Theor Appl Genet (2008) 117:1107–1118
DOI 10.1007/s00122-008-0848-6

ORIGINAL PAPER

Fine mapping and DNA fiber FISH analysis locates the tobamovirus resistance gene L^3 of *Capsicum chinense* in a 400-kb region of R-like genes cluster embedded in highly repetitive sequences

R. Tomita · J. Murai · Y. Miura · H. Ishihara · S. Liu · Y. Kubotera · A. Honda · R. Hatta · T. Kuroda · H. Hamada · M. Sakamoto · I. Munemura · O. Nunomura · K. Ishikawa · Y. Genda · S. Kawasaki · K. Suzuki · K. Meksem · K. Kobayashi

Received: 30 April 2008 / Accepted: 11 July 2008 / Published online: 29 July 2008

Abstract The tobamovirus resistance gene L^3 of *Capsicum chinense* was mapped using an intra-specific F2 population (2,016 individuals) of *Capsicum annuum* cultivars, into one of which had been introduced the *C. chinense* L^3 gene, and an inter-specific F2 population (3,391 individuals) between *C. chinense* and *Capsicum frutescence*. Analysis of a BAC library with an AFLP marker closely linked to L^3-resistance revealed the presence of homologs of the tomato disease resistance gene *I2*. Partial or full-length coding sequences were cloned by degenerate PCR from 35 different pepper *I2* homologs and 17 genetic markers were generated in the inter-specific combination. The L^3 gene was mapped between *I2* homolog marker IH1-04 and BAC-end marker 189D23M, and located within a region encompassing two different BAC contigs consisting of four and one clones, respectively. DNA fiber FISH analysis revealed that these two contigs are separated from each other by about 30 kb. DNA fiber FISH results and Southern blotting of the BAC clones suggested that the L^3 locus-containing region is rich in highly repetitive sequences. Southern blot analysis indicated that the two BAC contigs contain more than ten copies of the *I2* homologs. In contrast to the inter-specific F2 population,

R. Tomita and J. Murai contributed equally to this work.

Communicated by J.S. (Pat) Heslop-Harrison.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-008-0848-6) contains supplementary material, which is available to authorized users.

R. Tomita · J. Murai · Y. Miura · Y. Kubotera · A. Honda · R. Hatta · T. Kuroda · H. Hamada · M. Sakamoto · I. Munemura · K. Suzuki · K. Kobayashi (✉)
Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami, Iwate 024-0003, Japan
e-mail: kappei@ibrc.or.jp

H. Ishihara · S. Liu · K. Meksem
Department of Plant, Soil and Agricultural Systems, Southern Illinois University at Carbondale, Carbondale, IL 62901-4415, USA

O. Nunomura · K. Ishikawa · Y. Genda
Japan Horticultural Production and Research Institute, Matsudo, Chiba 270-2221, Japan

S. Kawasaki
National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan

Present Address:
J. Murai
Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Osaka 537-8511, Japan

Present Address:
Y. Miura
Nippon Flour Mills Co., Ltd, Central Laboratory, Atsugi, Kanagawa 243-0041, Japan

Present Address:
H. Ishihara
Max Planck Institute for Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam, Germany

Present Address:
T. Kuroda
Crop Center, Niigata Agricultural Research Institute, Nagaoka, Niigata 940-0826, Japan

Present Address:
H. Hamada
National Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan

Present Address:
K. Suzuki
School of Environmental Science, University of Shiga Prefecture, Hikone, Shiga 522-8533, Japan

no recombinant progeny were identified to have a crossover point within two BAC contigs consisting of seven and two clones in the intra-specific F2 population. Moreover, distribution of the crossover points differed between the two populations, suggesting linkage disequilibrium in the region containing the *L* locus.

Introduction

Mapping and cloning of plant disease-resistance genes (R-genes) are of great importance for several reasons (McDowell and Woffenden 2003): identification of genetic markers closely linked to R-genes are useful for breeding resistant cultivars; transgenic expression of R-genes can confer resistance against common pathogens to distantly related crop species; molecular analysis of R-gene products provides us with knowledge on plant defense mechanisms; and structural analysis of alleles of resistance genes is expected to help understand the co-evolution of pathogens and host R-genes.

Tobamovirus infection is one of the greatest threats for the cultivation of crops, especially peppers. *Capsicum* plants have genes, designated *L* genes, providing resistance against tobamoviruses. These genes have been introduced into pepper cultivars to protect the plants from tobamovirus infection. There are four allelic genes—L^1, L^2, L^3 and L^4—known to provide increased anti-tobamovirus resistance: L^1 confers resistance to P_0 pathotype viruses such as *Tomato mosaic virus* (ToMV); L^2 confers resistance to P_0 and P_1 pathotype viruses such as *Paprika mild mottle virus* (PaMMV) that overcomes L^1 resistance; L^3 confers resistance to P_0, P_1 and $P_{1,2}$ pathotype *Pepper mild mottle virus* (PMMoV) that overcomes L^2 resistance; L^4 confers resistance to P_0, P_1, $P_{1,2}$ and $P_{1,2,3}$ pathotype PMMoV that overcomes L^3 resistance (Boukema 1980, 1982, 1984). In addition to these classical *L* genes, another allelic gene with a different temperature sensitivity, L^{1a}, was recently identified (Sawada et al. 2004). Studies have identified the viral coat proteins (CPs) as elicitors of *L* genes-mediated resistance (Berzal-Herranz et al. 1995; de la Cruz et al. 1997; Gilardi et al. 2004; Matsumoto et al. 2008). Comparison of amino acid sequences revealed that the CPs of ToMV, PaMMV and PMMoV are distantly related to each other, but those from $P_{1,2}$ and $P_{1,2,3}$ pathotype PMMoV differ by only one or two amino acids (Garcia-Luque et al. 1993; Berzal-Herranz et al. 1995; Tsuda et al. 1998; Hamada et al. 2002; Hamada et al. 2007; Yamasaki et al. 2007). A recent study revealed that the coat protein of L^4 resistance-breaking $P_{1,2,3,4}$ pathotype of PMMoV differs in two amino acids from $P_{1,2}$ (Genda et al. 2007). These data support the idea that the interaction between *L* genes and tobamoviruses serves as a good system for studying the mechanism and evolution of virus perception by plants.

The emergence of *L*-genes mediated resistance-breaking strains of PMMoV implies that development of a new resistance gene with a wider resistance spectrum is needed. A promising approach would be to clone an *L* gene and cause it to evolve artificially to have a wider ability to recognize tobamovirus CPs. Such an approach was successful in *Potato virus X* resistance gene, *Rx* (Farnham and Baulcombe 2006). Alternatively, overexpression of a cloned *L* gene may help extend the resistance to higher order pathotype PMMoV, because L^3 gene-mediated resistance to $P_{1,2}$ pathotype PMMoV is reportedly gene dose-dependent: i.e., peppers homozygous for L^3 were resistant to $P_{1,2}$ pathotype PMMoV, but the heterozygous plants with a susceptible allele showed systemic necrosis upon infection with the virus (Hamada et al. 2002). It is important to clone the *L* genes and study the mechanisms by which they recognize tobamovirus CPs for establishing an effective control strategy against those viruses in peppers.

