parents only in *1640.6*. The sequence analysis showed that a SNP in *1640.7* gene causes a stop codon in the *1640.7* allele of susceptible parent. The *1640.7* allele of CM334 encodes a CNL type RX-CC-NBS-LRR like protein with a size of 899 aa, whereas the susceptible allele of ECW30R encodes 502 aa due to the nonsense mutation in the first exon. Two candidate genes *1640.6* and *1640.7* are proposed to be involved in resistance response for hypersensitive response (HR) and suppression of feeding site enlargement.

Keywords: Co-segregating markers, dominant locus, fine mapping, *Me7*, NBS-LRR, RenSeq, resistance gene

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LIST OF ABBREVIATIONS

AAA	ATPases associated with a variety of cellular activities
Avr	Avirulence
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
BSA	Bulked segregant analysis
CAPS	Cleaved amplified polymorphic sequences
CC	Coiled-coil
cDNA	Complementary DNA
CDS	Coding sequence
cM	centiMorgan
CNL	CC-NBS-LRR
CTAB	Cetyltrimethylammonium bromide
dai	Days after inoculation
DNA	Deoxyribonucleic acid
F_1	The first filial
F_2	The second filial
gDNA	Genomic DNA
GI	Galling index
HR	Hypersensitive reaction
HRM	High resolution melting
InDel	Insertions or deletions
J2	The second-stage juveniles
KRICT	Research Center for Biobased Chemistry, Korea Research Institute of Chemical Technology
MAS	Marker-assisted selection
NBS-LRR	Nucleotide-binding site and leucine-rich repeat
NICEM	National Instrumentation Center for Environmental Management
PacBio	Pacific BioSciences

PCR	Polymerase chain reaction
RenSeq	Resistance gene enrichment Sequencing
R genes	Resistance genes
RKN	Root-knot nematode
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RVT_2	Reverse transcriptase (RNA-dependent DNA polymerase) group 2
RX	Potato virus X resistance protein
SCAR	Sequence characterized amplified region
SMART	Single-Molecule Real Time
SNP	Single nucleotide polymorphisms
SSR	Simple sequences repeats
TIR	Toll/interleukin-1 receptor
UTR	Untranslated region
VCF	Variant Call Format

GENERAL INTRODUCTION

Root-knot nematode (RKN) *Meloidogyne* spp. is the most important sedentary plant-parasitic nematodes causes serious worldwide crop losses (Dukes et al., 1997). There are more than 98 species in the genus *Meloidogyne* spp. (Jones et al., 2013). Four of these, *Meloidogyne arenaria*, *M. incognita*, *M. hapla* and *M. javanica* are widely distributed with diverse host ranges and cause considerable yield losses in many crops (Jones et al., 2013; Wu et al., 2018). Among the *Meloidogyne* spp., *M. incognita* significantly reduces quality and yield of Solanaceae species worldwide (Eisenback and Triantaphyllou, 1991; Bernard et al., 2017). RKN manipulates the hosts' root vascular cells and induce specialized feeding cells.

RKN counteracts the plant defense response by injecting effector proteins into root cells and also reprogram the plant system through the molecular mimicry of host proteins by secreting pathogen proteins such as chorismate mutase, expansin-like proteins, CLAVATA3/ESR (CLE)-like proteins and annexins into host cell during feeding site development (Křeček et al., 2009; Gheysen and Michum, 2011; Kammerhofer et al., 2015). The feeding cells further expand and transformed into giant cells through synchronous nuclear divisions without cell division (Calilaud et al., 2008). The effector (s) also induces plant enzymes to modulate the cell wall composition to keep a larger amount of cytoplasm (Goellner et al., 2001).

Strategies to mitigate the RKN threat include biological and chemical control measures as well as the use of resistant cultivars/ rootstocks. Due to the inefficiency of chemical applications, as well as increasing awareness of food safety and environmental concerns, the application of nematicides has been restricted. Since nematode-resistant cultivars provide an efficient and environmentally safe alternative to chemical measures, much effort has been devoted to identifying

host resistance against RKN in cultivated or in wild species (Taylor and Sasser, 1978; Pegard et al., 2005).

In pepper, at least 10 dominant genes, *Me1* to *Me7*, *Mech1*, *Mech2* and *N* have been reported to confer resistance to *Meloidogyne* spp. (Wang and Bosland, 2006; Djian-Caporalino et al., 2007; Wang et al., 2009; Bucki et al., 2017). Several molecular markers linked to resistant loci have been developed for marker-assisted selection (MAS) and are used in breeding programs (Wang et al., 2009; Fazari et al., 2012; Uncu et al., 2015; Guo et al., 2016; Wang et al., 2018). Despite extensive mapping studies, the molecular aspects of pepper RKN resistance genes remain largely unexplored.

CM334 is a source of *Me7* mediated RKN resistance (Djian-Caporalino et al., 2007). The *Me7* locus was mapped on the long arm of pepper chromosome P9 in CM334 (Djian-Caporalino et al., 2007; Fazari et al., 2012), however, the exact physical location and the molecular basis of RKN resistance are poorly studied. To identify and clone the *Me7* gene, it is essential to construct a high resolution genetic map of the *Me7* locus and develop more closely linked markers.

C. annuum reference genomes including three versions of CM334 genome references; *C. annuum* 'CM334' versions v.1.5, v.1.55 (Kim et al., 2014), v.1.6 (Kim et al., 2017), UCD10X v.1.0 (Hulse-Kemp et al., 2018) and *C. annuum* 'Zunla-1' version v2.0 (Qin et al., 2014) were published by the several research groups. Recently sequenced *C. annuum* cv. Dempsey genome is also available to use internally. Moreover, BAC library (Yoo et al., 2003) also utilized to assist marker development in the complicated repetitive genomic regions or unreliable genome sequence assemblies (Jo et al., 2016).

Plant nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins are critical components of plant defense machinery encoded by one of the largest multigene families known

in plants (McHale et al., 2006). In this study, we applied Resistance gene enrichment Sequencing (RenSeq) method combined with bulked segregant analysis (BSA) for accurate prediction of NBS-LRR genes in the *Me7* locus.

Whole genome sequence data in important plants enabled the computational prediction of NBS-LRR genes in a genome-wide scale, which facilitated mapping of the previously uncharacterized of the NBS-LRR in crop breeding programs. Recent developments, such as RenSeq and Single-Molecule Real Time (SMART) RenSeq techniques hold great promise for yielding higher sequencing read depth for individual genes, and aid in accurate identification of sequence variations in plants with large complex genomes (Jupe et al., 2012, 2013; Witek et al., 2016).

Plant defense responses against RKN are often associated with gene-for-gene resistance (effector-triggered immunity). For instance, the well-known dominant *R* gene, *Mi-1* from *Solanum peruvianum* (Peruvian tomato), confers gene-for-gene resistance to some RKN *Meloidogyne* spp. *Mi-1* belongs to an *R* gene family that shares several canonical sequences and structural motifs of NBS-LRR domains (Milligan et al., 1998; Chen et al., 2007; Mao et al., 2015). The LRR domain induces hypersensitive reaction (HR) response like localized cell death (Hwang et al., 2000). Two RKN effectors, *MAP-1* from *M. incognita* and *Cg1* from *M. javanica*, have been suggested to be the cognate avirulence (*Avr)* genes of *Mi-1* (Gleason et al., 2008; Castagnone-Sereno et al., 2009). However, the interactions of these effectors with *Mi-1*, and *Mi-1*-mediated resistance, are not well understood. The potato cyst nematode RBP-1 and VAP1 effectors have been demonstrated to physically interact with their canonical R proteins, such as potato Gpa2 and a tomato Rcr3pim/Cf-2 and induce a foliar HR upon the transient co-expression (Sacco et al., 2009; Lozano-Torres et al., 2012). The *Me1* candidate gene (*CA09g16830*) was proposed to be a homolog of the resistance

protein R1A-3 which belong to the CNL type *R* gene (Wang et al., 2018). However, its functional role in RKN resistance need to be explored.

The aims of this study were to construct a high-resolution map of the *Me7* locus, predict and identify the candidate genes for RKN resistance. A total of 28 markers, including high-resolution melting (HRM) and cleaved amplified polymorphic sequences (CAPS) markers, linked to RKN resistance were developed and used for fine mapping of the *Me7* locus using the F_2 population derived from a cross between Early Calwonder 30R (ECW30R) and CM334. The tightly linked *Me7* markers identified in this study will be useful for marker-assisted selection for RKN resistance in pepper breeding programs. Characterization of candidate NBS-LRR type *R* genes in the *Me7* locus will be valuable source to uncover the molecular mechanisms of nematode resistance in plants.

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CHAPTER I

Fine Mapping of the Root-Knot Nematode (*Meloidogyne incognita*) Resistance Locus *Me7* in Pepper (*Capsicum annuum*)

ABSTRACT

M. incognita severely reduces yields of pepper worldwide. A single dominant locus conferring RKN resistance, *Me7*, was previously mapped on the long arm of pepper chromosome P9 in CM334. In the present study, a high resolution map of the *Me7* locus harbouring resistance to the *M. incognita* RKN was developed using an F_2 population of 714 plants derived from a cross between the RKN-susceptible parent *C. annuum* ECW30R and the RKN-resistant parent *C. annuum* CM334. The resistant parent, CM334 exhibits suppressed RKN juvenile movement, suppressed feeding site enlargement and significant reduction in gall formation compared with ECW30R. A 3:1 resistant to susceptible ratio in the F_2 population confirmed that resistance in CM334 to RKN was governed by a single dominant gene. Using the *C. annuum* CM334 reference genomes and BAC library sequencing, closely linked *Me7* markers were developed and subsequently fine mapping of *Me7* locus was performed. The *Me7* locus was delimited between two markers G21U3 and G43U3 covering a physical interval of approximately 394.7 kb on the CM334 chromosome P9. Nine markers co-segregated with the *Me7* gene. A cluster of 25 putative NBS-LRR-type *R* genes were predicted in the delimited *Me7* region. We propose that RKN resistance in CM334 is mediated by one or more of these NBS-LRR class *R* genes. The *Me7*-linked markers identified here will facilitate marker-assisted selection (MAS) for RKN resistance in pepper breeding programs, as well as functional analysis of *Me7* candidate genes in *C. annuum*.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are obligate phytopathogens causing significant economic losses in several crops including Solanaceae species (Eisenback and Triantaphyllou 1991; Bernard et al., 2017). There are more than 98 *Meloidogyne* species (Jones et al., 2013). Four of these, *M. arenaria*, *M. incognita*, *M. hapla* and *M. javanica*, are widely distributed with diverse host ranges and cause considerable yield losses in many crops (Jones et al., 2013; Noling 2014; Wu et al., 2018). Strategies to mitigate the RKN threat include biological and chemical control measures as well as the use of resistant cultivars/rootstocks. Due to the inefficiency of chemical applications, as well as increasing awareness of food safety and environmental concerns, the application of nematicides has been restricted. Since nematode-resistant cultivars provide an efficient and environmentally safe alternative to chemical measures, much effort has been devoted to identifying host resistance against RKN in cultivated or in wild species (Taylor and Sasser, 1978; Pegard et al., 2005).

Several dominant nematode resistance genes including *Mi1.1-2* and *Mi-9* have been reported in tomato (*Solanum lycopersicum*; Nombela et al., 2003; Seah et al., 2004; van der Vossen et al., 2005; Jablonska et al., 2007; Sanchez-Puerta et al., 2011; de Carvalho et al., 2015). In potato (*Solanum tuberosum*), the genes *Hero* and *Gpa2* confer resistance to *Globodera pallida* (white potato cyst nematode, van der Voort et al., 1999; van der Vossen et al., 2000; Ernst et al., 2002) and *Gro1-4* confers resistance to *Globodera rostochiensis* (yellow potato cyst nematode; Paal et al., 2004: Williamson and Kumar, 2006). In pepper, at least 10 dominant genes, *Me1* to *Me7*, *Mech1*, *Mech2* and *N* have been reported to confer resistance to *Meloidogyne* spp. (Wang and Bosland, 2006; Djian-Caporalino et al., 2007; Wang et al., 2009; Bucki et al., 2017). *Me1*, *Me3*,

Me7 and *N* confer resistance to a wide range of RKNs, including *M. arenaria*, *M. javanica* and *M. incognita*. *Mech1* and *Mech2* confer resistance to *M. chitwoodi*. These *R* genes originated from *C. annuum* accessions PI 201234 (*Me1* and *Mech2*), PI 322719 (*Me3* and *Me4*), CM334 (*Me7* and *Mech1*), and 'Missisippi Nemaheart' (*N*) (Hare, 1957). Each of these genes have been deployed in pepper breeding programs (Fery et al., 1998; Djian-Caporalino et al., 2007; Wang et al., 2009; Fazari et al., 2012; Celik et al., 2016; Wang et al., 2018).

These RKN resistance genes, including *Me1*, *Me3*, *Me4*, *Me7*, *Mech1*, *Mech2*, and *N* have been mapped on the P9 chromosome and are clustered in a 28 cM genetic interval (Djian-Caporalino et al., 2001, 2007; Fazari et al., 2012; Uncu et al., 2015). *N* is linked to the *Me1* and *Me3* loci, but is not allelic to them. *Me3* and *Me7* were originally mapped to different loci 12.1 cM apart (Djian-Caporalino et al., 2007) on chromosome P9, but were later found to be allelic (Thies and Ariss, 2009; Djian-Caporalino et al., 2011; Fazari et al., 2012). Several molecular markers linked to the *Me1*, *Me3*/ *Me7*, and *N* loci have been developed for marker-assisted selection (MAS) and are used in breeding programs (Wang et al., 2009; Fazari et al., 2012; Uncu et al., 2015; Guo et al., 2016; Wang et al., 2018). Despite extensive mapping studies, the molecular aspects of pepper RKN resistance genes remain largely unexplored.

CM334 is used by breeders as a source of resistance to a range of pathogens including several viruses, *P. capsici* (chili pepper blight) and RKN (Palloix et al., 1990; Dogimont et al., 1996; Djian-Caporalino et al., 2007). Molecular markers have been developed to map the *Me7* locus (Djian-Caporalino et al., 2007; Fazari et al., 2012), with the closest markers, HM2, SCAR_PM6a and SSCP_PM5 delimiting it to a 3.8 cM genetic interval (Fazari et al., 2012). However, the exact location of the *Me7* locus and the molecular basis of RKN resistance are poorly

understood. To identify and clone the *Me7* gene, it is essential to construct a fine genetic map of the *Me7* locus and develop more closely linked markers.

Our aims were to construct a high-resolution map of the *Me7* locus and predict candidate genes for RKN resistance. A total of 28 markers, including HRM and CAPS markers, linked to RKN resistance were developed and used for fine mapping of the *Me7* locus using the F_2 population derived from a cross between Early Calwonder 30R (ECW30R) and CM334. The tightly linked *Me7* markers we identified will be useful for marker-assisted selection for RKN resistance in pepper breeding programs as well as assist mapping for other RKN resistance loci on pepper P9 chromosome.

MATERIALS AND METHODS

Plant materials

An F_2 mapping population consisting of 714 plants, derived from a cross between *M. incognita* RKN-resistant CM334 and RKN-susceptible ECW30R lines from Horticultural Crops Breeding and Genetics Lab, Seoul National University, was used to fine-map the *Me7* locus. Phenotype screening of 504 of these F_2 plants was performed at the Research Center for Biobased Chemistry, KRICT, Daejoen, in 2014–2015 and phenotype screening of the other 210 F_2 plants was performed at Seoul National University, Seoul, Korea, in 2015.

Nematode inoculation

M. incognita race 1 was kindly provided by Prof. Young-Ho Kim (Clinical Plant Pathology and Nematology Laboratory, Seoul National University). *M. incognita* was propagated using susceptible tomato (*S. lycopersicum* cv. Micro-Tom). One-month old Micro-Tom plants were cultivated in pots with fresh commercial potting mixture (Hanarum, Minong Fertilizer, Korea) and sand in a 2:1 ratio were inoculated with 1,000 juvenile stage 2 (J2) of *M. incognita*. The inoculated plants were kept in the glasshouse condition with an average temperature of 26±2℃. The nematode egg masses were collected from approximately 55-day-old infected susceptible tomato roots. Briefly, infected roots were cleaned with water and cut into 1 cm pieces, and then stirred in 1% NaOCl solution for 5 min (Coyne and Ross, 2014). The suspension was passed sequentially through a stack of sieves of 250 µm, 180 µm and 25 µm, under running water, to remove any NaOCl residue. The eggs and J2 were captured on the 25 µm sieve with distilled water. The

suspension with egg masses and J2 stage were filtered through a tissue paper that was placed on a Baermann funnel glass at room temperature (25℃) for three to four days. Thus, the J2 collected were counted under a light microscope. Four-leaf stage plants of testing populations were inoculated with 1,000 freshly hatched J2 *M. incognita* as mentioned above. Inoculated plants were kept in the glasshouse maintained at 26±2℃.

Nematode resistance screening

The resistance phenotype was evaluated at 45 days after inoculation (dai). For egg mass production and viability study, four roots from each of the parental lines were uprooted and cleaned with tap water. Egg masses were handpicked from root system, and incubated in distilled water at room temperature for two hours before observing under the magnifying microscope (Carl Zeiss, Thornwood, NY). The root systems of each plant were examined, and the RKN resistance phenotype was scored using a root galling index (GI), rated on 0–4 scales, where 0 = 0–25%, 1 = 26-50%, 2 = 51-75%, 3 = 76-99% and 4 = ≥100% (Baker, 1985). The percentage was calculated by dividing the total number of galls from each root system by the mean of the number of galls from susceptible ECW30R. The plants were classified as resistant when GI = 0 and as susceptible when GI ≥1, a classification modified from Seo et al. (2014).

Analysis of histological responses

To study the histological response in the parental lines, infected roots as prepared above were harvested at 5, 10, and 15 dai for microscopic observation according to the procedure described by Seo et al. (2014). The specimens were sectioned by ultramicrotome (MT-X, RMC, Tucson, AZ, USA) to a thickness of 700 nm. The slides were stained with 1% toluidine blue O in 2% sodium

tetraborate. The root systems of both parents were observed for HR by staining with fuchsin-acetic acid solution (Bybd et al., 1983). Specimens were analyzed using a Carl Zeiss microscope (Carl Zeiss, Thornwood, NY).

Genomic DNA extraction

Total genomic DNA (gDNA) was extracted from young leaf tissues using the cetyltrimethylammonium bromide (CTAB) protocol (Kang et al., 2010). DNA concentration and quality were analyzed using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Analysis of previously reported *Me* linked markers

Previously reported PCR-based markers including sequence characterized amplified region (SCAR), simple sequences repeats (SSR) and CAPS linked to the *Me1*, *Me3*, *Me7*, and *N* loci (Djian-Caporalino et al., 2007; Fazari et al., 2012; Guo et al., 2016) were amplified from the ECW30R and CM334 parents and sequenced at the NICEM (Seoul National University, Korea). We analyzed these marker sequences for polymorphism and used BLAST to identify their physical positions in the CM334 genome version v.1.5 (scaffold) and v.1.55 (chromosome) (Kim et al., 2014). The SSR marker, CASSR37, linked to the *N* locus (Celik et al., 2016) and BAC-end markers PE43N9R, PE242G21R, PE11F6F, and PE25F15F, linked to the *Me3* locus derived from a double haploid pepper DH149 (Guo et al., 2016), were also used for marker development. The *Me*-loci and *N* linked markers are listed in **Table I- 1.**

Table I- 1. *Me* loci and *N* linked markers from previous studies.

Name of marker	Primer sequence	Linkage with locus	Reference	No. of recombinants in 192/714 F_2 individuals
SCAR_PM6a	F: ttcttacccctgtacatcacatcct	*Me3*, *Me7*, *N*	Fazari et al., 2012	0/0
	R: aacctggaaatttctggaggtatg			
SCAR_PM6b	F: cccggtctattttccctttt	*Me3*, *Me7*, *N*	Fazari et al., 2012	0/0
	R: tgtctaaattctcatggcagtg			
CASSR37	F: acatacccaaaaactctctcac	*N*	Celik et al., 2016	3/-
	R: gattgaccatgtttccgtat			
PE43N9R	F: agatttggctcacctgacc	*Me3*	Guo et al., 2016	not polymorphic
	R: gatggcataagagggctttaga			
PE242G21R	F: ggttgttaatattatactatc	*Me3*	Guo et al., 2016	not polymorphic
	R: acaacaacacacggttacaag			
PE11F6F	F: tgactcgcgcaaatacaag	*Me3*	Guo et al., 2016	not polymorphic
	R: gaagatttccttaagtccggc			
PE25F15F	F: tccctccttgctgtcatcac	*Me3*	Guo et al., 2016	not polymorphic
	R: tgttgtctgcacatgccttg			

Marker development

To develop additional markers for fine mapping *Me7*, PCR primers were designed from the v.1.55 chromosome and v.1.5 scaffold versions of the *C. annuum* 'CM334' genome (Kim et al., 2014). Amplified PCR products were sequenced at NICEM and Macrogen (https://dna.macrogen.com/eng/) to detect polymorphisms. Identified SNPs were converted into HRM or CAPS markers listed in **Table I- 2**.

Table I- 2. Newly developed markers used in the *Me7* locus mapping.

Name of marker	Primer sequence	Marker type	Sequence origin	No. of recombinants in 192 F_2 individuals	No. of recombinants in 714 F_2 individuals
Y35b	F: ggtggggtcctcgatgaca	HRM	v.1.55 chr P9	29	-
	R: cgcctgcactatctccccta				
Y58b	F: ggcacctttaacgtagatccca	HRM	v.1.55 chr P9	28	-
	R: acccttcatctgccatccca				
9217078971	F: cttgagaagagtgtctatcg	HRM	v.1.55 chr P9	6	-
	R: tggtggagaatagagatcag				
Me7B1a	F: cgtttaacttggcttcgtcg	HRM	v.1.55 chr P9	6	-
	R: ctacgtggccatctcacgtc				
NBLRR7b1	F: ggtcgaaaaagctttcccat	HRM	v.1.55 chr P9	3	5
	R: cggcaactctatccaccaaa				
Me7748b	F: cctttgcattgtactgcatctc	HRM	v.1.55 chr P9	3	5
	R: gggtcgatttctacagaccgt				
NBLRR6b	F: tggtgatgacctcggaaaga	HRM	v.1.55 chr P9	3	5
	R: cattagctggttattgatgggc				
CA63a	F: ctagagcagtggagttcacc	HRM	v.1.5 scaffold 1578	2	3
	R: gtgtcaaagagacttagtctcct				
6119403	F: accaaggacaatgtacactt	HRM	BAC611K18	1	2
	R: agtaaccactatgtcagttgg				
G21U3	F: acaaatgacaactttcctgc	HRM	BAC611K18	0	1
	R: acatggacaggagatacgaa				
G24U5	F: acaaccaagaacatatggct	HRM	BAC611K18	0	0
	R: agttcattgtttccgatgttg				
CA1-1b	F: gacgaaatttgcgctattca	HRM	BAC611K18	0	0
	R: gatccatgaagtccatctgc				
611109646	F: agaagcattgggtggaac	HRM	BAC611K18	0	0
	R: tcaacctgcttctccttaac				
SF164076	F: gggaggattgcccaagacaa	HRM	v.1.5 scaffold 1640	0	0
	R: gggatgattagtagtatccagtggc				
SF16406	F: cagtggctgatcgagagcac	HRM	v.1.5 scaffold 1640	0	0
	R: cagacatcagaggcatgcca				
SF164024	F: gcagcatcagcatccgaatctt	HRM	v.1.5 scaffold 1640	0	0
	R: tcccttattatgagtcgttgcct				
2111b1	F: tctgggccaaaaatgctacca	HRM	v.1.5 scaffold 1578	0	0

Name of marker	Primer sequence	Marker type	Sequence origin	No. of recombinants in 192 F_2 individuals	No. of recombinants in 714 F_2 individuals
	R: tggtttgactaacactcctgca				
G43U3	F: aagacgatcctgttagagtg	HRM	v.1.5 scaffold 1578	1	1
	R: tgggacttttacccactct				
G79U3	F: tgatgtcaagtttagggtca	HRM	v.1.5 scaffold 1677	1	1
	R: ttgaggagttcaaggaatga				
G80U5	F: taggtgaaatggtgtgaacg	HRM	v.1.5 scaffold 1677	2	2
	R: acagtgggctataacacatag				
G81U3	F: tcattctcaccttcgcatag	HRM	v.1.55 chr P9	3	3
	R: tcaatatctgcaacctcagt				
NBLRR10b	F: tggtggagcatgaaaaggga	HRM	v.1.55 chr P9	3	-
	R: agactgaatcgaaggatggttg				
551458	F: acgagagtgaatgaagttgctcc	HRM	v.1.55 chr P9	4	-
	R: ggtggtggtggtgttcgata				
551511	F: gcggttaatatgtcaccgaat	HRM	v.1.55 chr P9	4	-
	R: aagccggacacaccgataat				
NBLRR12	F: cggagaccatgattggtttg	HRM	v.1.55 chr P9	10	-
	R: gcatgttccatccacgaaag				
551177	F: gcttggcggacaactcctttt	HRM	v.1.55 chr P9	10	-
	R: tgtagcttatgcgtgttggct				
18660	F: ttggtgtcgagtctggtgta	CAPS	v.1.55 chr P9	13	-
	R: atgccttcgatcttctcagtg	(*Msp*I)			
SF164664	F: acgtgtgttagtatcccgagact	HRM	v.1.5 scaffold 1646	6/10*	-
	R: tgtgaaactgtaagcttctgca				

* 6 recombinants were detected out of 10 selected recombinant individuals from 192 F_2 population.

Genotype analysis

The HRM assays were performed as described (Liu et al., 2016) with slight modifications in the PCR conditions as follows: 95℃ for 4 min, followed by 50 cycles of denaturing at 95℃ for 20 s, touchdown annealing from 60–53℃ for 30 s, 72℃ extension for 25 s, and then hold at 25℃ for 30 s. The HRM used 0.1℃ increments between 65℃ and 90℃, with a Rotor-Gene^{TM} 6000 thermocycler (Corbett Research, Sydney, Australia). The HRM curve profiles of homozygous parents and heterozygous F_1 plants were used to assign the genotypes (**Figure I- 1**).

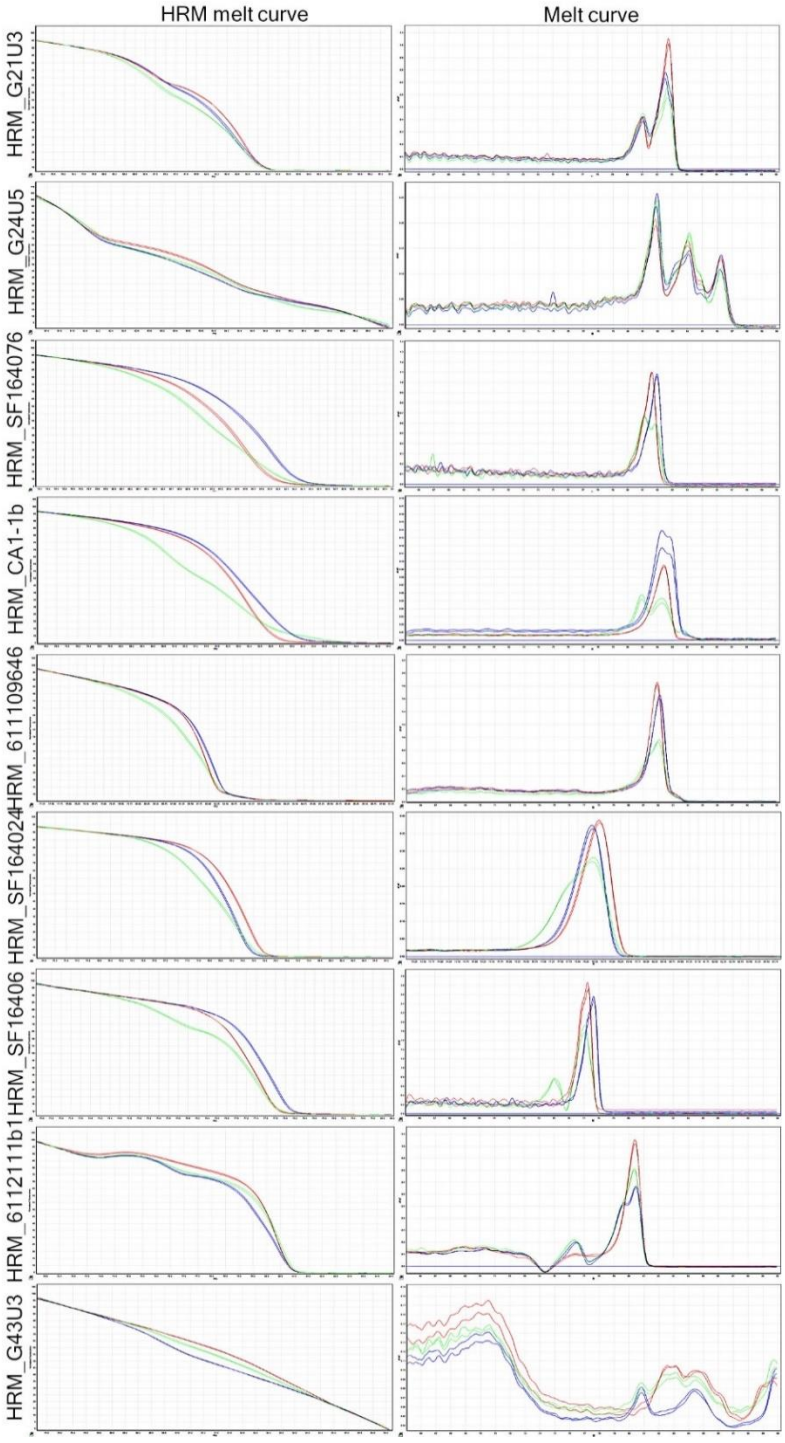

Figure I- 1. Example of HRM analysis of the *Me7* locus-linked markers. The analysis curves included HRM melt curves and melt curves. Blue = resistant (homozygous), red = susceptible and green = resistant (heterozygous).

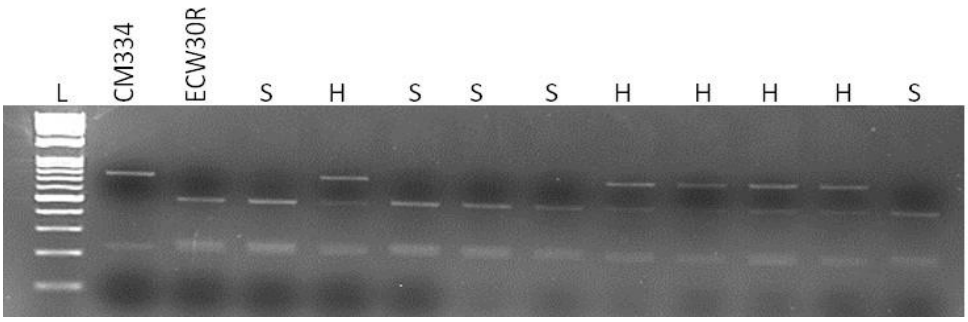

Figure I- 2. Restriction patterns of the CAPS marker, 18660. *Msp*I digested PCR product from susceptible and resistant lines show a clear band of 560 bp and 808 bp. Heterozygous resistant lines show both 560 and 808 bp bands. L indicate 1 kb DNA molecular size marker.

BAC library screening

To expand the distal ends of scaffolds 1640 and 1578, markers from these scaffolds were used to screen a BAC library (Yoo et al., 2003). A three-step BAC screening procedure was described by Jo et al. (2016). BAC ends of 13 positive BAC clones were sequenced using the universal primers T_7 and SP_6. The BAC end sequences were then BLAST searched against the *C. annuum* genome databases (http://passport.pepper.snu.ac.kr/?t=CAB). The selected BAC611K18 clones were PacBio (Pacific BioSciences) sequenced at Macrogen (http:// macrogen.com/kor/). The BAC sequence-derived SNPs and InDels were used for developing additional markers. The dot plot analysis of repeat sequences in the scaffold and BAC clones was performed using the NCBI server (http://blast.ncbi.nlm.nih.gov/ Blast.cgi).

Comparative analysis of the *Me7* linked markers and physical maps

To identify the most suitable sequence information to delimit the *Me7* locus, the total genome sequences from *C. annuum* 'CM334' version v.1.55, v.1.6 (Kim et al., 2014, 2017) and UCD10X v.1.0 (Hulse-Kemp et al., 2018) were used. CLC Main Workbench 8.1 (QIAGEN, Aarhus, Denmark) was used to obtain the physical position of the *Me7* linked markers, where map reads with ≥ 98% nucleotide similarity to the genomic sequence on chromosome P9 were used for comparative mapping.

Fine mapping and gene prediction

Genetic linkage analysis was performed using Carthagene ActiveTcl 8.4 (de Givry et al., 2005). The mapping distance was calculated using Kosambi's mapping function with the LOD threshold set at 3.0 and distance threshold at 0.5. The genetic linkage map was drawn using the MapChart

2.2 tool (Voorrips, 2002). The *Me7* locus was initially mapped using 192 F_2 plants and fine-mapped with 714 F_2 plants. The physical map data for the *Me7* locus were retrieved from the pepper genome database version v.1.6 (http://peppergenome.snu.ac.kr/download.php). The coding sequences (Annuum.v.2.0.CDS) from the genomic interval of the *Me7* locus were BLAST searched at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification of conserved domains and functional annotation.

RESULTS

Analysis of compatible and incompatible responses to RKN

To know the resistance response to RKN in our experimental conditions, *M. incognita* gall formation and egg mass production viability were observed in resistant *C. annuum* 'CM334' and susceptible *C. annuum* 'ECW30R' at 45 dai (**Figure I- 3A, Figure I- 4**). Viable egg masses with J2 were observed in both CM334 and ECW30R root systems (**Figure I- 4 B, D, E**), however, the root systems of CM334 formed only a few, small galls, while ECW30R roots had many, large galls (**Figure 3A, B**). We performed histological studies to investigate the intercellular resistance responses in the root systems. At 10 dai, no cell necrosis was noticed in ECW30R, whereas necrosis was observed in CM334 cells, as a result of a HR (**Figure I- 3C**), indicating that CM334 is highly resistant to *M. incognita*.

Cross-sections of the root system of RKN-infected plants revealed no morphological differences between susceptible ECW30R and resistant CM334 at five dai; however, juveniles of RKN were detected in both infected parents with no sign of feeding site formation (**Figure I- 3D; a-e**). At 10 dai, although giant cell development was initiated in both lines, the stele cells were slightly narrower and denser with enlarged feeding sites in ECW30R compared to CM334 (**Figure I- 3D; f-j**). At 15 dai, the infected cells in ECW30R grew much larger than in CM334 and caused increased compaction of cell layers in vascular tissues (**Figure I- 3D; k-o**). Overall, these results demonstrate that the RKN resistance of CM334 involves cell necrosis as well as the suppression of establishment and/or enlargement of feeding sites.

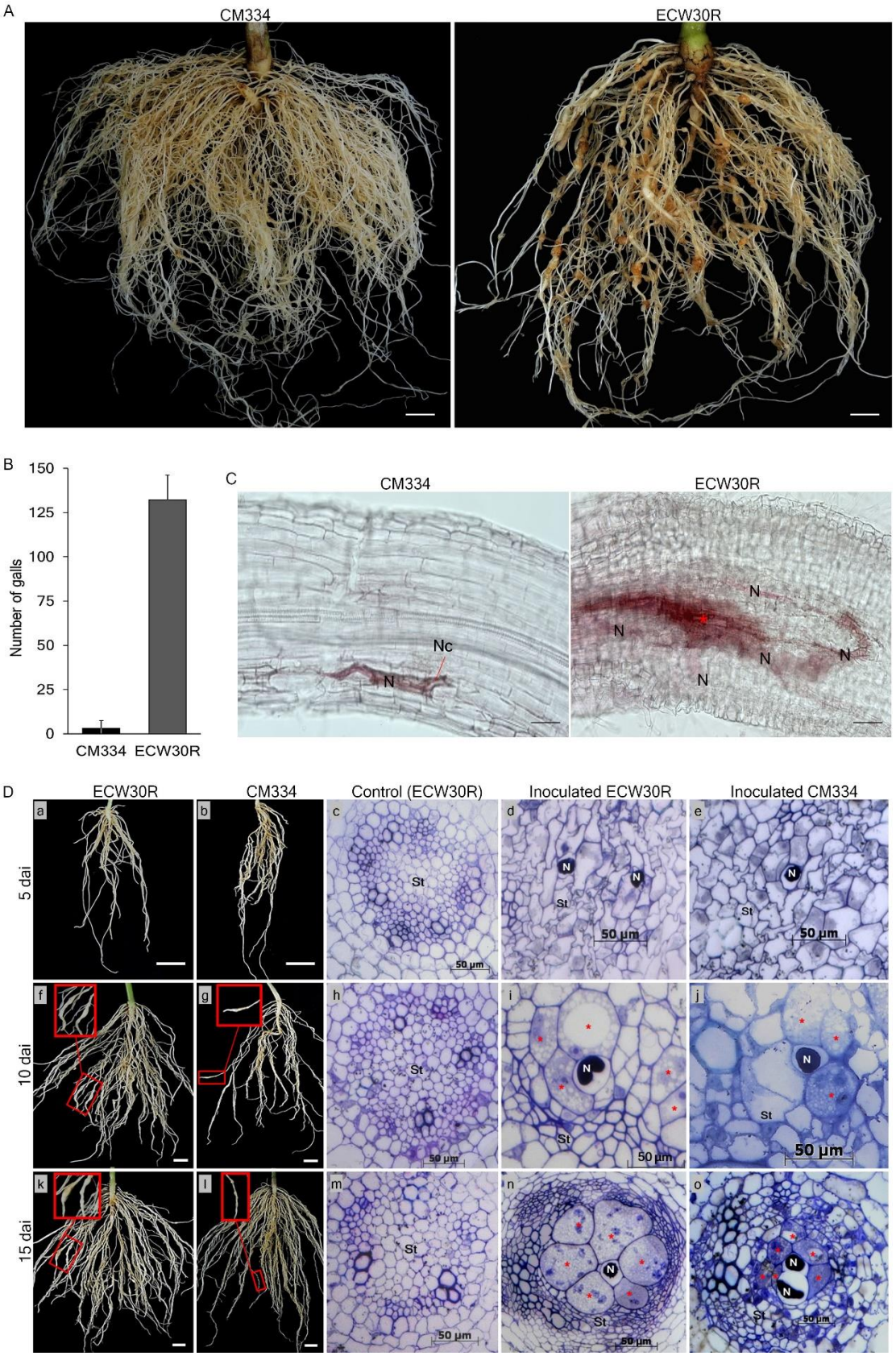
A
CM334
ECW30R
B
Number of galls
150
125
100
75
50
25
0
CM334 ECW30R
C
CM334
ECW30R
Nc
N
D
ECW30R
CM334
Control (ECW30R)
Inoculated ECW30R
Inoculated CM334
5 dai
10 dai
15 dai
St
50 µm

Figure I- 3. Comparison of resistant responses in the root systems of the parental lines. A) A resistant CM334 and a susceptible ECW30R observed at 45 dai infected with *M. incognita* juvenile 2 (J2). Bar = 1 cm. B) Average number of root galls observed at 45 dai from 18 plants of each parent. Bar = standard deviation. C) Resistance responses at 10 dai in CM334 and ECW30R root systems during juvenile penetration. Cell necrosis (Nc), nematode (N) and initiation of giant cell formation (red *). Bar = 100 μm. D) Root systems and cross-sections of susceptible ECW30R and resistant CM334. The inoculated roots were harvested at 5, 10 and 15 dai. Pictures in red boxes pointing to galls development on the infected roots in close view (f, g, k, l), Bar = 1 cm. The root systems of ECW30R and CM334 and gall formation were observed at 5 (a-e), 10 (f-j), and 15 (k-o) dai. Cross-sections of inoculated ECW30R, CM334 and non-inoculated ECW30R (control) at 5 (c-e), 10 (h-j), and 15 (m-o) dai. Nematode (N), enlarged multinucleate giant cells (red *) and stele (St). Bar = 50 μm.