The *L* genes were discovered in 1980s and have been used for the breeding of tobamovirus-resistant pepper cultivars. The *L* locus was mapped to the sub-telomeric region of pepper chromosome 11, at 4.0 cM from RFLP marker TG36 (Lefebvre et al. 2002), which was originally discovered in tomato (Livingstone et al. 1999). The region containing the *L* locus has synteny to a region of tomato chromosome 11 (Livingstone et al. 1999), which contains a *Fusarium* resistance gene *I2* (Ori et al. 1997; Simons et al. 1998; Grube et al. 2000). An AFLP marker has been developed that co-segregates with the L^3 gene (Sugita et al. 2005). The detailed location and structure of the *L* locus remains to be determined. It has been suggested that the region containing the *L* locus also contains many repetitive sequences and is less polymorphic than other parts of the pepper genome, which makes it difficult to analyze the region (Yoo et al. 2003).

Studies have revealed that R-genes are often found in a cluster of homologous genes (Michelmore and Meyers 1998). The size and arrangement of R-gene clusters vary within loci and plant species. The Dm3 locus of lettuce contains more than 24 genes in an approximately 4 Mb region, while the compact and tightly arranged *I2*-complex contains the *I2* gene and an additional six cognates within a 90 kb region with 8–10 kb intergenic regions. Huang et al. (2005) isolated the potato late blight resistance gene *R3a* using the synteny between the *R3a* and *I2* loci, and found that the *R3a* region is physically larger than the *I2* region: the *R3a* region contains more R-like genes with larger intergenic regions than *I2*. This fact indicates that the *L* locus may be larger and more complex than the *I2* and *R3a* loci.

In this report, we have characterized the L^3 locus for future cloning of this R-gene. Some resistance genes are

hard to clone by map-based methods, most likely due to the complex structure of the loci. For example, the Tm-1 tobamovirus resistance gene of tomato was mapped to chromosome 2, but could not be cloned by map-based methods. It was eventually identified by biochemical analysis (Ishibashi et al. 2007). It is important to characterize the resistance locus to determine if it is feasible to clone the resistance gene. We performed high resolution mapping of the L^3 gene using AFLP markers, markers made from BAC-end sequences and RGA (resistance gene analogs; Pflieger et al. 1999)-based markers in two mapping populations. Although we could not construct a single BAC contig encompassing the L^3 locus, analysis of BAC clones and DNA fiber FISH analysis located the locus in an approximately 400 kb region of *Capsicum chinense* chromosome 11. Analysis of the incomplete BAC contig revealed that this region contains multiple copies of RGAs, namely homologs of the tomato *I2* gene, and is rich in highly repetitive sequences included in the *Cot*-1 DNA fraction. The genetic distances from the L^3 locus to some markers were not consistent between the two mapping populations, suggesting linkage disequilibrium in the region containing the L^3 locus. The feasibility of L^3 gene isolation is discussed in the light of structure, inheritance and evolution of the *L* locus.

Materials and methods

Plant materials and DNA extraction

Capsicum annuum KOS, a susceptible cultivar, and NDN, a breeding line homozygous for the L^3 gene from PI152225, were bred in the Japan Horticultural Production and Research Institute. *C. chinense* PI159236, which is homozygous for L^3, and *Capsicum frutescens* LS 1839-2-4, which is homozygous for L^2, were obtained from Shinji Monma, National Institute of Vegetables and Tea Science, Mie, Japan. Two mapping populations used in this study, NK and YB populations, were generated by selfing the F1 between *C. annuum* KOS and NDN, and *C. chinense* PI159236 and *C. frutescens* LS 1839-2-4, respectively. DNA was extracted using a Nucleon PhytoPure plant DNA extraction kit (GE Health Care) or DNeasy plant maxi kit (QIAGEN) for large-scale preparation. Nucleon PhytoPure plant DNA extraction kit or DNeasy plant mini kit (QIAGEN) were used for small-scale preparation. For a smaller preparation (from cotyledons of YB population), a simplified CTAB procedure was used. Briefly, tissues were homogenized in 0.2 ml CTAB buffer (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 3% cetyltrimethylammonium bromide, 0.2% β-mercaptoethanol) using Tissue lyzer (Qiagen) with two 3 mm (diameter) zirconia beads, added with 0.3 ml CTAB buffer, incubated at 60°C for 30 min and extracted with chloroform. The aqueous phase (0.4 ml) was transferred to a new tube and DNA was precipitated with isopropyl alcohol. The DNA pellet was rinsed with 70% ethanol, air-dried, dissolved in TE buffer and used for PCR analysis.

Assay for the response of plants to PMMoV infection

Plants grown to 3–4 leaf stages were mechanically inoculated with 10 μg/ml PMMoV-Iw ($P_{1,2}$ pathotype; Hamada et al. 2007) on the first and the second true leaves and observed for the expression of necrotic symptoms on the inoculated leaves at 5 days post-inoculation. Alternatively, a cotyledon was cut from each plant, mechanically inoculated with 10 μg/ml PMMoV-Iw, kept in a moist chamber for 7 days and observed for the expression of necrotic symptoms. In some cases, virus spread was examined by hammer blotting immunostaining (Hamada et al. 2002) to confirm the response of plants to the virus infection.

Molecular markers

AFLP

AFLP markers were obtained by analyzing DNA from parental lines and PMMoV-resistant and -susceptible F2 progenies by the HEGS-AFLP method described previously (Kawaguchi et al. 2001), a modified procedure of AFLP protocol of Vos et al. (1995). Briefly, DNA was digested with *Eco*RI and *Mse*I, and the products ligated with *Eco*RI- and *Mse*I-adaptors. After preamplification with common adaptor primers, PCR products from 10 to 12 plants each of PMMoV-resistant and -susceptible F2 progenies were gathered together, respectively, for bulk analysis. From the bulked-PCR products derived from resistant and susceptible progenies, DNA fragments were PCR-amplified using 64 selective primers for both adaptor sequences (4,096 combinations of primers; all possible combination of selective extension of primers described by Vos et al. (1995); custom synthesized and purified by Fasmac, Atsugi, Japan) and analyzed on a polyacrylamide gel (5% stacking gel and 13% separation gel; 18 cm long and 1 mm thick) as described in detail by Kawaguchi et al. (2001). The primer combinations that gave polymorphic bands between resistant and susceptible plants were used for analyzing more PMMoV-resistant and -susceptible F2 progenies. Reproducibly polymorphic bands were gel-purified, cloned using a TOPO TA cloning kit for sequencing (Invitrogen) and sequenced. Based on the AFLP fragment sequence, sequence characterized amplified region (SCAR) markers that link to PMMoV-resistance were generated (Table 1 and Fig. 1).