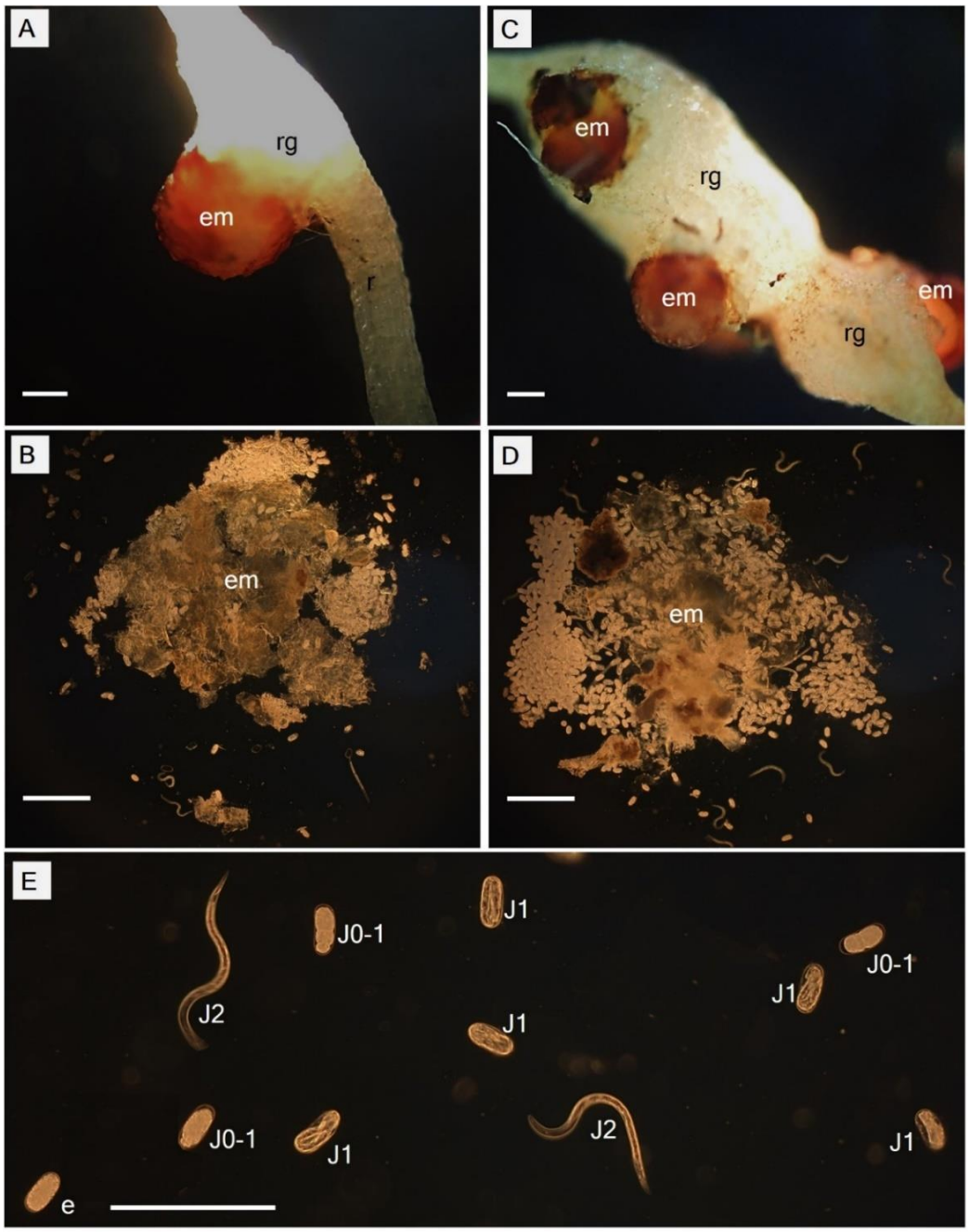

Figure I- 4. The RKN egg masses produced in CM334 and ECW30R roots, imaged at 45 dai. Eggs are exuded egg mass on the outside of the female RKN on roots of CM334 (A, B) and ECW30R (C, D). The egg mass from both CM334 (B) and ECW30R (D). Different developmental stages of RKN, from egg to hatching of J2 are indicated (E). e=egg, em= egg mass, r= root, rg= root gall, J0-1= egg developing undergo to J1. Bar = 0.5 mm.

Phenotyping and inheritance assay

The RKN resistance was evaluated at 45 dai. All tested CM334 and F_1 plants were resistant to RKN, while all ECW30R plants were susceptible. Out of 714 F_2 individuals, 558 plants showed resistance and 156 F_2 plants showed susceptible phenotypes, which fit a segregation ratio of 3 resistant:1 susceptible, as expected for the inheritance of a single dominant gene ($X^2 = 3.782$, $P = 0.0518$) (**Table I- 3**). These results are consistent with previous phenotyping studies (Djian-Caporalino et al., 2007; Fazari et al., 2012).

Table I- 3. Segregation analysis of RKN resistance in CM334, ECW30R, F_1 and F_2 mapping populations at 45 dai.

Parent lines and progenies	**Number of plants**			**Expected ratio (R:S)**	χ^2 **(df=1)**	***P=0.05***
	Total	**Resistant**	**Susceptible**			
CM334	37	37	0	1:0		
ECW30R	55	0	55	0:1		
ECW30R×CM334 F_1	30	30	0	1:0		
ECW30R×CM334 F_2	714	558	156	3:1	3.782	0.0518

Development of markers closely linked to the *Me7* locus

A high-density map for the *Me7* locus, was developed using the *Me* loci and *N* locus-linked markers from previous (**Table I- 1**), and new markers developed using genomic information from CM334 (**Table I- 2**). Among the previously reported markers, only SCAR_PM6a, SCAR_PM6b (linked to *Me3*, *Me7*, and *N* loci) and CASSR37 (linked to *N* locus) were found to be polymorphic and therefore used for mapping *Me7* in this study. No recombinants were found for SCAR_PM6a and SCAR_PM6b, whereas three recombinants were detected for CASSR37 in a subset of the F_2 population containing 192 individuals. These results indicate that the *N* and *Me7* loci are not allelic, which concurs with previous reports (Celik et al., 2016; Fazari et al., 2012). Four *Me3*-linked markers (PE43N9R, PE242G21R, PE11F6F, and PE25F15F) (Guo et al., 2016) were also tested; however, these markers were not polymorphic in our mapping population (**Table I- 1**).

To develop additional markers, we BLAST searched these *Me3*, *Me7*, and *N* linked marker sequences against the *C. annuum* CM334 genome (v.1.55) and scaffold (v.1.5) (Kim et al., 2014), and identified their genomic positions on chromosome P9 and unassigned scaffold sequences. Using the genomic information of CM334 and scaffolds (1640, 1646, 1677 and 1578) containing SCAR_PM6a, SCAR_PM6b, CASSR37, and the four *Me3*-linked markers, we developed 21 HRM markers and one CAPS marker (**Figure I- 5, Table I- 2**). In 192 F_2 individuals, four newly developed markers and two previous markers (SCAR_PM6a and SCAR_PM6b) co-segregated with the *Me7* locus (**Figure I- 5**) with a genetic interval of 0.9 cM on P9 (v.1.6) between G43U3/G79U3 and CA63a, which are located on scaffolds (1578, 1640, and 1677) (**Figure I- 5**, **Table I- 2**).

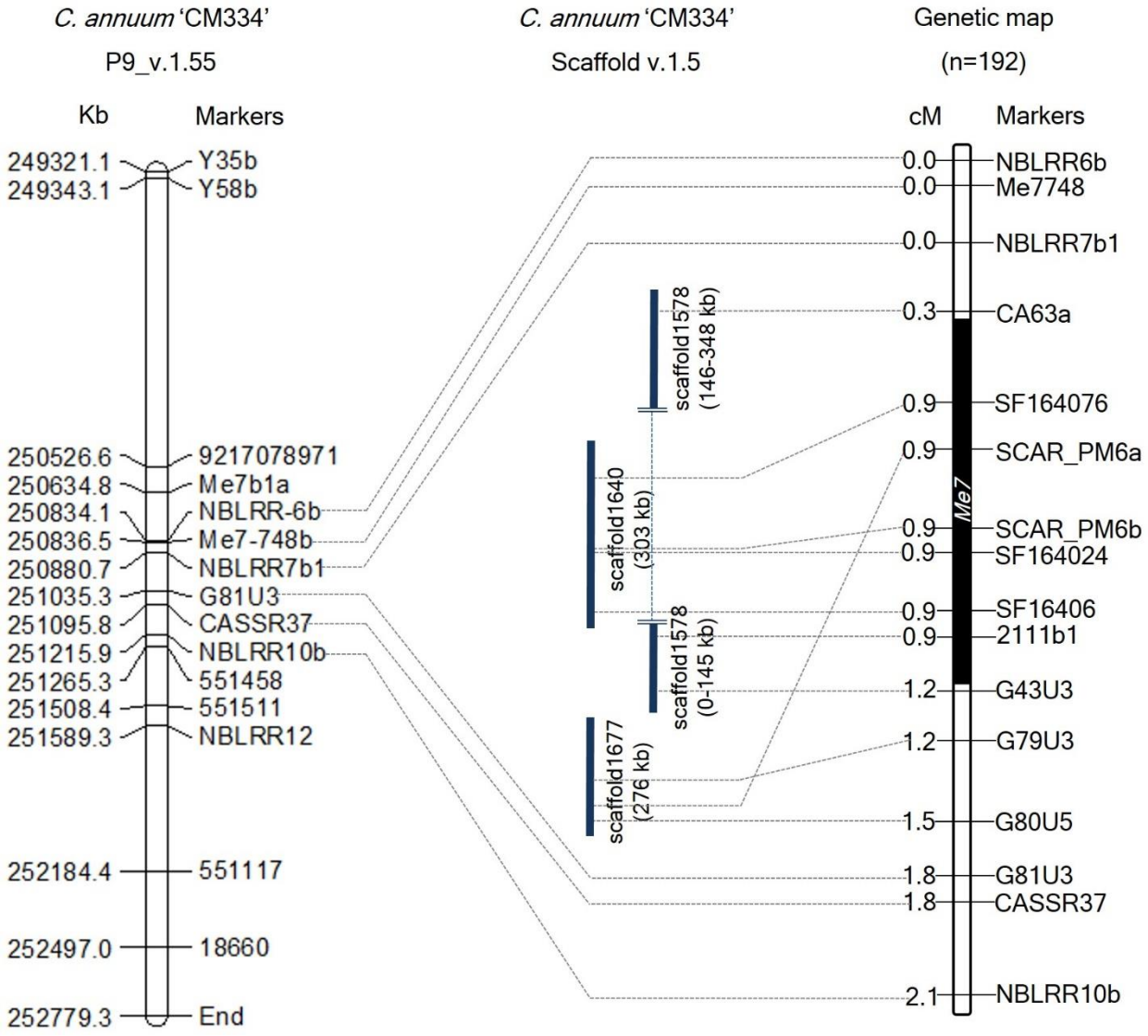

Figure I- 5. Comparative physical and genetic maps of the *Me7* locus developed from 192 F_2 plants. The left panel indicates the physical map of the *Me7* linked markers on the 'CM334' genome reference P9 (v.1.55). The right panel indicates the genetic map of the *Me7* locus. Six markers co-segregating with the *Me7* locus were detected on scaffolds (v.1.5). The *Me7* locus was delimited to a 0.9 cM region between the G43U3/G79U3 and CA63a markers. Physical and genetic distances between markers are indicated on the left side of the chromosome P9.

BAC library screening, sequence analysis and marker development

To extend the distal ends of scaffold 1640 and to identify the regions overlapping with scaffolds 1578, we performed BAC library screening using three markers: the CA63a marker from scaffold 1578 and the SF164076 and SF164024 markers from scaffold1640 (**Figure I- 6A**). BAC library screening with the marker CA63a detected the 760A3 clone, and both its BAC ends aligned to scaffold 1578 (**Figure I- 6A**). Screening with the markers G43U3 detected three BAC clones, 303A21, 668N6 and 739L20 (**Figure I- 6A**). BAC screening with the marker SF164024 identified five clones, 399A15, 765B13, 564D22, 694N7 and 714B12. Of these, only 765B13 and 714B12 clones overlapped with 611K18 and 514E15 (**Figure I- 6A**). BAC library screening with the SF164076 marker detected three BAC clones, 711J20, 514E15 and 611K18. BAC end sequences of the 611K18 clone aligned to the sequence of the scaffolds 1640 and 1578 (**Figure I- 6A**). Therefore, we selected the 611K18 clone for full-length sequencing and developed five markers (**Table I- 2**). Further BAC screening with the 6119403 marker developed from the 611K18 BAC clone detected a single clone, 742O5, which bridged the gap between the 760A3 and 611K18 clones (**Figure I- 6A**). Furthermore, the comparative dot plot analysis revealed an association between scaffolds 1640 and 1578 bridging and overlapping the BAC clones (**Figure I- 6B**). A total of five additional HRM markers (**Table I- 2**) were produced using genomic information of BAC clone 611K18 and a flanking marker indicated by G21U3 for fine mapping of the *Me7* locus.

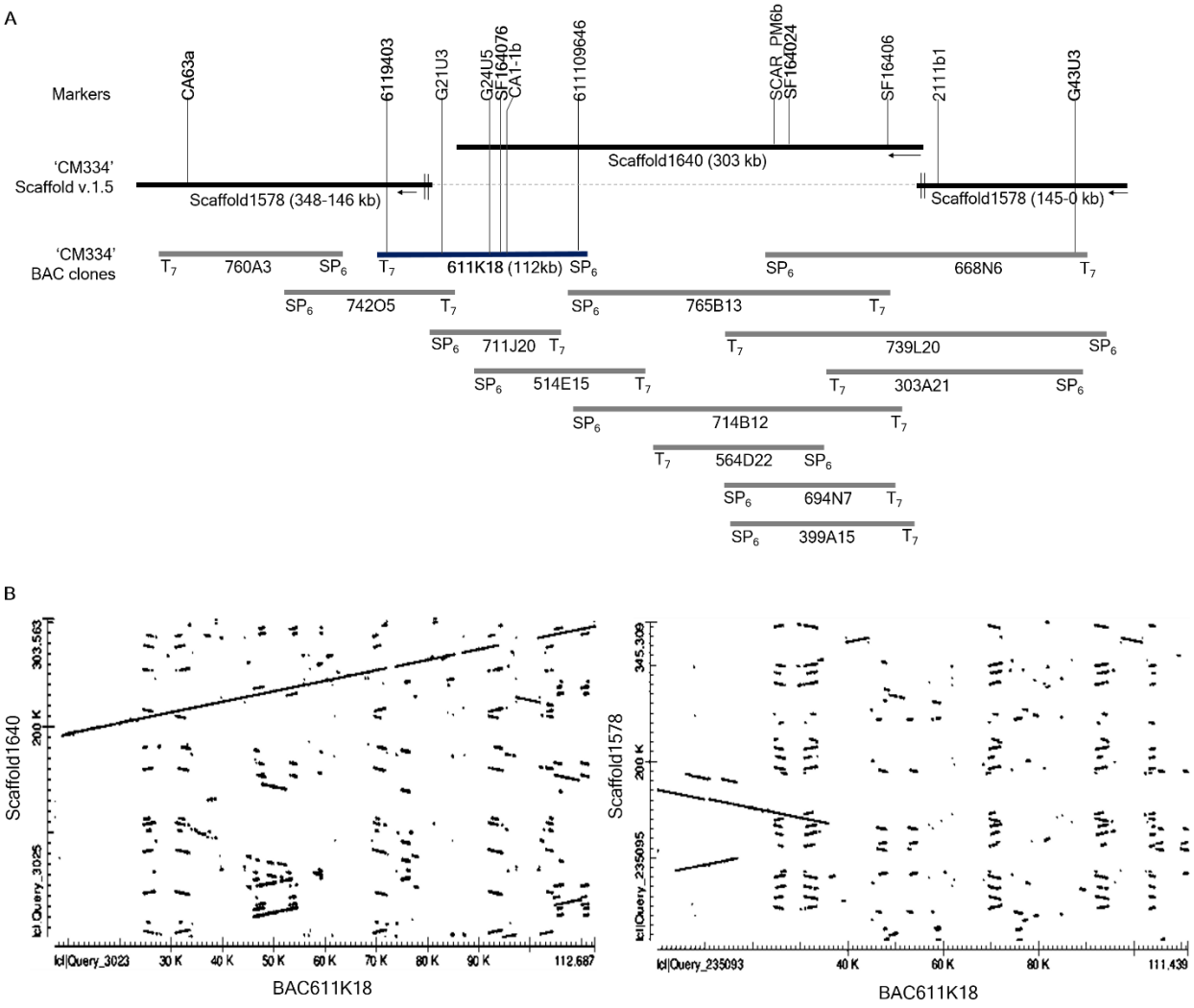

Figure I- 6. BAC library screening and alignment analysis. A) Five markers (CA63a, G43U3, 6119403, SF164076 and SF164024) derived from scaffold 1578, scaffold 1640 and BAC611K18 were used to screen the *C. annuum* 'CM334' BAC library (Yoo et al., 2003). BAC screening detected 13 positive clones which were aligned on scaffold 1640 and 1578. Full-length sequencing of the BAC clone 611K18 revealed that 611K18 overlapped and bridged the gap between scaffold 1640 and 1578. The *Me7* locus is covered by overlapping BAC clones, screened by the markers SF164076, SF164024, and G43U3. B) Dot plot analysis of full-length sequence revealed that 611K18 overlapped and bridged the gap between scaffold 1578 and 1640.

Fine mapping of the *Me7* locus

Initial mapping of the *Me7* locus in an F_2 population consisting of 192 individuals, placed SCAR_PM6a and SCAR_PM6b 0 cM from the *Me7* locus. We used 31 markers, including 28 newly developed ones, to narrow the target region and we increased the size of the mapping population to 714 plants (**Figure I- 7A**, **Table I- 1, 2**). This fine mapping analysis produced seven recombinant plants (**Figure I- 7A**; 38, 161, 175, 209, 300, 450, and 578); one recombinant for each of G21U3, G43U3, and G79U3, two recombinants for 6119403 and three recombinants for CA63a (**Figure I- 7A**). Three markers (CA63a, 6119403 and G21U3) were placed on the one side of the *Me7* locus, whereas two markers (G43U3 and G79U3) were placed on the other side (**Figure I- 7A**). Nine markers including seven newly developed markers and two reference markers (SCAR_PM6a and SCAR_PM6b) co-segregated with the *Me7* locus. The *Me7* locus was thus delimited to a 0.28 cM region with an interval of approximately 394.7 kb between G21U3 and G43U3 covered by BAC clone 611K18 and scaffolds 1640 and 1578 (**Figure I- 7A, B**).

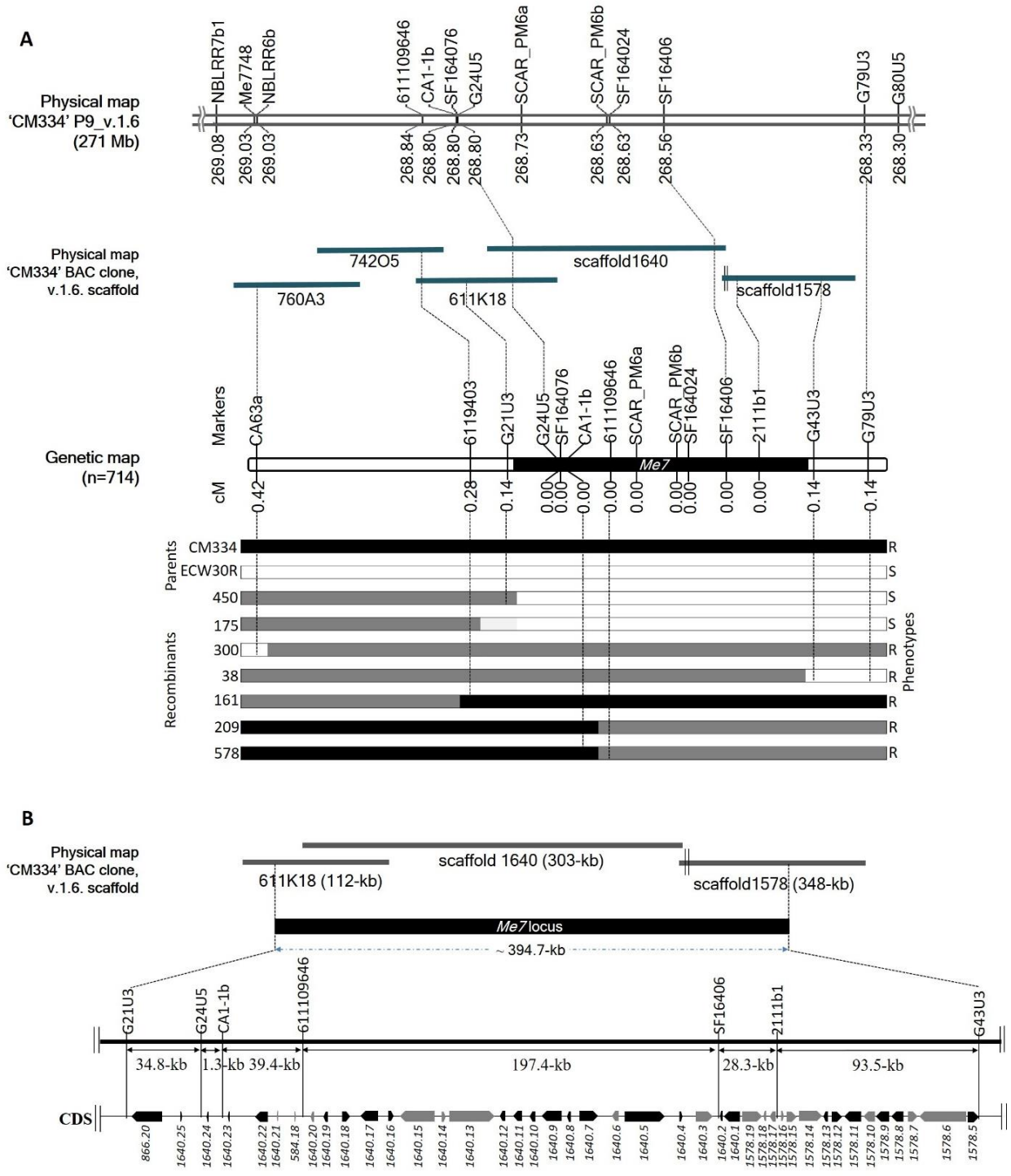
A
Physical map
'CM334' P9_v.1.6
(271 Mb)
NBLRR7b1
Me7748
NBLRR6b
611109646
CA1-1b
SF164076
G24U5
SCAR_PM6a
SCAR_PM6b
SF164024
SF16406
G79U3
G80U5
269.08
269.03
269.03
268.84
268.80
268.80
268.80
268.73
268.63
268.63
268.56
268.33
268.30
Physical map
'CM334' BAC clone,
v.1.6. scaffold
742O5
scaffold1640
760A3
611K18
scaffold1578
Genetic map
(n=714)
Markers
CA63a
6119403
G21U3
G24U5
SF164076
CA1-1b
611109646
SCAR_PM6a
SCAR_PM6b
SF164024
SF16406
2111b1
G43U3
G79U3
Me7
cM
0.42
0.28
0.14
0.00
0.00
0.00
0.00
0.00
0.00
0.00
0.00
0.00
0.14
0.14
Parents
CM334
ECW30R
Recombinants
450
175
300
38
161
209
578
R
S
S
S
R
R
R
R
R
Phenotypes
B
Physical map
'CM334' BAC clone,
v.1.6. scaffold
scaffold 1640 (303-kb)
611K18 (112-kb)
scaffold1578 (348-kb)
Me7 locus
~ 394.7-kb
G21U3
G24U5
CA1-1b
611109646
SF16406
2111b1
G43U3
34.8-kb
1.3-kb
39.4-kb
197.4-kb
28.3-kb
93.5-kb
CDS
866.20
1640.25
1640.24
1640.23
1640.22
1640.21
584.18
1640.20
1640.19
1640.18
1640.17
1640.16
1640.15
1640.14
1640.13
1640.12
1640.11
1640.10
1640.9
1640.8
1640.7
1640.6
1640.5
1640.4
1640.3
1640.2
1640.1
1578.19
1578.18
1578.17
1578.16
1578.15
1578.14
1578.13
1578.12
1578.11
1578.10
1578.9
1578.8
1578.7
1578.6
1578.5

Figure I- 7. Fine mapping of the *Me7* locus. A) The physical position of known and newly developed linked markers to the *Me7* locus on 'CM334' chromosome P9, scaffold v.1.6 and BAC clones is shown. Fine mapping of the *Me7* locus showing the genetic map constructed from 714 F_2 population and the introgression patterns. B) The *Me7* locus was delimited to a ~394.7 kb region (black area) between the G21U3 and G43U5 markers covered by the *C. annuum* 'CM334' BAC contig 611K18, scaffold 1640 and 1578. Gene prediction and BLAST search analysis against Annuum.v.2.0.CDS showed 42 genes in the target region. The genes colored in black and grey are indicating NBS-LRR and non-NBS-LRR genes, respectively.

Prediction of the *Me7* gene candidates and coding sequence (CDS) analysis

A total of 42 CDS were identified from the ~394.7 kb target region (**Figure I- 7B**) covered by scaffold 1640, scaffold 1578, and BAC611K18. BLAST analysis of the corresponding predicted proteins revealed that 25 belonged to the NBS-LRR family and the other 17 were unknown hypothetical proteins (**Table I- 4**). The NBS-LRR candidates contained four different types of other conserved domains, including RX, AAA, RVT_2, or RT (**Table I- 4**). Among the 25 predicted NBS-LRR family genes, many of them are truncated, lack both TIR and CC motifs. Only six NBS-LRR family genes (*1578.8*, *1578.12*, *1640.1*, *1640.7*, *1640.17*, and *1640.22*) were found to be related to CNL type *R* gene class. Furthermore, these NBS-LRR proteins shared sequence similarity with putative late blight resistance proteins including R1A-3, R1A-4, R1A-10, R1B-8, R1B-14, R1B-16, and R1B17 (**Table I- 4**). We thus consider these tightly linked NBS-LRR class resistance (*R*) genes as strong candidates to underlie the RKN resistance conferred by the *Me7* locus.

Table I- 4. Predicted genes from the ~394.7 kb *Me7* locus by BLAST alignment from the predicted CDS Annuum.v.2.0.CDS.

Gene ID	CDS size (bp)	NCBI Conserved domain hits	NCBI Blastn hits: *C. annuum*	Query cover (%)	E value	Identity (%)	GenBank ID
866.20	2634	PLN00113, STKc_IRAK, RNase_HI_RT_Ty1	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 (LOC107856496), mRNA	85	0	92	XM_016701500.1
1640.25	333	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-14 (LOC107840730), mRNA	94	2.00E-68	82	XM_016684605.1
1640.24	333	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-14 (LOC107840730), mRNA	94	2.00E-68	82	XM_016684605.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107841050), mRNA	92	8.00E-63	81	XM_016685060.1
			PREDICTED: putative late blight resistance protein homolog R1A-10 (LOC107840989), transcript variant X1, mRNA	78	1.00E-61	83	XM_016684974.1
1640.23	336	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107876981), mRNA	10	3.00E-156	96	XM_016723782.1
*1640.22**	1908	RX-CC, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107877461), mRNA	99	0	99	XM_016724105.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852551), transcript variant X2, mRNA	99	0	99	XM_016697588.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852551), transcript variant X1, mRNA	99	0.00E+00	99	XM_016697587.1
1640.21	192	PLN02365	-	-	-	-	-
584.18	771	RNase_HI_RT_Ty1	PREDICTED: serine/threonine-protein kinase At5g01020-like (LOC107877496), transcript variant X3, mRNA	18	5.00E-53	93	XM_016724148.1
			PREDICTED: uncharacterized mitochondrial protein AtMg00810-like (LOC107845361), partial mRNA	13	7.00E-47	100	XM_016689638.1
1640.20	525	-	PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107854603), mRNA	99	9.00E-177	88	XM_016699613.1
			PREDICTED: putative late blight resistance protein homolog R1A-10 (LOC107840990), transcript variant X2, mRNA	99	1.00E-174	88	XM_016684976.1
1640.19	669	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107841011), transcript variant X5, mRNA	89	0	94	XM_016684997.1
			PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107841011), transcript variant X4, mRNA	89	0	94	XM_016684996.1
1640.18	1251	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-8 (LOC107852210), mRNA	67	0	94	XM_016697263.1
			PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107841054), mRNA	28	1.00E-113	94	XM_016685063.1
1640.17	2835	RX-CC, NBS-LRR, NBS	PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107854603), mRNA	94	0	88	XM_016699613.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107840770), transcript variant X2, mRNA	94	0	88	XM_016684673.1
*1640.16**	816	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852538), mRNA	100	0	100	XM_016697576.1
1640.15	975	CYCLIN, Cyclin_N2	PREDICTED: uncharacterized LOC107844017 (LOC107844017), ncRNA	18	1.00E-84	98	XR_001666539.1

Gene ID	CDS size (bp)	NCBI Conserved domain hits	NCBI Blastn hits: *C. annuum*	Query cover (%)	E value	Identity (%)	GenBank ID
			PREDICTED: uncharacterized LOC107852165 (LOC107852165), transcript variant X3, ncRNA	47	2.00E-83	98	XR_001669306.1
1640.14	636	TIP49	PREDICTED: uncharacterized LOC107852165 (LOC107852165), transcript variant X3, ncRNA	99	0	99	XR_001669306.1
1640.13	678	-	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852540), mRNA	87	0	100	XM_016697578.1
1640.12	636	NBS-LRR	PREDICTED: late blight resistance protein R1-A-like (LOC107852560), transcript variant X2, mRNA	99	2.00E-156	98	XM_016697593.1
1640.11	1245	AAA, NBS-LRR	PREDICTED: late blight resistance protein R1-A-like (LOC107852560), transcript variant X1, mRNA	76	0	99	XM_016697592.1
*1640.10**	867	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107840741), transcript variant X4, mRNA	29	6.00E-68	85	XM_016684638.1
1640.9	960	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-8 (LOC107852210), mRNA	42	1.00E-159	92	XM_016697263.1
1640.8	450	NBS-LRR	-	-	-	-	-
1640.7	2805	RX-CC, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852559), mRNA	96	0	98	XM_016697590.1
1640.6	333	HemeO	PREDICTED: heme oxygenase 1, chloroplastic-like (LOC107840774), mRNA	98	2.00E-152	96	XM_016684676.1
1640.5	810	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852561), transcript variant X2, mRNA	84	0	100	XM_016697595.1
1640.4	480	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852561), transcript variant X2, mRNA	100	0	99	XM_016697595.1
1640.3	840	-	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852561), transcript variant X2, mRNA	67	0	100	XM_016697595.1
1640.2	363	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107840741), transcript variant X4, mRNA	30	2.00E-33	90	XM_016684638.1
1640.1	2274	RX-CC, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1A-3 (LOC107840762), mRNA	99	0	94	XM_016684661.1
1578.19	1542	HAD_like	PREDICTED: calcium-transporting ATPase, endoplasmic reticulum-type (LOC107839342), transcript variant X7, mRNA	77	0	96	XM_016682783.1
1578.18	447	PPR_2	PREDICTED: pentatricopeptide repeat-containing protein At5g08510-like (LOC107839343), mRNA	100	0	93	XM_016682784.1
1578.17	813	PPR_2	PREDICTED: pentatricopeptide repeat-containing protein At5g08510-like (LOC107839343), mRNA	68	0	95	XM_016682784.1
1578.16	444	-	PREDICTED: probable membrane-associated kinase regulator 6 (LOC107879248), mRNA	99	1.00E-163	90	XM_016726325.1
			PREDICTED: uncharacterized LOC107877479 (LOC107877479), transcript variant X7, ncRNA	52	2.00E-112	98	XR_001676380.1
1578.15	426	TIP49	PREDICTED: uncharacterized LOC107877479 (LOC107877479), transcript variant X7, ncRNA	88	7.00E-107	99	XR_001676380.1
1578.14	1317	CYCLIN, Pox_A6	PREDICTED: uncharacterized LOC107844017 (LOC107844017), ncRNA	22	4.00E-144	98	XR_001666539.1
1578.13	1128	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852212), mRNA	95	0	94	XM_016697264.1
1578.12	2667	RX-CC, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852562), transcript variant X1, mRNA	61	0	98	XM_016697596.1

Gene ID	CDS size (bp)	NCBI Conserved domain hits	NCBI Blastn hits: *C. annuum*	Query cover (%)	E value	Identity (%)	GenBank ID
1578.11	2622	AAA, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1B-8 (LOC107852210), mRNA	48	0	91	XM_016697263.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107852531), mRNA	25	0	97	XM_016697573.1
*1578.10**	1875	-	PREDICTED: putative late blight resistance protein homolog R1B-17 (LOC107852564), mRNA	100	0	100	XM_016697598.1
1578.9	1218	RVT_2, NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1A-10 (LOC107852537), mRNA	84	0	100	XM_016697575.1
			PREDICTED: putative late blight resistance protein homolog R1B-16 (LOC107877460), mRNA	75	0	98	XM_016724104.1
1578.8	3441	RT, RX-CC, NBS-LRR, NBS	PREDICTED: putative late blight resistance protein homolog R1B-17 (LOC107852536), mRNA	49	0	99	XM_016697574.1
1578.7	909	PPR_2	PREDICTED: pentatricopeptide repeat-containing protein At5g08510-like (LOC107839343), mRNA	100	1.00E-158	93	XM_016682784.1
1578.6	2289	HAD_like	PREDICTED: calcium-transporting ATPase, endoplasmic reticulum-type (LOC107839342), transcript variant X7, mRNA	57	0	95	XM_016682783.1
1578.5	1377	NBS-LRR	PREDICTED: putative late blight resistance protein homolog R1A-4 (LOC107852554), mRNA	46	0	100	XM_016697589.1

*Partial CDS

DISCUSSION

Understanding the molecular and genetic mechanisms of RKN resistance is of utmost importance for the development of RKN-resistant pepper lines. Although the RKN resistance gene *Me7* from CM334 was previously mapped to a 3.8 cM genetic interval, its identity remained unknown. The primary aim of current study was to develop a high-resolution map of the *Me7* locus and predict candidate genes. We were able to narrow down the *Me7* locus to an approximately 394.7 kb region covered by the BAC clone 611K18 and unassigned scaffolds 1640 and 1578 (Kim et al., 2014) containing 42 predicted genes. Among these, 25 genes were predicted to belong to the NBS-LRR family of disease resistance genes. The *Me1* gene (*CA09g16830*), which is a homolog of the resistance protein R1A-3 (Wang et al., 2018) belong to the CNL type *R* gene was found to be located approximately 3.3 and 1.1 Mb from the *Me7* locus based on the CM334 v.1.6 (Kim et al., 2017) and UCDv1.0 (Hulse-Kemp et al., 2018) reference genomes, respectively (**Figure I- 8**).