Table 1 Molecular markers used in this study

Markers[a]	Methods/types[b]	Sequences[i]	Target sequence[j]
A214 (NK)	AFLP[c] → BAC-end	5′-ACCGTTATGGTATGGGACAG-3′	AB442177
	→ SCAR[d]	5′-GAAGGGCCTTTTGGTCATCT-3′	
A214 (YB)	AFLP → BAC-end	5′-ATGAGAAAAAGATCTGTGTTTTC-3′	AB442177
	→ SCAR	5′-CATGGTTTTAGGGTTGGGATA-3′	AB442196
A339 (NK)	AFLP → SCAR[e]	5′-TCTCGGTAGGCCATTTTGCT-3′	DD410853
		5′-GTAAGTTGCTATGCCCACCA-3′	
A339 (YB)	AFLP → SCAR	5′-GTTTTACATGAAACGCGTTC-3′	DD410853
		5′-GAAGATAGTGGTGGAGAAAA-3′	AB442209
YB2A25 (NK and YB)	AFLP → SCAR	5′-ATGTCCGTCACGGCCAGGCA-3′	AB442167
		5′-CGGCAAATTCCCCGTTAGTT-3′	AB442211
YB2A14 (NK)	AFLP → SCAR	5′-GCGCTTCTGATGATAAGCCA-3′	AB442169
		5′-GCATCTGATTCTCACGTTGA-3′	
YB2A19 (NK)	AFLP → SCAR	5′-TATGCTGCTGCGGGAAAATG-3′	AB442168
		5′-CACCGAACAAATCTACGAGC-3′	
IH6-06 (NK)	RGA[f], SCAR	5′-CTTCTCCTGTCATCTTGTAAG-3′	AB442187
		5′-CTTGGATCCCACTAGCACATA-3′	
IH1-04 (NK and YB)	RGA, SCAR	5′-GGAAGTAAGATCATTGTGACGACTA-3′	AB442185
		5′-CCCTATTTTCTAGAGAATGTCGT-3′	AB442188–AB442191
IH1-18 (YB)	RGA, SNPs	5′-TTTTTCTCAACATAAAGGAGAAGTCG-3′	AB442186
	(*Taq* I, Cf)[g]	5′-CCACAGAAGTTGCAGGTGTTCT-3′	AB442192–AB442195
189D23M (NB)	BAC-end[h], SCAR	5′-ATTGTCAGAGTCGGGAAGCA-3′	AB442178
		5′-TACTATGCACAGGGTCTAGG-3′	
189D23M (YB)	BAC-end, SCAR	5′-ATTGTCAGAGTCGGGAAGCA-3′	AB442178
		5′-AACGACAAGGGTTTATTGTATGC-3′	AB442197–AB442199
21L24M (YB)	BAC-end, SNPs (HpyCH4 IV, Cc)	5′-AAAACACAACTTACTGTCTAGAAAAC-3′	AB442181
		5′-ACTCCTGCAATAATAAATGGAT-3′	AB442205–AB442208
197AD5R (YB)	BAC-end, SNPs (*Fok* I, Cc)	5′-TCAAACTTTCAGAAACTTCGGA-3′	AB442179
		5′-GCAATACCTTGACGGCTATAAA-3′	AB442200–AB442204
253A1R (YB)	BAC-end, SCAR	5′-GCTTCTCCCCAAATGTAGCA-3′	AB442182
		5′-CTCAAACGAGTCATGGCTGA-3′	
213E3R (YB)	BAC-end, SCAR	5′-TCATTGGACATGGTGGCTAT-3′	AB442180
		5′-GGGCTCGTGACGACCTATTA-3′	

[a] Name of molecular markers with mapping population in brackets

[b] Methods used to develop the molecular markers and types of the molecular markers

[c] AFLP gel photos are shown in electronic supplementary material, Fig. S3

[d] Molecular markers developed from end sequences of BAC clone identified using AFLP marker

[e] Molecular markers first developed by AFLP and then converted to SCAR marker by the analysis of AFLP fragments

[f] Analysis of resistance gene analogs

[g] Restriction enzymes used in PIRA-PCR and parental lines from which cleavable versions of polymorphisms were derived: *Cc Capsicum chinense* PI159236, *Cf Capsicum frutescens* LS 1839-2-4

[h] Analysis of BAC-end sequences

[i] Sequences of PCR primers used for the detection of the molecular markers. Mismatches introduced into PIRA-PCR primers are underlined

[j] Accession No. of sequences used for the development of the markers

RGA

Degenerate PCR primers were designed based on the alignment of reported sequences for *I2* and its cognate (Accession No. AF004878 and AF004879) and sequences obtained in the analysis of BAC clones (Accession No. AB442166, AB442172, AB442173, AB442174, AB442175 and AB442176; alignment is shown in electronic supplementary material Fig. S1; primer sequences are in Table 2 and their positions in CC-NBS-LRR protein domain map are

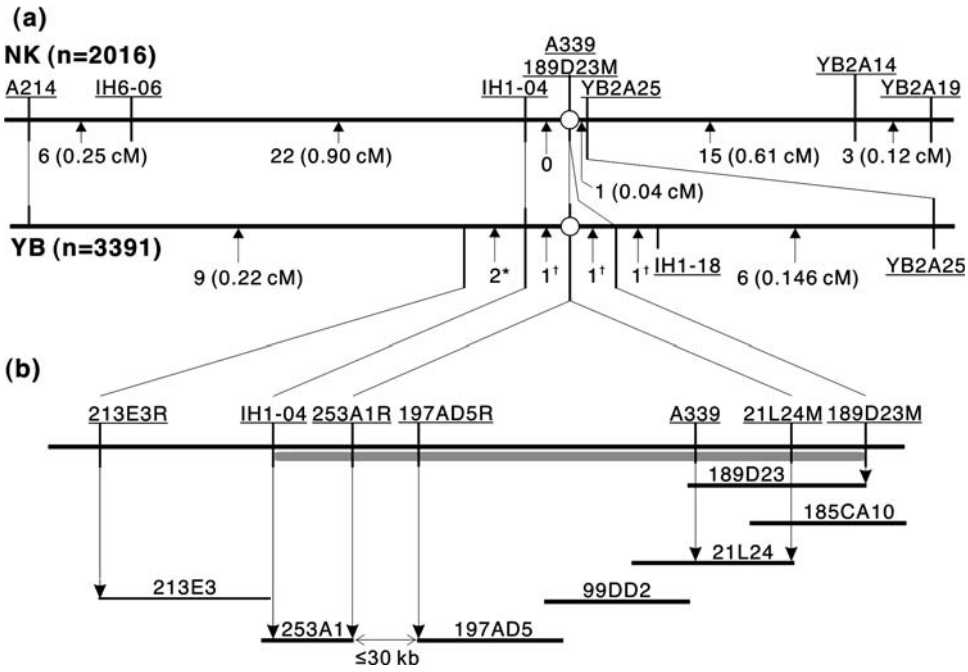

Fig. 1 Genetic (**a**) and physical (**b**) map of the L^3 region. **a** The L^3 region was mapped with two different F2 populations, NK and YB. *Open circles* indicate the position of the L^3 gene. Names of molecular markers are *underlined*. *Numbers with brackets* indicate the number of recombinant progenies with Cosambi's distances in brackets. *0.05 cM; †0.02 cM. Note that some markers are common to both mapping populations. **b** BAC contigs constructed in the L^3 region (*gray bar*) are shown. *Lines with clone names* indicate the positions and size of BAC clones. Refer to Fig. 3 for the gap