During phenotype screening, we found galls and egg masses of *M. incognita* in the root system of the resistant CM334 variety, in contrast to the previous studies of Pegard et al. (2005), who did not observe egg masses at six weeks after inoculation. In the present study, inoculations were done with 1000 J2 instead of 300 to 600 J2 used in the previous works (Djian-Caporalino et al., 1999, 2001, 2007; Pegard et al., 2005), which may explain the development of egg masses in our experiment. Similarly, occasional egg masses were also reported to be produced on HDA149 pepper line, carrying *Me3* (an allele of *Me7*) after inoculation with >1,000 J2 or ≥3,000 eggs (Castagnone-Sereno et al., 1996; Thies and Ariss, 2009). Thus, evidence from this study and previous research suggest that high inoculum densities (J2 ≥1,000 or eggs ≥3,000), has potential for resistant breaking in populations carrying *Me* (*s*) genes, such

as *Me3* and *Me7* (Castagnone-Sereno et al., 1996; Thies and Ariss, 2009; Djian-Caporalino et al., 2011).

The ability of the nematode to overcome the plants early HR is highly correlates with RKN virulence levels. For instance, in *Me3*-resistant PM687 and HDA149 peppers formation of giant cells are no longer be prevented if cell necrosis in the outer barriers (epidermis and root cortex) has been overcome (Castagnone-Sereno et al., 1996). By contrast, in *Me1* carrying peppers, PM217 and HDA330 defense reactions could takes place even later infection stages, at near or inside the vascular tissues and thus blocks nematode development (Castagnone-Sereno et al., 1996). In the present study, CM334 root tissue cross-sections suggested that it has at least two kinds of resistance to *Meloidogyne* spp.: firstly, resistance that supresses penetration by J2 and secondly, resistance that blocks development after penetration (Pegard et al., 2005). Furthermore, in our histological study, we also observed inhibition of feeding site enlargement and minimal vascular tissue damage in CM334 resistant line, suggesting an extra layer of resistance in CM334 that suppressed feeding site enlargement.

To fine-map the *Me7* locus in pepper, we utilized information from previous mapping studies (Celik et al., 2016; Fazari et al., 2012) in combination with comparative mapping results (**Figure I- 8**) from different versions of pepper genome databases (Kim et al., 2014, 2017; Hulse-Kemp et al., 2018). However, gaps in the genome sequence data and assembly errors due to multiple homologs with high nucleotide similarity hindered marker development and subsequent mapping of the *Me7* locus. BAC library screening has previously been integrated to assist marker development in complicated repetitive genomic regions or unreliable genome sequence assemblies (Jo et al., 2016). Here, by screening a BAC library, we were able to fill gaps between scaffold sequences (1640 and 1578) with a bridge from the BAC611K18 clone, which aided the development of tightly linked *Me7* markers. Furthermore, the release of the

latest pepper genome update (version v.1.6, Kim et al., 2017) also partially allowed the integration of clone 611K18 and unassigned scaffolds 1640 and 1677 into chromosome P9.

A number of genes conferring resistance to parasitic plant nematodes encode NBS-LRR domains. Notably, the cyst nematode-resistance gene *Hero* from tomato (Ernst et al., 2002), *Gro1-4* (Williamson and Kumar, 2006) and *Gpa2* (Sacco et al., 2009; van der Voort et al., 1999) from potato and the RKN-resistance genes, *Mi-1, 2, and 9* from tomato (Milligan et al, 1998; Seah et al., 2004; Jablonska et al., 2007), pepper RKN-resistance genes, *CaMi* which is homologous to *Mi1.2* (Chen et al., 2007) and *Me1,* the homolog of putative late blight resistance protein R1A-3 gene (Wang et al., 2018) and *Ma* from *Prunus* spp. (Claverie et al., 2011) are reported to encode NBS-LRR proteins (Milligan et al., 1998; van der Vossen et al., 2000; Paal et al., 2004; Claverie et al., 2011). Similarly, we identified a cluster of 25 genes that were predicted to belong to the NBS-LRR *R* gene family, and which are strong candidates to be the *Me7* gene. Several plant NBS-LRR class *R* genes cluster at specific genomic locations due to tandem and/or segmental duplications in the course of evolution (Hulbert et al., 2001; Leister, 2004; Huang et al., 2005; McDowell and Simon, 2006). For instance, dominant *R* genes such as *Ph-3* from tomato, *R1* from potato, and *RpsUN1*, *RpsUN2*, and *Rpg1-b* from soybean (Ballvora et al., 2002; Ashfield et al., 2003; Zhang et al., 2014; Li et al., 2016) belong to NBS-LRR domain-encoding complex *R* gene clusters. *R* gene clusters could be sources of novel *R* genes, as such clusters enhance the possibility of structural and copy number variation arising through various molecular mechanisms (Michelmore and Meyers, 1998; Torii, 2004; Nagy and Bennetzen, 2008). The RKN resistance-related *R* gene clusters identified in this study could be associated with RKN resistance in CM334. However, further studies are required to reveal the specific NBS-LRR gene encoding the *Me7* gene.

In conclusion, we successfully delimited the *Me7* locus to a genomic region of approximately 394.7 kb. We developed a total of 28 markers linked to the *Me7* locus and

identified nine markers co-segregating with RKN resistance. Notably, the newly-delimited *Me7* target region included a cluster of 25 NBS-LRR class candidate *R* genes. The tightly linked *Me7* markers will facilitate MAS in pepper breeding programs as well as assisting with mapping of other nematode-resistant genes in *C. annuum*.

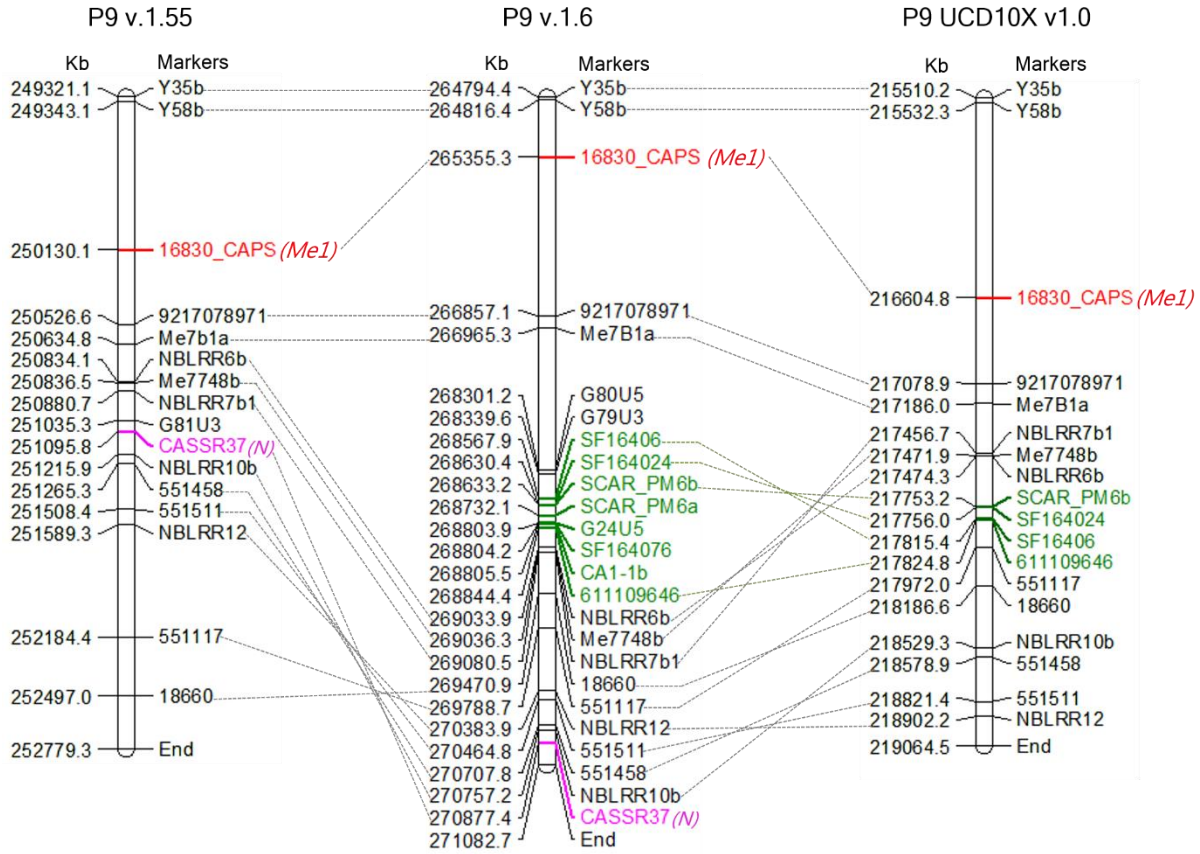

Figure I- 8. Comparative analysis of the *Me7* linked markers on P9 from three genome references. The physical position of the *Me7* linked markers on 'CM334' P9 version v.1.55, v.1.6 and UCD10X v1.0 (Kim et al., 2014, 2017; Hulse-Kemp et al., 2018) is shown. BLAST alignment was performed using CLC Main Workbench 8.1 (QIAGEN, Aarhus, Denmark), where map reads with ≥ 98% nucleotide similarity are shown. Markers that co-segregated with the *Me7* locus in the screening with 714 F_2 individuals are represented in green, *Me1* gene based markers presented in red and the *N* locus linked marker showed in pink.

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CHAPTER II

Bulked Segregant RenSeq Analysis Pinpoints Candidate Genes for the Root-Knot Nematode (*Meloidogyne incognita*) Resistance Gene *Me7* in Pepper (*Capsicum annuum*)

ABSTRACT

C. annuum CM334 carries the *Me7* locus for the resistance to root knot nematode (RKN) *M. incognita*. Fine mapping of the *Me7* locus delimited it to a 394.7 kb region on chromosome 9 of *C. annuum*. Among the 42 genes identified from the *Me7* target region in CM334, only 30 genes were predicted in the corresponding region of *C. annuum* Dempsey. In the present study, RenSeq analysis was performed to detect the sequence variations of NBS-LRR like sequences captured using baits designed from 755 NBS-LRR genes of *Capsicum* CM334 reference (v.1.55). BSA using RenSeq baits enabled to detect significant Δ(SNP-index) value <-0.3 linked to the *Me7* locus. A total of 492 SNPs/InDels were detected in 224.92 – 270.94 Mb on P9 at the significance level of 99%. Among the variants, 104 SNPs/InDels were located in 16 candidate genes in the target region. The sizes of candidate genes ranged 0.192 kb to 2.835 kb. Among the 16 candidate genes, two CNL gene types (*1640.1* and *1640.7*) contained missense, nonsense and InDel mutations, whereas three non-CNL genes (*1640.6, 1640.15* and *1640.21*) had only missense mutations. RT-PCR analysis of the *1640.6* gene enhanced expression in the resistant plant. The sequence analysis of the full-length CDS revealed a SNP in the *1640.7* gene causing a stop codon in the susceptible parent. In addition, a 30 bp in-frame InDel was detected in exon 1 in the susceptible parent. The *1640.7* allele of CM334 encodes a CNL type RX-CC-NBS-LRR like protein with a size of 899 aa, whereas the susceptible allele of ECW30R encodes 502 aa due to the nonsense mutation in the first exon. Further functional characterization study of these candidate genes via transgenic or gene silencing approaches is required to confirm their clear roles in RKN resistance.

INTRODUCTION

To understand the mechanism of the *Me7* gene mediated resistance, cloning of *Me7* will be a key step. However, the complexity of large genome size and genome assembly errors in the current reference genomes hinder precise identification of disease resistance genes (Jupe et al., 2012). Whole genome sequence information of the important plants enabled the prediction of NBS-LRR genes on a genome-wide scale, which aided in the mapping of the NBS-LRR genes to a considerable extent in crop breeding for disease resistance. Recent development of targeted sequencing methods using RenSeq and SMART-RenSeq sequencing methods hold a great promise in accurate identification of disease resistance genes in plants with large complex genomes (Jupe et al., 2012, 2013; Witek et al., 2016).

RenSeq allows genome complexity reduction and enrichment of NBS-LRR type disease resistance genes in fully or partially assembled genomes (Jupe et al., 2012, 2013; Witek et al., 2016) and has been utilized for reannotation and identification of NBS-LRR type *R* genes of Solanaceae plants, such as tomato, potato, and tobacco (Jupe et al., 2013; Andolfo et al., 2014). For instance, the number of NB-LRRs identified increased from 438 to 755 the RenSeq analysis in potato (Jupe et al., 2013). The RenSeq method exploits biotinylated 120-mer RNA baits to capture fragmented gDNA sequence of the NBS-LRR complements in a plant. The captured gDNA library can be sequenced with sufficient depth for NBS-LRR genes.

BSA is an effective method to identify the target region on the genome, which is responsible for a given phenotype (Giovannoni et al., 1991; Michelmore et al., 1991). Combined use of BSA and next-generation sequencing technologies leads to acceleration of the identification of candidate genes controlling traits of interest in various crops (Trick et al., 2012; Livaja et al., 2013; Takaji et al., 2013; Win et al., 2017). Combining BSA with NBS

profiling through RenSeq analysis is a powerful tool to locate the genomic position as well as of structure of *R* locus, thus a starting point for cloning of the gene (Michelmore et al., 1991; van der Linden et al., 2004; Pel et al., 2009).

In this study, we used RenSeq method combined with BSA for accurate prediction of NBS-LRR genes in the *Me7* locus. The sequence variations identified through RenSeq were validated by Sanger sequencing and subsequently reannotated some putative *Me7* candidate genes. Two candidate genes *1640.6* and *1640.7* are proposed to be involved in RKN resistance response, including programmed cell death (*1640.7*) and suppression of feeding site enlargement (*1640.6*). Further functional characterization of these candidate genes via transgenic approaches using Micro-Tom as the heterologous expression host is warranted to elucidate their clear roles in RKN resistance.

MATERIALS AND METHODS

Comparative analysis of the *Me7* locus

Syntenic analysis of the *Me7* locus was performed using the previous syntenic map of the *Me7* locus in **Chapter I** (CM334 v.1.6; Kim et al., 2017) with a new *C. annuum* genome reference 'Dempsey' (v1.0, unpublished), which shared the sequence similarity with the susceptible parent ECW30R. CLC Main Workbench 8.1 (QIAGEN, Aarhus, Denmark) was used to obtain the physical position of the *Me7* linked markers (**Chapter I, Table I- 2**), where map reads with ≥ 96% nucleotide similarity to the genomic sequence on Dempsey reference genome were used for comparative mapping. The genetic linkage map was drawn using the MapChart 2.2 tool (Voorrips, 2002). BLAST search physical positions of the absent genes were performed against three reference genomes (Zunla-1 v2.0; Qin et al., 2014, CM334 UCD10X v.1.0; Hulse-Kemp et al., 2018 and Dempsey v1.0)

RenSeq library construction

F_2 plants derived from the cross between 'ECW30R' and 'CM334' were genotyped using three co-segregated markers (SCAR_PM6a, SF164076, and SF164024) and three linked markers (Me7748b, Me7b1a, and 551511) (**Chapter I, Table I- 1, I- 2**). Resistant and susceptible plants were selected based on genotyping analysis of the F_2 population. Equal molar amount of high quality DNA from 20 resistant and 20 susceptible plants were pooled. CM334 and ECW30R were used as the references. The RenSeq library preparation and enrichment were carried out at Macrogen (https://dna.macrogen.com/eng/). Briefly, Illumina MiSeq libraries were prepared using the NEBNext Ultra DNA library prep kit (NEB, Massachusetts, USA) according to the manufacturer's protocol. NBS-LRR sequences were captured using 120-mer baits tiled across

each locus with a 60-mer overlap designed from 755 *Capsicum* NBS-LRR in *Capsicum* database (v.1.55; Seo et al., 2016).

Reference-based SNPs/InDels calling and candidate genes selection

To identify sequence variations in NBS-LRR loci, RenSeq were mapped to the 'CM334' genome v.1.6 sequence (Kim et al., 2017. For variant calling and filtering, the GWTK Unified Genotyper v3.3-0 was utilized (DePristo et al., 2011). SNPs from RenSeq sequence data were filtered with a minimum sequence quality of 30 and a minimum read depth of 20. The variations were processed as Variant Call Format (VCF) output file provided with the SNPs/InDels physical position and read depth on the genome reference. The ratio of total SNPs or Indels with >0.7, 0.3-0.7, and <0.3 were scored as paternal, heterozygous and maternal genotypes, respectively (Han et al., 2016). The variations were further investigated using the VCF output data (annotated with the CM334 Annuum.v.2.0.CDS data; Annuum.v.2.0.CDS.gff3.gz; http://peppergenome.snu.ac.kr/download.php) by SnpEff program (http://snpeff.sourceforge.net/SnpEff_manual.html#input) as described by Cingolani et al. (2012).

Gene structure prediction

The gene coding sequences of CM334 (Annuum.v.2.0.CDS) were retrieved from the pepper genome data v.1.6 (Kim et al., 2017). For selected candidate genes at the *Me7* locus, gene structures were predicted using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). The potential annotated gene based on RenSeq sequence alignment were predicted using AUGUSTUS gene prediction program (Stanke and Morgentern, 2005).

RNA extraction and complementary DNA (cDNA) synthesis

Four-leaf stage resistance and susceptible parental lines, CM334 and ECW30R, were inoculated with 1,000 freshly hatched J2 *M. incognita* as mentioned in **Chapter I**. The non-inoculated parental lines were used as controls. Inoculated plants along with controls were kept in the glasshouse maintained at 26±2℃. Root samples were collected at five and seven days after inoculation (dai). Total RNA was isolated from roots of both inoculated and non-inoculated plants using the miniBEST plant RNA extraction kit (Cat. no. 9769, TAKARA, Shiga, Japan) according to the manufacturer's procedures.

Two µg of total RNA was reverse transcribed to synthesize cDNA using the SMARTScribe^{TM} Reverse Transcriptase (Cat. no. 639536, TAKARA, California, USA). cDNA samples were diluted four-fold with sterile water for the subsequent experiments.

Semi-quantitative RT-PCR analysis

Gene specific primers to amplify the selected candidate genes were designed based on the CDS sequence from the CM334 v.1.6 (Kim et al., 2017). The specificity of primers was tested with gDNA PCR prior to RT-PCR analysis. For RT-PCR analysis, *CaActin* was used a positive internal control (**Table II- 1**). The RT-PCR assays were performed as described by Liu et al. (2016). The PCR and RT-PCR products were analyzed on 1% agarose gels.

Table II- 1. List of primers used in gDNA PCR/RT-PCR analyses.