Table 2 Degenerate primers used in an exhaustive search for *I2* homologs

Primer names	Sequences[a]	Pair/condition
PI2H-B1F1	5′-GAGATTGGCTTCGCARTYGG-3′	PI2H-B2R1 and -B2R2 Normal PCR[b]
PI2H-B1F2	5′-TGTTYTCTTTGAYAGGCTTGCT-3′	PI2H-B2R1 and -B2R2 Normal PCR
PI2H-B2R1	5′-TCRAAYAAYGATCTTGATCTCAA-3′	PI2H-B1F1 and -B1F2 Normal PCR
PI2H-B2R2	5′-CTGAYACARAGTTTTGAAGATG-3′	PI2H-B1F1 and -B1F2 Normal PCR
PI2H-B2F1	5′-TGTATAACTTAGAGACACTTCTC-3′	PI2H-B3R1 and -B3R2 Touch-down PCR-1[c]
PI2H-B2F2	5′-GAGYTRCCGCYRCAGATGG-3′	PI2H-B3R1 and -B3R2 Touch-down PCR-1
PI2H-B3R1	5′-TAYGTCTCTTTCAGTTTGNGAAT-3′	PI2H-B2F1 and -B2F2 Touch-down PCR-1
PI2H-B3R2	5′-TRTGGGCYTAGCTCATCAAGT-3′	PI2H-B2F1 and -B2F2 Touch-down PCR-1
PI2H-B3F1	5′-ATGYCNGARTGGAAGCARTG-3′	PI2H-B3R1 and -B3R2 Touch-down PCR-2[d]
PI2H-B3F2	5′-TTGCCGRAAATTGAAATTGGA-3′	PI2H-B3R1 and -B3R2 Touch-down PCR-2
PI2H-B4R1	5′-CAGTAYTCCYCCTTGTCRAA-3′	PI2H-B2F1 and -B2F2 Touch-down PCR-2
PI2H-B4R2	5′-TGAGCAATTTYTGRCCAGTA-3′	PI2H-B2F1 and -B2F2 Touch-down PCR-2

[a] Sequences of primers: Y, C or T; R, A or G; N, any nucleotide

[b] PCR condition was as follows: 94°C for 4 min; 30 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 30 s

[c] PCR condition was as follows: 94°C for 2 min; 15 cycles of 94°C for 30 s, 65–51°C (1°C reduction every cycles) for 30 s and 72°C for 30 s; 25 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 30 s

[d] PCR condition was as follows: 94°C for 2 min; 15 cycles of 94°C for 30 s, 65–51°C (1°C reduction every cycles) for 90 s and 72°C for 30 s; 25 cycles of 94°C for 30 s, 63°C for 90 s and 72°C for 30 s

in Fig. 2a). The primers were used for isolating RGA sequences from *C. chinense* PI159236 under the conditions described in the footnote of Table 2. Thirty-five RGAs were sequenced in both *C. chinense* PI159236 and *C. frutescens* LS 1839-2-4 and those which exhibited polymorphism between the parental lines were used for the generation of

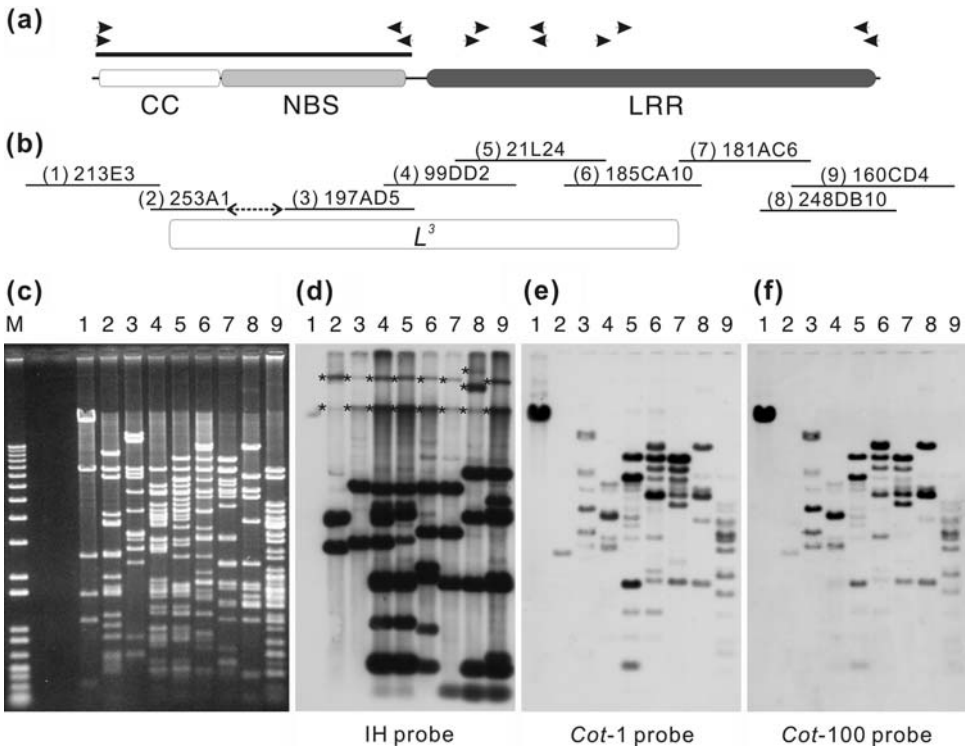

Fig. 2 Analysis of *I2* homologs in the L^3 region. **a** Schematic representation of the *I2* protein structure: *open*, *light gray* and *dark gray boxes* indicate coiled-coil (*CC*), nucleotide binding (*NBS*) and leucine-rich repeat (*LRR*) domains. *Arrowheads* indicate the positions of PCR primers used in the exhaustive search for *I2* homologs (Table 2). *Thick lines* indicate the position of Southern blotting probes used in **d**. **b** Schematic representation of BAC contigs analyzed in **c**–**f**. *Open boxed labeled* "L^3" indicates the L^3 region. *Numbers in brackets* with BAC clone names correspond to lane numbers in **c**–**f**. BAC DNAs were digested with *Hin*d III, separated on an agarose gel (**c**; ethidium bromide staining), transferred to a nylon membrane and probed with *I2* homologs (**d**), *Cot*-1 (**e**) or *Cot*-100 DNA (**f**)

SCAR or SNP markers (Table 1). The SNPs were detected by primer-introduced restriction analysis (PIRA)-PCR as described (Haliassos et al. 1989; Ke et al. 2001).

Generation of *C. chinense* BAC library, chromosome walking and partial sequencing of BAC clone 21L24

Capsicum chinense PI159236 nuclei were isolated, capsulated with agarose and partially digested with *Hin*dIII. The digested DNA was separated by pulse-field gel electrophoresis and DNA fragments larger than 100 kb were purified and inserted into pBeloBAC11. The BAC library, which consisted of 100,224 independent clones with an average insert size of 100 kb, were PCR-screened as follows: first screen, PCR analysis of 261 pools of DNA each consisting of 384 independent clones; second screen, PCR analysis of four pools of 96 clones each; third screen, PCR analysis of 96 clones (for detail, see electronic supplementary material, Fig. S2). BAC end sequences were determined for positive clones and used for designing PCR primers for the next cycle of screening as above and for linkage analysis. Pairs of PCR primers were designed in the BAC-end sequence, tested for polymorphism in the parental lines to generate BAC-end makers. When no polymorphism between the parental lines was detected by the primer pairs, the homologous DNA fragment was isolated from susceptible parent and sequenced, then SCAR or SNP markers were generated based on the alignment of sequences from the parental lines. A BAC clone, 21L24, was sequenced by a shotgun sequencing procedure. Briefly, the BAC DNA was sheared by sonication to 1–2 kb, end-repaired using DNA blunting kit (Toyobo) and cloned using a Zeroblunt TOPO DNA cloning kit for sequencing (Invitrogen). Sequences were determined in 1,200 clones and assembled using AutoAssembler version 2.1 (ABI) to give eight contigs.