Gene name	Variant	Name of marker	Primer sequence	gDNA expected size	cDNA expected size
1640.1	Nonsense	CAPS_NLR2_F	F: ATCGCAAAGGCATACGTGAA	2110 bp	1958 bp
		SF1677-16_R	R: TCAAGTTGCTGCAGATGGAC		
1640.6	Missense	1640.6start_F	F: ATGGGATTAGAAAGGTCAGAG	100 bp	100 bp
		1640.6ex1_R	R: TATAGCTGACACCAGGAGTT		
1640.7	Nonsense, InDel	1640.7e_F	F: TAGAACGGGTTCGTTTCAAA	1342 bp	1342 bp
		CAPS_NLR16_F	R: ATGGATCGTATCTGGAAAGA		
1640.15	Missense	1640.15start_F	F: ATGTATGATGTTTTTGTTAACTT	98 bp	98 bp
		1640.15ex1_R	R: TAGTTCACATCTGCAGCA		
1640.21	Missense	1640.21_F	F: ATGGCTGGAGACAGTGAT	162 bp	162 bp
		1640.21_R	R: AGTGCCTGCTTTTTGGAC		
	positive internal	*CaActin**	F: AGGAATGGTTAAGGCTGGATTT	-	200 bp
	control	*CaActin*	R: TCTTCTCCATATCGTCCCAGTT		

F= forward, R= reverse,*= Koeda et al., 2012

Sequence validation and full-length gene sequencing

According to the RenSeq analysis, gene-specific primers for the putative candidate genes were designed (**Table II- 2**). The PCR assays were performed as described by Jo et al. (2016). The PCR products were purified using PCR Purification Kit (Cosmogenetech, Seoul, Korea). The identified SNPs were verified thought sequencing of the gDNA and cDNA PCR products using Sanger sequencing at Macrogen (https://dna.macrogen.com/eng/).

The candidates which fail to obtain the sequence information from the PCR product sequencing were TA cloned. The primers used for amplification of the target regions are listed in **Table II- 2**. The PCR cycling conditions were performed as described (Jung et al., 2019) with modifications of annealing temperature of 55℃ and extension for 2 min. The PCR products were TA cloned using Mighty TA-cloning Kit (Cat. #6028, TAKARA, Shiga, Japan). The ligation products were transformed to Trans5α chemically competent cell (TransGen Biotech, Beijing, China). Blue/white colony screening and colony PCR were performed for selection of positive clones. The positive colonies were sequenced using universal primer M13F-puc and M13R-puc at Macrogen (http:// macrogen.com/kor/).

The primers used in full-length amplification and sequencing are listed in **Table II- 3**. The full-length gene was obtained by aligned the gene short sequences using SeqMan program (SeqMan NGen® . Version 12.0. DNASTAR. Madison, WI.)

Table II- 2. Primers used in sequence analysis of the *Me7* candidate genes.

Gene ID	Name of marker		Primer sequence	Size (gDNA)	Sequencing
1640.1	HRM_268557578_F	F:	TTACAGTATGCCCGTGACTG	630 bp	PCR product
	SF1677-16_R	R:	TCAAGTTGCTGCAGATGGAC		
1640.6	1640.6U5b_F	F:	TCAACAGTAGCGGTACCT	462 bp	PCR product
	1640.6ex1_R	R:	TATAGCTGACACCAGGAGTT		
1640.7	1640.7utr3_F	F:	ACATGGGAGATCGAGGTCTT	3180 bp	Plasmid
	HRM_G36U5_R	R:	GTTGCATAACTGACCTGAAC		

F= forward, R= reverse

Table II- 3. Primers used in full-length investigation and full sequence analysis of the candidate gene *1640.7*.

Purpose	Name of marker		Primer sequence	Amplicon size
Full-length primer set 1	CAPS_NLR2_F	F:	ATCGCAAAGGCATACGTGAA	3039 bp
	1640.7utr3_F	R:	ACATGGGAGATCGAGGTCTT	
Full-length primer set 2	CAPS_NLR2_F	F:	ATCGCAAAGGCATACGTGAA	3053 bp
	G36exon3_F	R:	AGGGTTATGAACTAGCATCA	
Full-length primer set 3	CAPS_NLR2_F	F:	ATCGCAAAGGCATACGTGAA	3268 bp
	G36U3_F	R:	TAGACCCTGGTCCAATAGAT	
Walking primer	M13F_pUC*	F:	GTTTTCCCAGTCACGAC	~1000 bp
Walking primer	M13R_pUC*	R:	CAGGAAACAGCTATGAC	~1000 bp
Walking primer	Pr1-4_F	F:	GTTTGTGCCTGGGCTACTGT	~1000 bp
Walking primer	Pr1-5_F	F:	TTTGAAACGAACCCGTTCTA	~1000 bp
Walking primer	1640.7in_F	R:	AAACCCCATGTGACTCCCTT	~1000 bp

* universal primers

Identification of suitable susceptible host for heterologous expression of the *Me7* candidate genes

To identify the suitable host for functional characterization of the candidate genes by transgenic approach, *Nicotiana benthamiana, S. lycopersicum* cv. Micro-Tom, and commercial tomato lines were inoculated with RKN. The nematode inoculation and resistance screening were performed as described in **Chapter I**. The average number of galls were calculated from roots of three plants.

RESULTS

Comparative analysis of the *Me7* locus

The BLAST result showed that the *Me7* linked markers on chromosome P9 (v1.6; Kim et al., 2017) corresponding to Dempsey superscaffold 10 (v1.0) (**Figure II- 1**). Moreover, the markers linked to CM334 scaffolds 1578 and 1640, and BAC clone 611K18 were also found in this scaffold (**Figure II- 1, Chapter I; Table 1- 2**). The number of linked markers were increased from 24 markers on CM334 P9 to 29 markers on Dempsey scaffold 10. The two *Me7* flanking markers, G21U3 and G43U5, were aligned to the scaffold 10 at the positions of 268,206 kb and 268,370 kb, respectively (**Figure II- 2A**), delimiting the *Me7* locus to ~198 kb (**Figure II- 1, Figure II- 2A**).

The comparative sequence analysis between CM334 and Dempsey reference genomes were performed for the presence and absence of predicted genes at the *Me7* locus. When 42 candidate genes were detected at the ~394.7 kb target region of CM334 chromosome P9, only 30 genes were predicted at the corresponding Dempsey region spanning about 198 kb. Therefore, 12 candidates predicted in CM334 were missing in Dempsey (**Figure II- 2B**). The 12 absent genes were further investigated to identify their locations in three *C. annuum* reference genomes, 'Dempsey v0.1', 'CM334 UCD10X', and 'Zunla-1 v2.0', and all 12 genes were located at different physical positions (**Table II- 4**), indicating that the additional genes could be potentially inserted in the *Me7* locus ~394.7 kb region of CM334 possibly by erroneous annotation or miss assembly of similar sequences. Therefore, these 12 genes were ruled out being the *Me7* candidate genes.

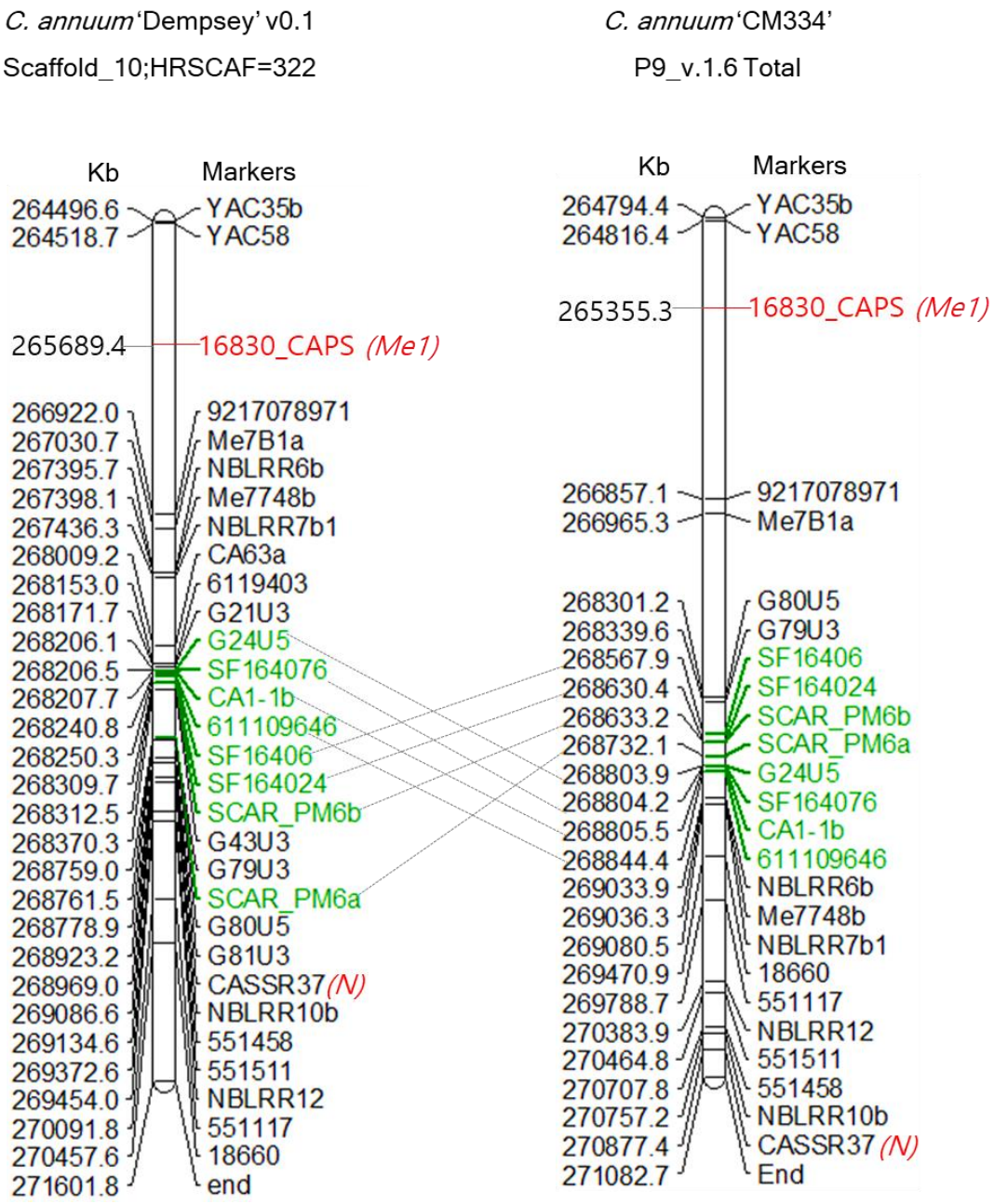

Figure II- 1. Comparative analysis of the *Me7* linked marker positions between CM334 and Dempsey reference genomes. The physical positions of the *Me7* linked markers on Dempsey v1.0 scaffold 10 and 'CM334' P9 v.1.6 (Kim et al., 2017) are shown. BLAST alignment was performed using CLC Main Workbench 8.1 (QIAGEN, Aarhus, Denmark), where map reads with ≥ 96% nucleotide similarity are shown. Markers that co-segregated with the *Me7* locus in the 714 F_2 population are represented in green, the *Me1* gene-based markers presented in red, and *(N)* is the marker linked to the *N* gene.

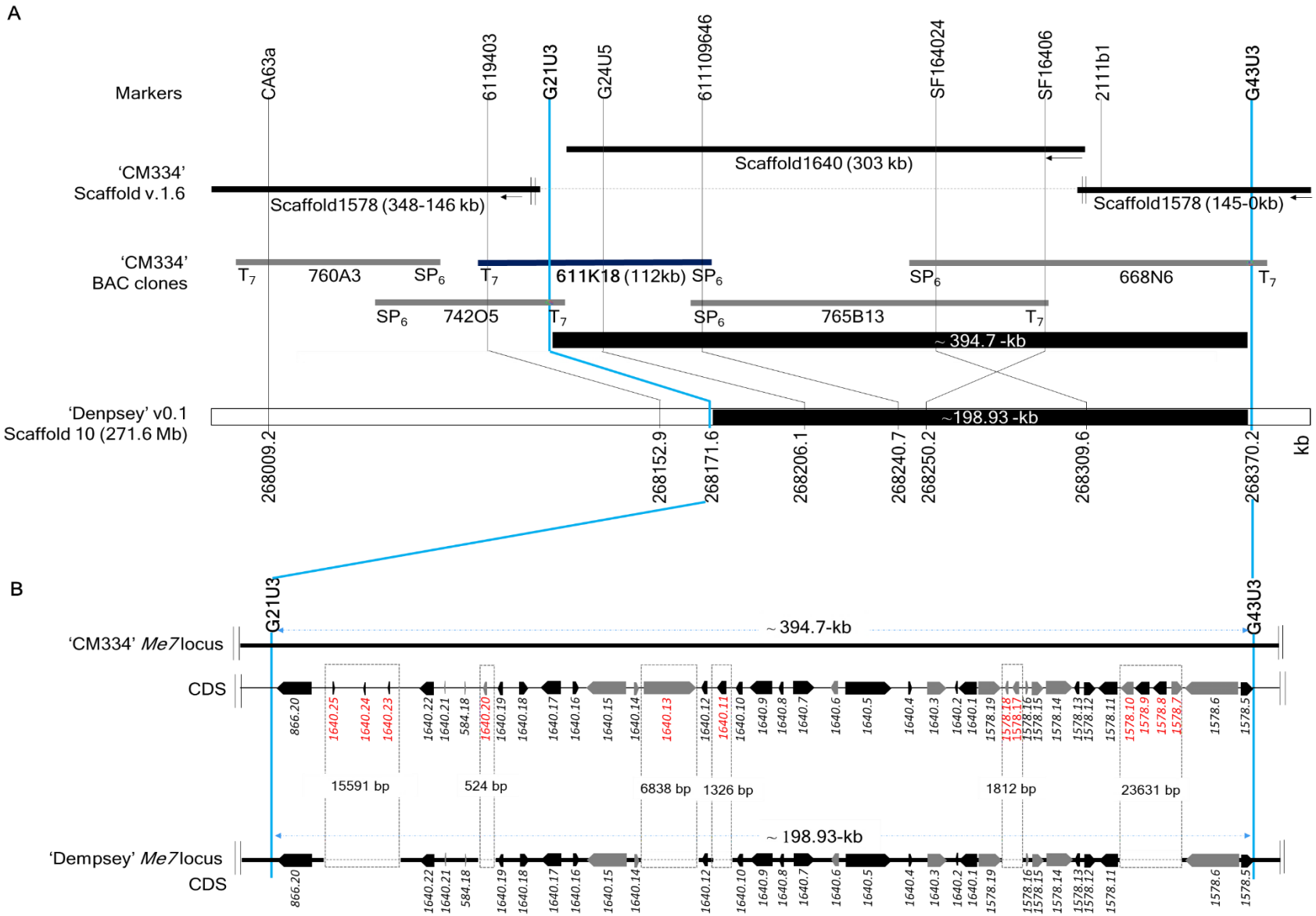
A
Markers
CA63a
6119403
G21U3
G24U5
611109646
SF164024
SF16406
2111b1
G43U3
'CM334' Scaffold v.1.6
Scaffold1640 (303 kb)
Scaffold1578 (348-146 kb)
Scaffold1578 (145-0kb)
'CM334' BAC clones
T7
760A3
SP6
T7
611K18 (112kb)
SP6
SP6
668N6
T7
SP6
742O5
T7
SP6
765B13
T7
~ 394.7 -kb
'Denpsey' v0.1 Scaffold 10 (271.6 Mb)
~198.93 -kb
268009.2
268152.9
268171.6
268206.1
268240.7
268250.2
268309.6
268370.2
kb
B
G21U3
G43U3
'CM334' Me7locus
~ 394.7-kb
CDS
866.20
1640.25
1640.24
1640.23
1640.22
1640.21
584.18
1640.20
1640.19
1640.18
1640.17
1640.16
1640.15
1640.14
1640.13
1640.12
1640.11
1640.10
1640.9
1640.8
1640.7
1640.6
1640.5
1640.4
1640.3
1640.2
1640.1
1578.19
1578.18
1578.17
1578.16
1578.15
1578.14
1578.13
1578.12
1578.11
1578.10
1578.9
1578.8
1578.7
1578.6
1578.5
15591 bp
524 bp
6838 bp
1326 bp
1812 bp
23631 bp
'Dempsey' Me7locus
~ 198.93-kb
CDS
866.20
1640.22
1640.21
584.18
1640.19
1640.18
1640.17
1640.16
1640.15
1640.14
1640.12
1640.10
1640.9
1640.8
1640.7
1640.6
1640.5
1640.4
1640.3
1640.2
1640.1
1578.19
1578.16
1578.15
1578.14
1578.13
1578.12
1578.11
1578.6
1578.5

Figure II- 2. The sequence alignment of the *Me7* locus of CM334 and Dempsey reference genomes. A). The comparative physical mapping of the *Me7* locus among CM334 scaffolds, BAC contigs and Dempsey scaffold 10. B) BLAST alignment of the 42 predicted candidate genes from the *Me7* locus of ~394.7 kb region against ~198 kb corresponding region on Dempsey scaffold 10. Dotted boxes indicate genes present and absent.

Table II- 4. BLAST search of the 12 absent genes against three reference genomes.

	Genome	**Dempsey**		**UCD10X**		**Zunla-1**	
No.	CDS	Location	Identity (ID %)	Location	Identity (ID %)	Location	Identity (ID %)
1	*1578.7*	Scaffold_10	95.364	Chr00	98.57	Chr00	99.84
2	*1578.8*	Scaffold_10	94.918	Chr00	99.14	Chr00	99.38
3	*1578.9*	Scaffold_4	97.947	Chr00	100	Chr00	100
4	*1578.10*	Scaffold_4	97.947	Chr00	100	Chr00	99.81
5	*1578.17*	Scaffold_10	97.388	Chr00	95.18	Chr00	93.36
6	*1578.18*	Scaffold_10	97.704	Chr00	95.37	Chr07	95.55
7	*1640.11*	Scaffold_10	94.359	Chr00	99.29	Chr00	99.93
8	*1640.13*	Scaffold_4	91.442	Chr00	99.82	Chr00	99.80
9	*1640.20*	Scaffold_10	97.976	Chr00	98.67	Chr00	99.94
10	*1640.23*	Scaffold_4	95.276	Chr00	99.68	Chr07	95.11
11	*1640.24*	Scaffold_4	98.05	Chr00	98.07	Chr07	98.05
12	*1640.25*	Scaffold_10	99.46	Chr00	97.80	Chr09	99.46

Bulked segregant analysis using RenSeq

RenSeq data of CM334, ECW30R, F_2 resistant and susceptible bulks together with CM334 v.1.6 references were used to find significant SNPs between two bulks. The Δ(SNP-index) value less than 0 were used to detect the significantly different SNP-index regions. At the 95% significance level, two genomic regions on P2 and P9 were detected (**Figure II- 3**). At the 99% significance level, only one genomic region on P9 (224.92 – 270.94 Mb) was detected with less than -0.3 indicating the position is correlated with the *Me7* resistance (**Figure II- 3**). A total of 492 SNPs were detected at the region of approximately 46.02 Mb with an average of 11 SNPs/ Mb (**Table II- 5**).