Stretched DNA fiber FISH analysis

Capsicum chinense PI159236 nuclei were isolated and stretched DNA fibers were prepared on Poly-Prep Slides (Sigma-Aldrich) by the dragging method as described previously (Fransz et al. 1996; Li et al. 2005; Walling et al. 2005). Selected BAC clones were labeled with biotin or digoxygenin using biotin High prime or DIG High prime labeling kits (Roche), respectively, accordingly to manufacturer's protocols, with 6 h of reaction time. Blocking DNA, Cot-1 and Cot-100 DNA were prepared as described previously (Budiman et al. 2004; Walling et al. 2005). Stretched DNA fibers were denatured at 80°C for 5 min in 10 µl/slide of hybridization buffer containing about 50 ng each of labeled probes, 1 µg Cot-1 DNA, 2 µg Cot-100 DNA and 10 µg herring testes DNA. After washing twice in 0.1 × SSC containing 0.1% SDS at 45°C for 10 min, slides were blocked with TBST-BB (20 mM Tris–HCl, pH 7.2, 0.5 M NaCl, 0.05% Tween-20, 1% blocking reagent (Roche), 2% BSA), probed sequentially with a mixture of 0.4 µg/ml Alexa FluorR 488-streptavidin (Molecular

probe) and 0.1 μg/ml anti-DIG mouse monoclonal antibody (Roche), with a mixture of 0.1 μg/ml biotinylated goat anti-streptavidin (Vector) and 0.67 μg/ml Alexa FluorR 568-Rabbit anti-Mouse IgG (Molecular probe), and then with a mixture of 0.4 μg/ml Alexa FluorR 488-streptavidin and 0.67 μg/ml Alexa FluorR 568-goat anti-Rabbit IgG (Molecular probe). Incubation was done for 10–16 h at 4°C. Fluorescent images were taken under a BX51 fluorescent microscope equipped with a DP70 digital camera using DP controller software (version 2.2.1.227) and processed using DP manager software (version 2.2.1.195; Olympus).

Southern blot analysis of BAC clones

Southern blot analysis of BAC clones was performed by a standard procedure (Sambrook et al. 1989). Probes for *I2* homologs were prepared as follows: *I2* homologs were PCR-amplified from *C. chinense* PI159236 total DNA using degenerate primers, PI2H-B1F2 and PI2H-B2R1 (Table 2), which amplified at least 19 different *I2* homologs as revealed during the development of RGA markers; the DNA fragments from multiple numbers of pepper *I2* homologs were gel purified and then labeled using Hi-Prime DIG DNA labeling kit (Roche). *Cot*-1 and *Cot*-100 DNA were also labeled using the same kit.

Results

Primary mapping of L^3 gene

We first mapped the L^3 gene using the NK population, an intra-specific F2 population derived from a *C. annuum* cultivar and its derivative containing an introduced L^3 gene from *C. chinense* PI152225. Three rounds of AFLP analysis on bulk DNA from PMMoV-resistant and -susceptible F2 progenies revealed eight polymorphic DNA fragments amplified by seven combinations of selective PCR primers. Two SCAR markers were generated from them. Marker A339 did not segregate from the L^3 gene and marker A214 was mapped to 1.15 cM from the L^3 gene (from the analysis of 2,016 individuals from NK population (Fig. 1a). To increase the number of molecular markers available for the mapping experiments, we took two different approaches. Firstly, we generated and analyzed the BAC contig containing the A339 marker and secondly we analyzed the YB population by AFLP. We generated a BAC contig with 13 independent clones (designated as contig 339), generated 6 SCAR markers including 189D23M, but these markers did not segregate from the L^3 gene (Fig. 1a). AFLP analysis of the YB population revealed 19 polymorphic DNA fragments that exhibited linkage to the L^3 gene. We successfully generated 13 SCAR markers based on the sequences of the polymorphic DNA fragments from NDN and KOS. Nine of them were mapped further than or at the same location as A214 from the L^3 gene. A marker designated YB2A26 did not segregate from the L^3 gene and another three (YB2A25, YB2A14 and YB2A19) were mapped on the opposite side of the L^3 gene to A214 (Fig. 1a). Thus we obtained multiple markers on either side of the L^3 gene. However, none of them identified a BAC contig that encompassed the entire L^3 gene, most likely due to interference by repetitive sequences in the BAC screening.

Discovery of pepper *I2* homologs and development of RGA markers

Sequence analysis of BAC clone 21L24, which was identified by screening with the A339 marker, revealed three pseudogenes homologous to *I2*, in keeping with previous reports. Screening of a cDNA library and PCR amplification with primer pairs based on *I2* sequence identified three additional *I2* homologs. Southern analysis of flanking clones revealed the presence of more *I2* homologs (Fig. 2c) in contig 339. These findings led us to develop molecular markers from the *I2* homologs. PCR primers were designed from four highly conserved regions, such that poorly conserved regions between them could be amplified (Fig. 2a). Amplification of *I2* homologs with four different PCR primer pairs targeting three regions gave rise to the identification of 29 *I2* homolog fragments. Specific PCR primer pairs for each of them were designed and used to isolate homologs from *C. frutescens* LS 1839-2-4. Polymorphism was observed in four different *I2* homolog fragments between *C. annuum* NDN and KOS. One of them was mapped between A214 and A339 (IH6-06; Fig. 1a) and the others did not segregate from the L^3 gene (IH1-04 in Fig. 1a and two others that are not shown). Between *C. chinense* PI159236 and *C. frutescens* LS 1839-2-4, 17 out of 35 *I2* homolog fragments exhibited polymorphism. Two of them (IH1-04 and IH1-18, obtained by using PI2H-B1F1 and -B2R1) did not segregate from the L^3 gene and were used for fine mapping described below.

Fine mapping of the L^3 gene in the YB population

Because recombinants from the NK population failed to map newly developed RGA markers, we used the YB population for fine mapping of the L^3 gene. Preliminary mapping of the L^3 gene in the YB population indicated that A339 co-segregated with the L^3 gene and that A214 and YB2A25 were the closest molecular markers that flank the L^3 gene. The screening strategy of the YB population was as follows: all F2 plants were first

examined for A214, A339 and YB2A25; recombinant plants that had crossover points between A214 and YB2A25 were then tested for PMMoV resistance and used for mapping of newly developed markers. Analysis of 3,391 F2 plants identified 20 recombinants and enabled us to map the L^3 gene between two RGA markers, IH1-04 and IH1-18, and a BAC-end marker 189D23M further narrowed down the L^3 region (Fig. 1a). IH1-04 but not IH1-18 identified a specific BAC clone (Fig. 1b). The results indicated that the L^3 gene is contained in the pepper chromosome region included in five BAC clones and a single blank region between BAC clones 253A1 and 197AD5. Our BAC library did not have any positive clone for the 253A1R marker besides BAC clone 253A1 itself, and 197AD5R was not useful for BAC screening because it includes repetitive sequences and generated false positive clones. Therefore, the physical distance between 253A1 and 197AD5 could not be determined by the analysis of the BAC library.