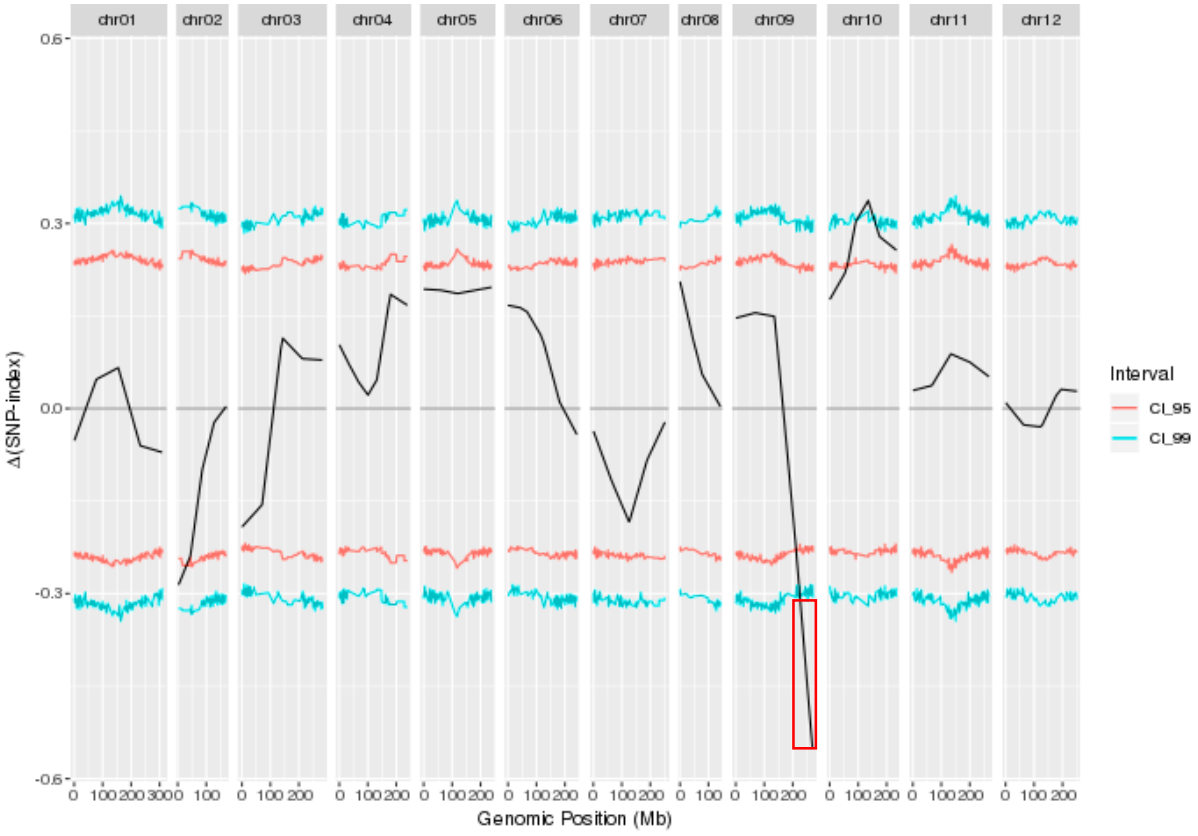

Figure II- 3. The SNP-index graphs of BSA using RenSeq on pepper chromosomes. The Δ(SNP-index) less than 0 indicates the allele contributing to the trait of the reference parents, while > 0 indicates alternate alleles. Red rectangular box shows P9 distal end passed the 99% confidence interval thresholds.

Table II- 5. SNPs detected at pepper P9 long arm from BSA using RenSeq.

Location	Start	End	Length	No. of SNPs	Avg. SNPs/ Mb	Confidence
chr09	213,676,586	270,941,986	57,265,400	501	9	95%
chr09	224,921,242	270,941,986	46,020,744	492	11	99%

Identification and analysis of SNPs and InDels in RenSeq reads

The 30 candidate genes for *Me7* were further analyzed to identify significant SNPs or InDels. A total of 104 significant SNPs/InDels variations were detected in only 16 candidate genes. Among the genes, 13 genes are belonging to NBS-LRR type *R* gene (**Table II- 6**). BLAST search results confirmed all of the candidates shares sequence similarity to the 755 pepper NBS-LRR genes (**Table II- 6)**.

The candidate genes were evaluated for the presence of SNPs and InDels that causing amino acid sequence variations. Three candidate genes including *1640.1, 1640.7* and *1640.16* contained SNPs leading to missense or nonsense mutations. The nonsense mutations were found in the candidate *1640.1* and *1640.7* located at 268,557,685 and 268,633,326, respectively. The gene *1640.7* additionally contained InDels at the position 268,632,957 (**Table II- 7**). The two candidate genes *1640.1* and *1640.7* are encoding CNL gene type *R* gene containing RX-CC-NBS-LRR conserved domains. These two CNL candidate genes contained nonsense considered as the strong candidate genes for RKN resistance.

Three non-CNL gene types (*1640.6, 1640.15* and *1640.21*) were found to have missense mutations (**Table II- 6, 8)**. The BLAST analysis of these genes revealed three different types of conserved domains including HemeO (Heme oxygenase-1), CYCLIN (Clyclin_N2) and PLN02365 (2-oxoglutarate-dependent dioxygenase), respectively **(Table II-6)**. HemeO is known as a rate-liming enzyme in the degradation of heme to biliverdin IXα, carbon monoxide (CO), and free iron ions (Fe^{2+}), oxidative stress response and cellular signaling. CYCLIN functions in cell-cycle and transcription control. PLN02365 is involved in various oxygenation/ hydroxylation reactions, such as cell wall protein synthesis. These three candidate genes could be involved in the suppression of establishment and/or enlargement of feeding sites.

Table II- 6. SNPs in the candidate genes and conserved domains in the genes.

Gene ID (RenSeq)	Number of SNPs	Gene ID (Annuum.v.2.0.CDS)	CDS size	NCBI Conserved domain hits	NBS-LRR gene models[b]						
					Hit	Type	Group	Class	Hit length (bp)	E-value	Identity (%)
1578.5	2	*1578.5*	1377	NBS-LRR	*CA00g73680*	NBS	NGpG2	S	603	0	100
1640.1	9	*1640.1*	2274	RX-CC, NBS-LRR	*CA09g17570*	CC-NBS-LRR	G2	C2	1377	0	90.81
1640.2	13	*1640.2*	363	NBS-LRR	*CA06g00850*	CC-NBS	NGpG2	C74	216	0	90.28
					CA06g01050	CC-NBS	NGpG2	C74	216	0	90.28
1640.3	7	*1640.3*	840	-	*CA09g17570*	CC-NBS-LRR	G2	C2	565	0	96.64
1640.4	3	*1640.4*	480	NBS-LRR	*CA00g76310*	NBS	NGpG2	C66	480	0	100
-		*1640.5*	810	NBS-LRR	*CA07g21630*	NBS-LRR	G2	C2	460	0	98.04
1640.5-1640.6	2	-	-	-		-	-			-	-
1640.6	5	*1640.6*	333	HemeO	*CA09g16920*	CC-NBS-LRR	G10	C18	24	8.16E-03	91.67
1640.7	26	*1640.7*	2805	RX-CC, NBS-LRR	*CA00g76340*	CC-NBS-LRR	G2	C2	2697	0	100
1640.7-1640.8	2	-	-	-		-	-			-	-
1640.8	1	*1640.8*	450	NBS-LRR	*CA00g76360*	CC-NBS	NGpG2	S	450	0	100
1640.9	4	*1640.9*	960	NBS-LRR	*CA07g00840*	NBS-LRR	G2	C2	412	0	99.71
1640.15	4	*1640.15*	975	CYCLIN, Cyclin_N2	*CA07g08810*	NBS	GT	C95	20	0.3	95
1640.16	12	*1640.16*[a]	816	NBS-LRR	*CA00g76440*	NBS	G2	C2	816	0	100
1640.17	2	*1640.17*	2835	RX-CC, NBS-LRR, NBS	*CA09g17710*	CC-NBS-LRR	G2	C2	2677	0	87.59
1640.18	2	*1640.18*	1251	NBS-LRR	*CA00g76460*	NBS-LRR	G2	C2	1251	0	100
1640.18-1640.19	3	-	-	-		-	-			-	-
1640.19	6	*1640.19*	669	NBS-LRR	*CA00g76470*	NBS	NGpG2	S	669	0	100
1640.21	1	*1640.21*	192	PcbC	*CA03g02140*	NBS-LRR	G10	C12	15	0.67	100
					CA02g10730	CC-NBS-LRR	Ngfull	C58	15	0.67	100

[a]Partial CDS; [b]Seo et al., 2016

Table II- 7. Sequence variations causing amino acid mutations in the CNL type candidate genes.

Candidate	Position (P9)	SNP	Strand	CM334	ECW30R	Bulked-R	Bulked-S	Variant
1640.1	268,557,685	C/A	+	0/0	1/1	0/0	1/1	Stop codon
				(721/0)	(4/1388)	(1164/3)	(100/1172)	(GT/AD)
	268,557,759	G/A	+	0/0	1/1	0/0	0/1	Stop codon
				(717/0)	(0/291)	(1363/7)	(108/300)	(GT/AD)
1640.7	268,633,326	G/A	-	0/0	1/1	0/0	1/1	Stop codon
				(899/1)	(3/2192)	(1581/57)	(73/1535)	(GT/AD)
	268,632,957	TATGTCTAAATTCTCATGGCAGTGCAATGA	-	0/0	1/1	0/0	0/1	a 30 bp InDel
				(785/0)	(0/872)	(1426/35)	(98/848)	(GT/AD)
1640.16	268,761,448	C/T	-	0/0	1/1	0/0	0/1	Stop codon
				(766/0)	(6/660)	(1566/14)	(124/564)	(GT/AD)

Homologous= 0/0 and 1/1, Heterozygous= 0/1, GT/AD= Genotype/ Alignment depth

Table II- 8. Missense variations of non-CNL type candidate genes

Candidate	Position (P9)	SNP	Strand	CM334	ECW30R	Bulked-R	Bulked-S	Variation
1640.6	268,629,837	C/G	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.6	268,630,297	T/C	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.6	268,630,298	T/G	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.6	268,630,305	A/G	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.6	268,630,447	A/T	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.15	268760,162	C/T	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.15	268,760,190	C/T	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.15	268,760243	G/T	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.15	268,760,306	T/G	+	0/0	1/1	0/0	1/1	downstream gene variant
1640.21	268,812,769	T/C	-	0/0	1/1	0/0	1/1	downstream gene variant

Homologous: 0/0, 1/1; Heterozygous: 0/1

Confirmation of sequence variations in candidate genes

The candidate gene specific primers (**Table II-1**) were designed to for the five selected candidate genes (*1640.1*, *1640.6*, *1640.7*, *1640.15* and *1640.21*). Primer specificity was tested by gDNA PCR and expected sizes of amplicons were obtained (**Figure II- 4A**). RT-PCR analysis revealed that only four candidate genes were expressed (*1640.1*, *1640.6*, *1640.7* and *1640.21*). *1640.1* and *1640.7* did not show expression differences between the resistant line CM334 and the susceptible line ECW30R, whereas *1640.6* was highly expressed in CM334 in both non-inoculated and RKN inoculated plants, and *1640.21* was expressed only in ECW30R (**Figure II- 4B**).

Sequencing results revealed a 30 bp InDel at the position -169 from the start codon of the *1640.6* gene (**Figure II- 4C**, **Table II- 2**). Predicted SNPs and InDels in the candidate genes *1640.1* and *1640.7* were confirmed by sequence analysis of gDNA amplicons and a 30 bp InDel mutations were also confirmed in the candidate gene *1640.7*. By contrast, *1640.1* had only nonsynonymous mutations (**Figure II- 4C, Table II- 2**). These analyses prompted us to propose that the CNL type *1640.7* and non-CNL type *1640.6* could be the strong candidates for RKN-mediated resistance and suppression of feeding site enlargement, respectively, in CM334.

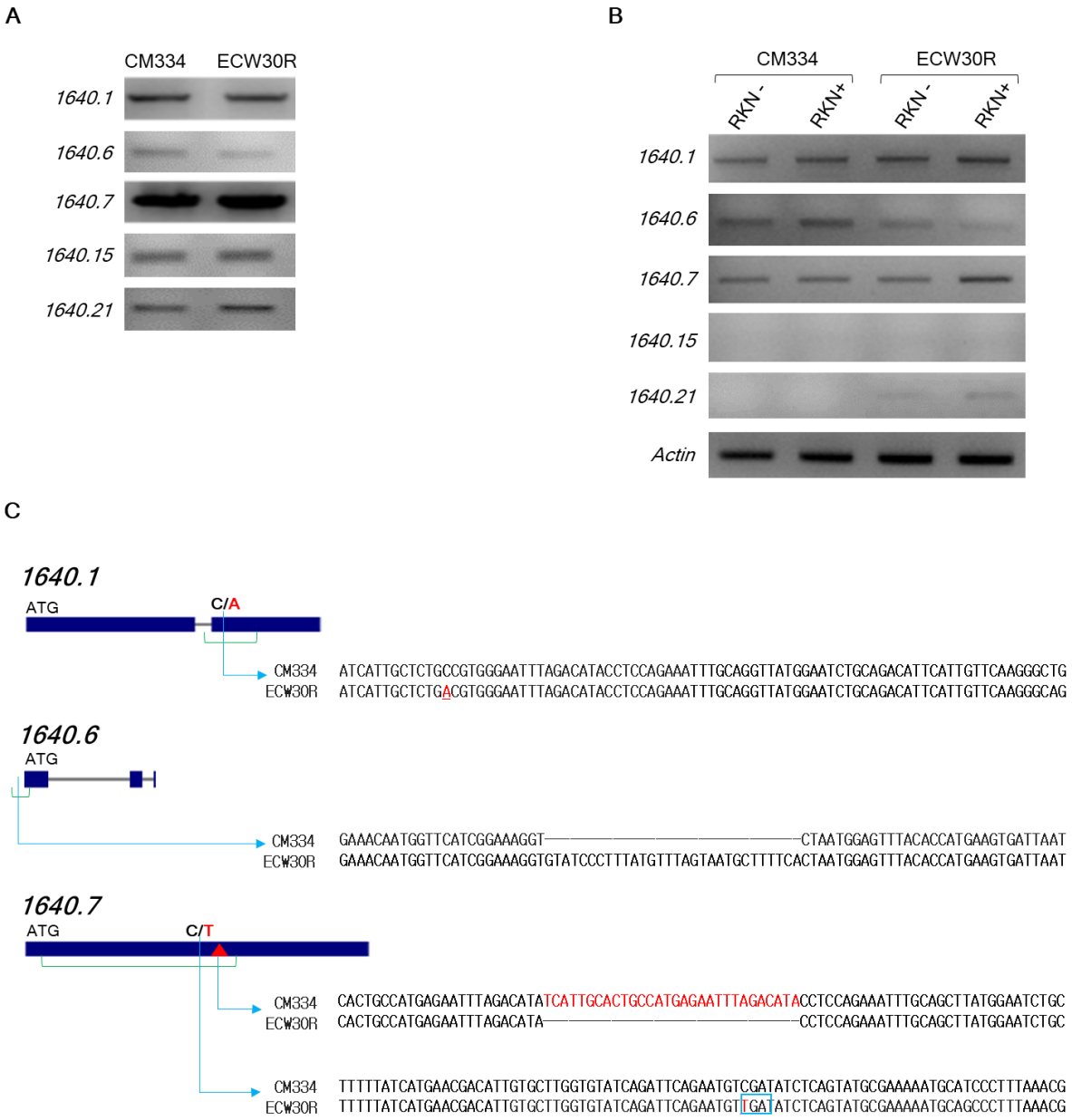

Figure II- 4. Validation of the missense, nonsenses and InDels mutations detected in candidate genes through PCR, RT-PCR and sequencing. A) gDNA PCR analysis of the candidate genes. B) RT-PCR expression analysis of the candidate genes. C) The sequences validation of nonsense SNPs/InDels using gDNA. The SNPs in red and underlined are predicted SNPs variations led stop codon according to RenSeq data. Stop codon confirmed is shown in the blue rectangular box. Sequence deletions are represented with dashes. The green line indicated the amplified fragment.

Reannotation of the candidate genes and full-length investigation

The gene reannotation analysis of the two strong candidate genes (*1640.6* and *1640.7*) were performed using three different gene prediction methods (**Figure II- 5**). The candidate *1640.7* was detected with an alternative gene structure named as *g3903* (**Figure II- 5**). We further validated the gene structure of the previously predicted gene (*1640.7*) and the reannotated gene (*g3903*). The gene*1640.7* is consisted of a single exon, while the gene *g3903* was predicted to contain three exons as per the RenSeq analysis (**Figure II- 6A**). Three sets of full-length primers were used to amplify gDNA as well as cDNA sequences (**Figure II- 6A, Table II- 3**). Amplicons were obtained with all three primer sets when gDNA was used as templates (**Figure II- 6B**), whereas cDNA amplicons were obtained only with primer set 2 (**Figure II- 6C**). These gene validation analyses indicated that the full-length *1640.7* gene size is larger than previously predicted but smaller than the reannotated gene *g3903* only having two exons.

The full-length gene cloning and sequencing of *1640.7* using primer set 2 were further performed (**Figure II- 6C, Table II- 3**). The full-length CDS size of the *1640.7* gene were found to be 2,751 bp and 2,820 bp, respectively from CM334 and ECW30R genomes. In ECW30R, a nonsense mutation at the position of 1,507 bp, and an in-frame InDel (30 bp) at position 1,830 bp in the first exon were detected. The *1640.7* gene is predicted to encode a protein with amino acid size of 899 aa and 502 aa in CM334 and ECW30R, respectively (**Figure II- 7**).