Stretched DNA fiber FISH analysis for determination of the blank length in a BAC contig encompassing the L^3 gene

We employed stretched DNA fiber FISH analysis to assess the physical distance between BAC clones 253A1 and 197AD5. Because BAC clones consisting of contig 339 contain numbers of highly repetitive sequences (Fig. 2e, f), it was difficult to obtain unambiguous results in the analysis of this region by stretched DNA fiber FISH. DNA probes prepared from BAC clones 197AD5 and 99DD2 gave numerous short fluorescent signals, in addition to stretches of successive fluorescent signals in the absence of *Cot*-100 blocking DNA, and in its presence, gave only the short signals (data not shown). We found that probes prepared by an elongated labeling reaction are slightly more tolerant to blocking by *Cot*-100 DNA and generate longer signals than probes prepared by the standard 1 h reaction. Reproducible patterns of long fluorescent signals for BAC clones 253A1, 197AD5 and 99DD2 were eventually obtained (Fig. 3). The insert sizes of these clones were calculated from an electropherogram of *Hin*dIII-digested DNA (Fig. 2c) to be about 50, 80 and 90 kb, respectively. Based on these results, the blank region between BAC clones 253A1 and 197AD5 was assessed to be about 30 kb or shorter. Partial sequencing, restriction analysis and Southern analysis (data not shown) suggested that the overlapping lengths between each adjacent pair of BAC clones are as follows: 197AD5 and 99DD2, 15 kb; 99DD2 and 21L24, 40 kb; 21L24 and 185CA10, 25 kb. The L^3 gene was thus located within a pepper chromosome 11 region of about 350–400 kb.

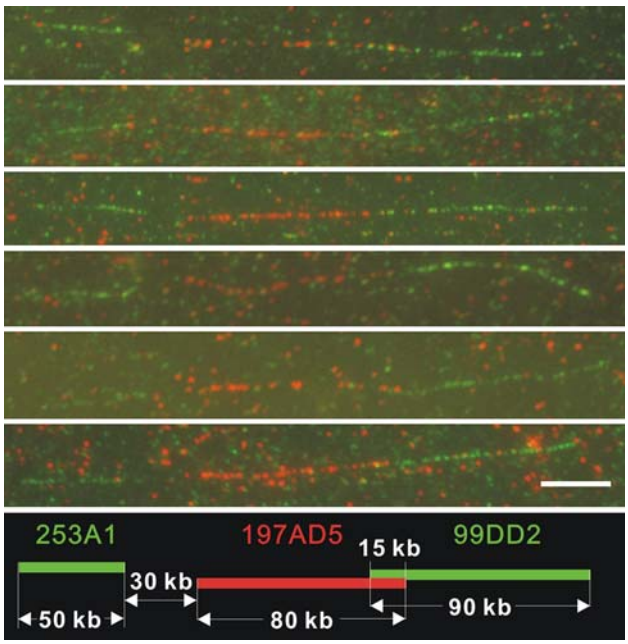

Fig. 3 Stretched DNA fiber FISH analysis to estimate the gap size between two BAC contigs. Stretched DNA fibers were probed with BAC clones 253A1, 197AD5 and 99DD2, fluorescently labeled *green*, *red* and *green*, respectively. Representative micrographs with apparent continuous signals are shown. As 197AD5 and 99DD2 contain highly repetitive sequences, only the continuous signals should be taken into account. The *scale bar* indicates 10 μm, which corresponds to 30 kb. Sizes of the BAC clones and the overlapping region between 197AD5 and 99DD2 were estimated from agarose gel electrophoresis, while the gap was estimated by stretched DNA fiber FISH analysis

An incomplete BAC contig encompassing the L^3 gene contains highly repetitive sequences and multiple copies of *I2* homologs

The *I2* locus was reported to contain seven compactly arranged genes, called the *I2* complex, homologous to the CC-NBS-LRR-class of R-gene within the region of 90 kb of the tomato chromosome. The pepper chromosome region containing L^3 reportedly has synteny to the tomato chromosome region containing *I2* and we identified three pseudogenes homologous to *I2* in BAC clone 21L24. To explore if the L^3 region codes for more *I2* homologs than those we had found in BAC clone 21L24, we analyzed five BAC clones that corresponded to the L^3 region and an additional four proximal clones, by Southern blotting. BAC clones digested with *Hin*dIII were analyzed with an *I2* homolog probe, revealing more than ten *I2* homologs within the L^3 region (Fig. 2d). Unlike the compactly arranged *I2* complex, they seemed to be sparsely distributed in this region. *I2* homologs were also detected outside the L^3 region (Fig. 2d, lanes 7, 8 and 9), suggesting that the L^3 region is part of a large R-gene cluster.

We then probed the identical Southern blot with *Cot*-1 and *Cot*-100 DNA to assess how much repetitive sequence exists in this region. A number of fragments were detected by the repetitive sequence probes with a slight difference in band patterns between the two probes (Fig. 2e, f). BAC clone 213E3, which is adjacent to the L^3 region, contained a large fragment heavily labeled by the repetitive sequence probes, revealing a large cluster of repetitive sequence adjacent to the L^3 region (Fig. 2e, f, lane 1). Although located in different *Hin*dIII fragments, other BAC clones (namely 21L24, 185CA10 and 181AC6) also contained large numbers of repetitive sequences (Fig. 2e, f, lanes 5, 6 and 7). These results are consistent with a previous report that demonstrated a molecular marker linked to the *L* locus that gave numerous positive clones when used in BAC library screening. Collectively, our results indicate that the L^3 gene is encoded within a pepper chromosome 11 region containing both large numbers of repetitive sequences and R-gene homologs including some pseudogenes.

Discussion

In this study, we mapped the L^3 gene to a pepper chromosome 11 region of about 400 kb. Although *L* genes have been known for a long time, they have not been precisely mapped, probably because of the lower density of molecular markers developed in this region. We used three different types of molecular markers for the mapping of the L^3 gene, AFLP, BAC-end and RGA markers. RGA markers were the most useful in this case, probably because the L^3 region is rich in repetitive sequences, is less polymorphic, and therefore, other types of markers are difficult to obtain in this region as suggested previously (Yoo et al. 2003). RGA markers were used to efficiently map quantitative trait loci (QTLs) related to several disease resistances (Pflieger et al. 1999), suggesting, together with our results, that RGA markers are useful in the mapping of resistance genes.