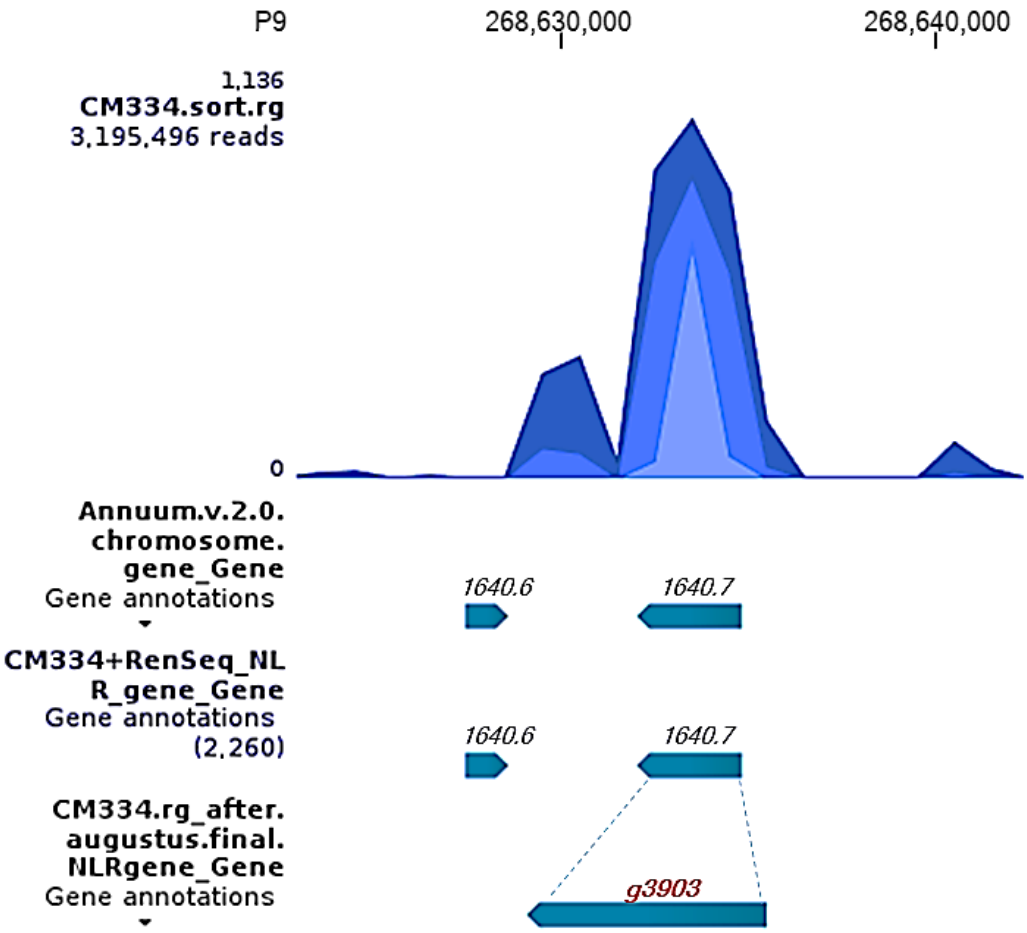

Figure II- 5. Reannotation of the predicted candidate genes. The blue graph represented the read depth of RenSeq sequence on the CM334 (v.1.6) reference P9. The reannotation of the candidate genes *1640.6* and *1640.7* using three gene prediction sources (Annuum.v.2.0, CM334and RenSeq and AUGUSTES NLR) presented on the left panel.

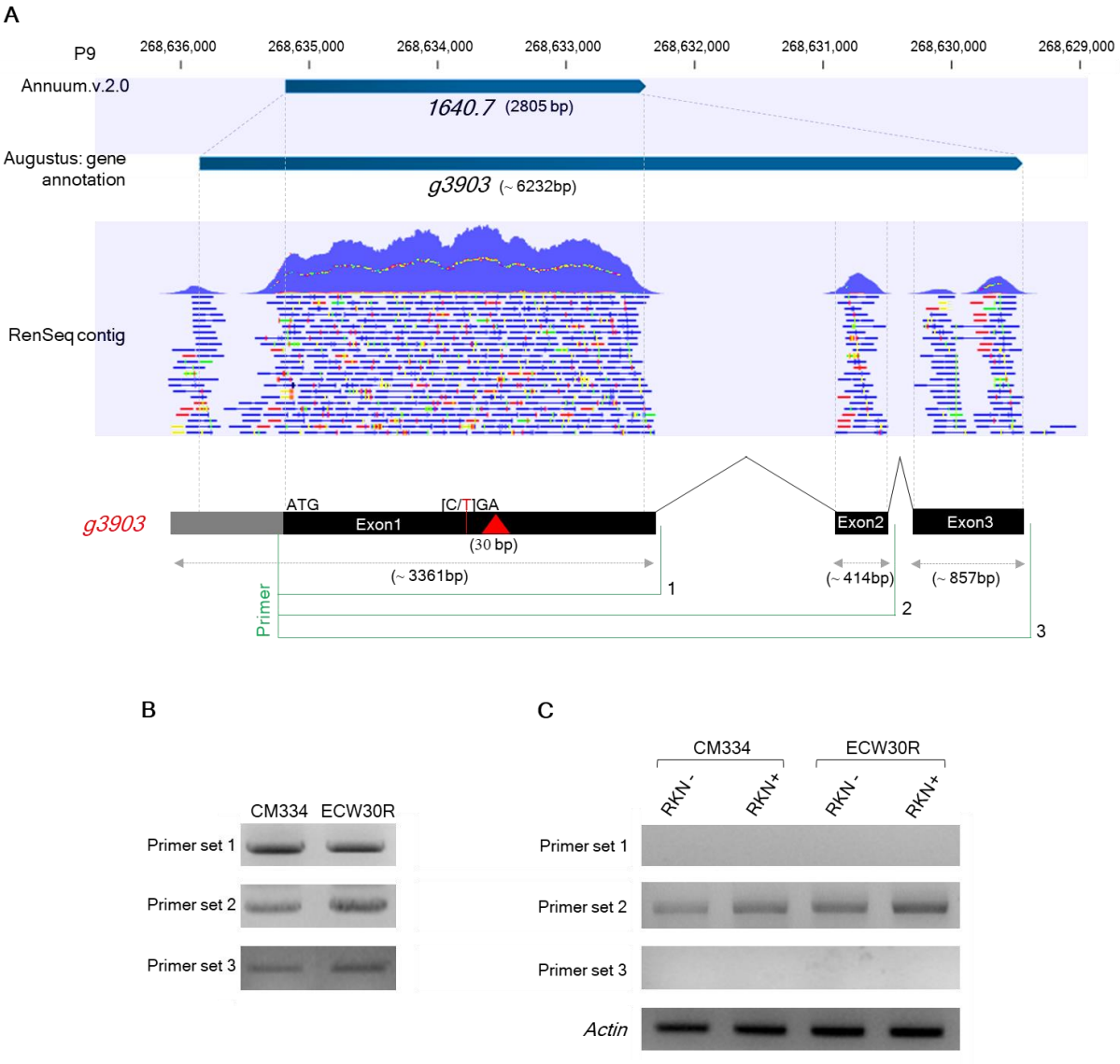

Figure II- 6. Validation of the *Me7* reannotated candidate gene full-length. A) The gene structure prediction of the reannotation *g3903*. The grey box predicted to be part of 5' untranslated region (UTR), which could be the part of the reannotated gene *g3903*. Exons and introns are represented by black filled boxes and V–shaped lines, respectively. Red triangle indicated InDels location. The number under the boxes is exon size (bp) obtained from the RenSeq fragment sequences. Three primer sets binding sites and amplicon sizes are shown in green lines. Validation of predicted full-length gene size through PCR (B) and RT-PCR (C).

Figure II- 7. Amino acid sequence alignment of the candidate gene *1640.7* from resistant CM334 and susceptible ECW30R. The asterisk (*) symbol under the amino acid indicated no variation and shaded regions with the grey color is amino acid variants.

Hosts for heterologous expression of *Me7* candidate genes for functional studies

For functional characterization of the *Me7* candidate genes, plants were screened for RKN susceptibility. Micro-Tom inoculated with RKN showed small to heavy galls (70.67±11.06), whereas *N. benthamiana* exhibited small and few heavy galls (28±9.84) thought the root systems. By contrast, only a few galls were observed in commercial tomato cultivars (5.67±2.08) (**Figure II- 8**). These results demonstrate that the most suitable host for the *Me7* candidate genes functional analysis is Micro-Tom, while *N. benthamiana* can an alternative option.

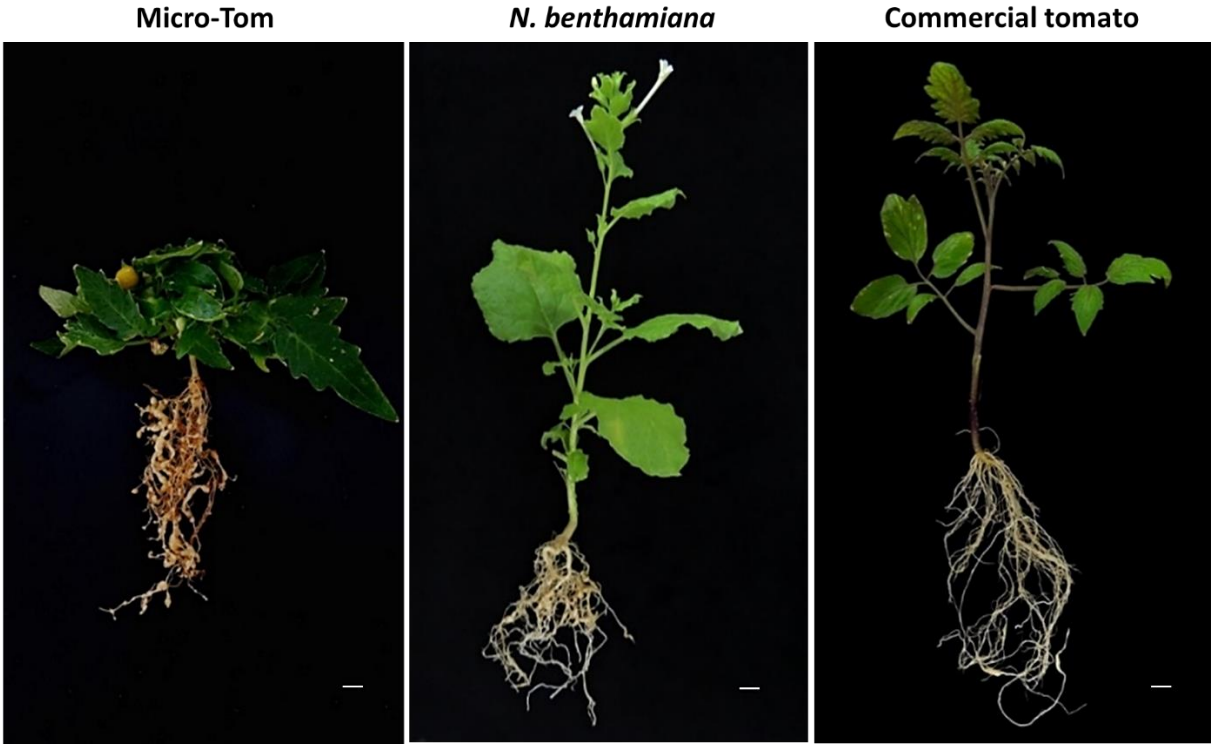

Figure II- 8. Susceptible hosts screening. Micro-Tom, *N. benthamiana* and commercial tomato cultivar were infected with 1,000 J2 *M. incognita* and observed at 45 dai. Bar = 1 cm.

DISCUSSION

Plant NBS-LRR genes are belonged to a multigene family and have ancient origins. NBS-LRR genes are critical components of plant defense machinery and are encoded by one of the largest gene families known in plants (McHale et al., 2006). NBS-LRR genes are generally classified according to the presence or absence of different conserved domains, such as TIR and CC domains in the N-terminal region. The core part of NBS-LRR is comprised of NB-ARC domain, which binds to nucleotide and hydrolyzes ATP (Leipe et al., 2004). The most variable region of NBS-LRR genes is the C-terminal LRR region, which functions in protein-protein interactions in plant defense responses (Lee and Yeom, 2015).

RenSeq has been used for the identification of SNP markers linked to the disease resistance. For instance, molecular markers for *Phytophthora infestans* resistance were developed using RenSeq in potato (Jupe et al., 2013). Similarly, RenSeq was utilized for development of the closest markers for the *Rpi-rzc1* resistance in tomato within a cluster of resistance genes (S ́liwka et al., 2012). In the present study, we performed BSA using RenSeq and identified candidate genes associated with RN resistance within a cluster of NBS-LRR genes.

Mostly, known RKN resistance genes in Solanaceae family are belonged to the NBS-LRR class *R* genes. For instance, RKN-resistance genes *Mi1.2* and *Mi-9* from tomato (Milligan et al., 1998; Seah et al., 2004; Jablonska et al., 2007), and *CaMi* (Chen et al., 2007) and *Me1* (Wang et al., 2018) from pepper have been reported to encode NBS-LRR proteins. The cyst nematode-resistance gene, such as *Hero* from tomato (Ernst et al., 2002), *Gro1-4* (Williamson and Kumar, 2006) and *Gpa2* (Sacco et al., 2009; van der Voort et al., 1999) from potato also encode NBS-LRR proteins. The gene *1640.7* is a CNL gene type *R* gene that contained RX-

CC-NBS-LRR conserved domains and share 98% sequence similarity with putative late blight resistance protein homolog R1B-16 (XM_016697590.1). Rx domain in *Gpa2* gene is known to trigger HR response and localized cell death (van der Voort et al., 1999; van Ooijen et al., 2007; Mazourek et al, 2009; Sacco et al., 2009). The *1640.7* gene could be associated with resistance response in CM334 through the programmed cell death pathway.

Genes associated with hormone biosynthesis and regulation are also known to be involved in defense responses. For example, cyst nematode hormone-based defense genes, *JASMONATE RESISTANT 1*, and *jasmonate-isoleucine synthase* (*JAR-1*) from Arabidopsis trigger defense responses through suppression of jasmonic acid biosynthesis and signaling during early syncytium (feeding site) formation (Wasternack and Hause, 2013; Kammerhofer et al., 2015; Regis et al., 2015). *PR-1*, *-2* and *-5* are associated with inhibition of salicylic acid biosynthesis pathways in tobacco nematode feeding site (Antoniw et al., 1980; van Loon, 1981; Sels et al., 2008; Hamanmouch et al., 2011). In this study, identified a candidate gene *1640.6* which share 96% sequence similarity with heme oxygenase 1 (HemeO gene type), chloroplastic-like (LOC107840774), mRNA (XM_016684676.1). Hemo oxygenase-1 can be induced by diverse stimuli that trigger stress response by inhibiting growth-promoting hormones synthesis, such as auxins (e.g. indole-3 acetic acid) and gibberellin (Xuan et al., 2008; Wu et al., 2016). Therefore, the *1640.6* gene could be associated with suppression of feeding sites enlargement by inhibiting auxin and gibberellin synthesis.

One of the setbacks of RenSeq is difficulty in assembly of full-length genes with a greater degree of confidence due to the large number of gene sub-families and the high sequence homology between paralogs (Jupe et al., 2013; Andolfo et al., 2014). Therefore, we performed direct sequencing of gDNA and cDNA PCR amplicons to confirm the SNPs and InDel identified by the RenSeq. Through direct sequencing, we were able to validate the SNPs and InDels identified through RenSeq analysis. However, comparing the target genes from the

region of the repetitive sequence with several reference genomes were necessary (Han et al., 2019). In this study, we successfully obtain the full-length gene *1640.7* using three different gene prediction methods.

In conclusion, we compared the *Me7* locus specific sequence from CM334 (v.1.6) and Dempsey (v1.0), and using BSA-RenSeq analysis we were able to identify a total of 492 SNPs with 99% significance level associated with the *Me7* locus. SNPs/InDels variation analysis detected two strong candidates *1640.6* and *1640.7* involved in resistance response for HR (*1640.7*) and suppression of feeding site enlargement (*1640.6*). Physical distance between *1640.6* and *1640.7* in CM334 (v.1.6) and Dempsey (v1.0) are only 7.432 kb and 7.364 kb, respectively, which indicate that these genes apparently inherit together. The *1640.6* gene was highly expressed in resistant plant CM334 and a 30 bp InDel was detected in 5′ UTR region of CM334. Full-length CDS sequencing of the gene *1640.7* found a nonsense mutation, which creates a premature stop codon in susceptible plant ECW30R. Further studies of functional characterization of these candidate genes via transgenic approaches using Micro-Tom as the heterologous expression host would reveal its clear roles in RKN resistance.

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ABSTRACT IN KOREAN

뿌리혹선충(*Meloidogyne incognita*)은 전세계적으로 고추(*Capsicum annuum*) 생산에 심각한 피해를 끼치는 병원균이다. 기존 연구에 의하면, 단일우성 뿌리혹선충 저항성 유전자 *Me7* 은 염색체 9 번의 장완 부분에서 존재하는 것으로 보고되었다. 본 연구에서는 뿌리혹선충 저항성인 *C. annuum* CM334 와 이병성인 *C. annuum* ECW30R 을 교배하여 얻은 714 개의 F_2 집단을 이용하여 *Me7* 유전자의 정밀 유전자 지도를 작성하였다. 뿌리혹선충은 CM334 에서 뿌리혹선충의 유충기가 억제되고 먹이 공급 부분의 비대화가 억제되며, 뿌리혹의 형성이 현격히 줄어드는 것으로 보인다. F_2 집단에서의 뿌리혹선충의 병리검정을 통해 558 개의 저항성 개체와 156 개의 이병성 개체를 확인하였으며, 이는 선충저항성 유전자가 단일우성일 때 관찰되는 저항성 개체:이병성 개체 (3:1) 의 비율과 통계적으로 유의미하게 일치하였다. *C. annuum* CM334 표준유전체 서열과 BAC 라이브러리 검정을 통해 *Me7* 의 정밀 지도 작성을 수행하였고, *Me7* 유전자좌를 두 개의 분자표지 (G21U3, G43U3) 범위로 줄였다. 이 구간은 물리적 거리로 환산하였을 때, CM334 염색체 9 번의 약 394.7 kb 이며, Dempsey scaffold 10 번의 약 198 kb 구간으로 확인되었다. 총 9 개의 마커가 이 *Me7* 후보 지역과 공분리되었다. RenSeq 방법을 이용한 분리형별 혼합분석을 수행하여 *Me7* 유전자좌와 동일한 대립유전자를 가지는 값인 -0.3 보다 작은 유의미한 Δ(SNP-index) 값을 관찰할 수 있었다. 염색체 9 번 (224.92 ~ 270.94 Mb) 에서 99%의 신뢰도를 가지는 SNP/InDel 은 총 492 개가 확인되었다. 그 중 104 개의 SNPs/InDels 이 16 개의 후보유전자에 분포하였으며, 후보유전자의 길이는 0.192 kb 에서 2.835 kb 까지

다양하게 확인되었다. 16 개의 후보유전자 중에서 두 개의 CNL type 의 유전자(*1640.1, 1640.7*) 에서는 missense, nonsense, InDel 로 인한 염기서열 변이가 확인되었다. 다른 세 개의 non-CNL type 의 유전자 (*1640.6, 1640.15, 1640.21*) 에서는 오로지 missense 변이만이 확인되었다. 또한 오직 *1640.6* 유전자에서만 RT-PCR 분석을 통해서 저항성과 이병성 개체 간 발현량 차이를 확인할 수 있었다. 추가 염기서열분석을 통해 *1640.7* 에 존재하는 SNP 만이 감수성 식물체에서 종결코돈을 형성하는 것을 확인할 수 있었다. 유전자 *1640.7* 는 CNL type 의 RX-CC-NBS-LRR-like 단백질을 암호화하며, 그 아미노산의 길이는 nonsense 변이로 인하여 CM334 에서는 899 aa, ECW30R 에서는 502 aa 으로 확인되었다. 두 개의 후보 유전자 *1640.6* 과 *1640.7* 은 HR 반응을 통한 저항성 반응에 관여하며 먹이 공급 부분의 비대화를 억제할 것이라 예측된다.