We analyzed BAC clones corresponding to the L^3 region and found that, as suggested previously, the L^3 region contained a cluster of R-like genes and also highly repetitive sequences (Grube et al. 2000; Yoo et al. 2003). Southern blotting suggested there are about ten copies of R-like genes in the 400 kb region. The result suggests that, like the *R3a* region, the L^3 region is larger and more sparsely arranged than the *I2* region, although the physical distance between common markers (e.g., TG105 and TG26 in tomato and potato; Ori et al. 1997; Simons et al. 1998; Huang et al. 2005) is not available in peppers. One of the *I2* homologs in potato [*I2GA-SH194-1* in Huang et al. (2005)] was shown to have an inserted retrotransposon. We found repetitive sequences in the Southern blotting of the BAC contigs and also identified a pair of similar sequences of about 2.3 kb in the partially sequenced 21L24 BAC clone (Accession No. AB442183 and AB442184). Although we have not identified any sequences typical to transposons in our BAC contigs, the insertion of transposons would be one of the mechanisms for physical expansion in the *R3a* and L^3 loci. It is interesting that the potato *R3a* locus is more similar in its R-like gene arrangement to the L^3 locus of the distantly related pepper than it is to the *I2* from the closely related tomato. Each of the resistance loci may have evolved independently during speciation, even if they share an ancestral origin. It is possible that, like the *R3a* gene, the L^3 gene is one of the *I2* homologs in pepper. However, this cannot be determined until the L^3 gene has been isolated (see below).

The structural features of the L^3 region provided insight into the structure of the *L* locus. As mentioned before, allelic genes—L^1, L^2, L^3 and L^4—provide increasing anti-tobamovirus resistance. There are some models for the hierarchical interaction between *L* genes and tobamoviruses (Fig. 4). One is that each allele consists of a single R-protein coding gene, which can confer increasing spectrum of resistance (Fig. 4a) and another is that each allele consists of increasing numbers of protein-coding genes that can confer resistance to either of the tobamovirus pathotypes (Fig. 4b). Our finding of multiple copies of R-like genes is consistent with both models. In addition to these models, it is possible that multiple genes have roles in the L^3-mediated resistance in a mutually dependent manner (Fig. 4c), analogous to the *Arabidopsis* RPP2A and RPP2B genes (Sinapidou et al. 2004). Although segregation of multiple genes involved should be assumed in the second model, such a phenomenon was not observed. Nevertheless, the possible linkage disequilibrium discussed below could explain the allelism of *L* genes in the second model.

Mapping of the L^3 gene in two different mapping populations revealed a striking difference between the two populations in the distribution patterns of recombination crossover points, suggesting linkage disequilibrium within the region encompassing the *L* locus. For example, only one recombinant was identified to have a crossover point between YB2A25 and A339 markers in the NK population, but there were eight recombinants in the YB population. On the other hand, there were 28 recombinants between the L^3 gene and marker A214 in the NK population but only 12 in the YB population, although the latter population was about 1.7 times larger. A possible explanation of the difference in the recombination patterns is that the distribution of repetitive sequences in the region is not conserved among the three species used in this study. *C. chinense* is the origin of the L^3 gene, *C. annuum* KOS has a susceptible gene (L^+), NDN has the L^3 gene derived from *C. chinense* and *C. frutescens* has the L^2 gene. Given that the

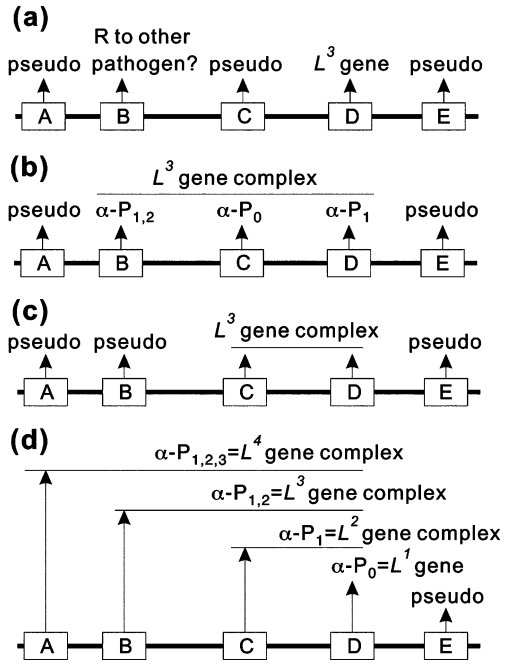

Fig. 4 Models for the structure of *L* genes, alleles of which exhibit an increasing spectrum of resistance to tobamoviruses. **a** Increasing spectrum of resistance is produced by a single R-protein coding gene that confers resistance to different pathotypes of tobamoviruses: e.g., L^3 gene to P_0, P_1 and $P_{1,2}$. Other R-like genes in L^3 region are either pseudogenes or R-genes to other pathogens. **b** Increasing spectrum of resistance is produced by increasing numbers of different R-protein coding genes, each of which confers resistance to one of the tobamovirus pathotypes: e.g., the L^3 gene consists of three R-genes, α-P_0, -P_1 and -$P_{1,2}$. **c** Increasing spectrum of resistance is produced as in **a**, but multiple copies of R-like genes are required for the conferral of resistance (e.g., L^3 requires two or more mutually dependent genes to confer the resistance to P_0, P_1 and $P_{1,2}$). **d** Increasing spectrum of resistance is produced by the additional components, which cannot function by themselves: e.g., L^3 gene confers the resistance to $P_{1,2}$ pathotype of tobamoviruses by an additional component B, function of which depends on two components, C and D, that confer the resistance to P_1. The resistance to P_1 pathotype is produced by the component C, function of which depends on the component D that confers the resistance to P_0

sequences, numbers and distribution patterns of repetitive sequences in the *L* loci of these three species are diverse from each other, the differences would lead to different recombination frequencies in some sub-regions of the *L* locus among these species. Another possible explanation is that the presence of repetitive sequence induces heterochromatin formation in the region (Lamb et al. 2007). However, this is not very likely because the region contains at least a single active gene that confers tobamovirus resistance.

In addition to the difference between the two mapping populations in the distribution patterns of recombination points, the sequence in closer proximity to the L^3 gene exhibited a strong tendency to be suppressed in recombination. In the YB population, there were three recombinants identified between 213E3R and 253A1R, which were generated from BAC-end sequences of two contiguous clones and separated by about 150 kb, but there was no recombination between 253A1R and 21L24M, which are about 300 kb away from each other. Moreover, no recombinant was identified in the NK population within the region encompassing the L^3 gene within 500 kb. As mentioned for the regions distantly linked to the L^3 gene, differences in highly repetitive sequences could have a role in the suppression of recombination in the L^3 region. Taken together, it is possible that each allele of the *L* genes contains a different number of R-genes that behave as a single locus, supporting the model b (Fig. 4).

Moscone et al. (2007) analyzed the karyotype of a number of wild and crop species from genus *Capsicum* by several techniques including fluorochrome staining and physical mapping of telomeric sequences. Based on the results, they proposed that *C. chacoense*, which harbors the L^4 gene, was the first to branch off the ancestral *Capsicum* plant, then the cluster of *C. annuum*, *C. chinense* and *C. frutescens* branched off, and thereafter others, including *C. baccatum*, were speciated. We analyzed sequences of SRC2 homologs from these five species, because a *C. chinense* gene similar to soybean SRC2 was induced during the resistance response (HH et al., unpublished result). Comparison of SRC2 homologs (Accession No. DQ465394, AB442165, AB442214, AB442215 and AB442216) supported the phylogenetic relationship proposed by Moscone (data not shown). We have tested several accessions belonging to these five species for the resistance to tobamoviruses and found that only *C. chacoense* has the L^4 gene (except for those with artificially introduced L^4 genes, e.g., L^4-cultivars of *C. annuum*). These facts led us to another model, in which ideas of models in Fig. 4b and c are combined (Fig. 4d). This model assumes that the L^4 gene complex consists of at least four components that are all required for the resistance to $P_{1,2,3}$ pathotype tobamovirus in the ancestral *Capsicum* plant and *C. chacoense*. During speciation, one of the components would have lost their function by a mutation: loss of gene A in Fig. 4d resulted in the generation of L^3 resistance; loss of gene B resulted in L^2 resistance (meanwhile, gene A became neutral for selection and eventually lost its functional requirements); loss of gene C resulted in L^1 resistance with eventual loss of function in genes A and B; and loss of gene D resulted in the susceptible phenotype with eventual loss of function in genes A, B and C. Under this model, a cross of the L^4 and susceptible L^+ plants would result in segregation of the component genes leading to the generation of all types of resistance in the F2 generation. Suppression of recombination in the *L* locus as discussed above might have

maintained the L^4 gene complex during the artificial crosses for breeding of L^4 cultivars.

In this study, we mapped L^3 gene to a 400 kb region by identifying five BAC clones that partially cover the region and revealed the gap in the BAC contig to be about 30 kb by stretched DNA fiber FISH. Although abundant repetitive sequences could interfere with obtaining contiguous sequence data, it is feasible to obtain sequences for protein coding genes from several independent contigs. R-gene homologs, especially *I2* homologs, would be good candidates for the L^3 gene. Functional analysis for identifying L^3 gene might be difficult for two reasons: more than two genes could be required for the L^3 resistance as discussed above, and the L^3 gene could be non-functional in heterologous species. Although *Agrobacterium*-mediated transient expression in pepper is quite inefficient in our hands, we have established a protocol for stable transformation of a tobamovirus-susceptible cultivar of *C. annuum* (JM and KS, unpublished results). Transgenic screening of the candidate genes would lead to the isolation of L^3 gene, which is expected to reveal the structure of *L* locus and the molecular basis of the hierarchical interaction between *Capsicum L* genes and tobamoviruses of different pathotypes.

Acknowledgments We thank Kazue Obara, Harumi Takahashi and Nozomi Takahashi for assistance with plant growth and technical issues. This study was supported in part by Iwate prefecture government and in part by a Grant-in-Aid for Scientific Research (C) (18580047) from the Japan Society for the Promotion of Science.

References

Berzal-Herranz A, De La Cruz A, Tenllado F, Diaz-Ruiz JR, Lopez L, Sanz AI, Vaquero C, Serra MT, Garcia-Luque I (1995) The Capsicum L3 gene-mediated resistance against the tobamoviruses is elicited by the coat protein. Virology 209:498–505

Boukema IW (1980) Allelism of genes controlling resistance to TMV in *Capsicum* L. Euphytica 29:433–439

Boukema IW (1982) Resistance to a new strain of TMV in *Capsicum chacoense* Hunz. Capsicum Newsl 1:49–51

Boukema IW (1984) Resistance to TMV in *Capsicum chacoense* Hunz. is governed by allele of the L-locus. Capsicum Newsl 3:47–48

Budiman MA, Chang SB, Lee S, Yang TJ, Zhang HB, De Jong H, Wing RA (2004) Localization of jointless-2 gene in the centromeric region of tomato chromosome 12 based on high resolution genetic and physical mapping. Theor Appl Genet 108:190–196

De La Cruz A, Lopez L, Tenllado F, Diaz-Ruiz JR, Sanz AI, Vaquero C, Serra MT, Garcia-Luque I (1997) The coat protein is required for the elicitation of the Capsicum L2 gene-mediated resistance against the tobamoviruses. Mol Plant Microbe Interact 10:107–113

Farnham G, Baulcombe DC (2006) Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. Proc Natl Acad Sci USA 103:18828–18833

Fransz PF, Alonso-Blanco C, Liharska TB, Peeters AJ, Zabel P, De Jong JH (1996) High-resolution physical mapping in *Arabidopsis* thaliana and tomato by fluorescence in situ hybridization to extended DNA fibres. Plant J 9:421–430

Garcia-Luque I, Ferrero Ml, Rodriquez JM, Alonso E, De La Cruz A, Sanz AI, Vaquero C, Serra MT, Diaz-Ruiz JR (1993) The nucleotide sequence of the coat protein genes and 3′ non-coding regions of two resistance-breaking tobamoviruses in pepper shows that they are different viruses. Arch Virol 131:75–88

Genda Y, Kanda A, Hamada H, Sato K, Ohnishi J, Tsuda S (2007) Two amino acid substitutions in the coat protein of pepper mild mottle virus are responsible for overcoming the L4 gene-mediated resistance in *Capsicum* spp. Phytopathology 97:787–793

Gilardi P, Garcia-Luque I, Serra MT (2004) The coat protein of tobamovirus acts as elicitor of both L2 and L4 gene-mediated resistance in Capsicum. J Gen Virol 85:2077–2085

Grube RC, Radwanski ER, Jahn M (2000) Comparative genetics of disease resistance within the solanaceae. Genetics 155:873–887

Haliassos A, Chomel J, Kruh JC, Kitzis A (1989) Detection of minority point mutations by modified PCR technique: a new approach for a sensitive tumor-progression markers. Nucleic Acid Res 17:8093–8099

Hamada H, Takeuchi S, Morita Y, Sawada H, Kiba A, Hikichi Y (2002) Amino acid changes in Pepper mild mottle virus coat protein that affect L3 gene-mediated resistance in pepper. J Gen Plant Pathol 68:155–162

Hamada H, Tomita R, Iwadate Y, Kobayashi K, Munemura I, Takeuchi S, Hikichi Y, Suzuki K (2007) Cooperative effect of two amino acid mutations in the coat protein of Pepper mild mottle virus overcomes L3-mediated resistance in Capsicum plants. Virus Genes 34:205–214

Huang S, Van Der Vossen EA, Kuang H, Vleeshouwers VG, Zhang N, Borm TJ, Van Eck HJ, Baker B, Jacobsen E, Visser RG (2005) Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. Plant J 42:251–261

Ishibashi K, Masuda K, Naito S, Meshi T, Ishikawa M (2007) An inhibitor of viral RNA replication is encoded by a plant resistance gene. Proc Natl Acad Sci USA 104:13833–13838

Kawaguchi M, Motomura T, Imaizumi-Anraku H, Akao S, Kawasaki S (2001) Providing the basis for genomics in Lotus japonicus: the accessions Miyakojima and Gifu are appropriate crossing partners for genetic analyses. Mol Genet Genomics 266:157–166

Ke X, Collins A, Ye S (2001) PIRA PCR designer for restriction analysis of single nucleotide polymorphisms. Bioinformatics 17:838–839

Lamb JC, Yu W, Han F, Birchler JA (2007) Plant chromosomes from end to end: telomeres, heterochromatin and centromeres. Curr Opin Plant Biol 10:116–122

Lefebvre V, Pflieger S, Thabuis A, Caranta C, Blattes A, Chauvet JC, Daubeze AM, Palloix A (2002) Towards the saturation of the pepper linkage map by alignment of three intraspecific maps including known-function genes. Genome 45:839–854

Li L, Yang J, Tong Q, Zhao L, Song Y (2005) A novel approach to prepare extended DNA fibers in plants. Cytometry A 63:114–117

Livingstone KD, Lackney VK, Blauth JR, Van Wijk R, Jahn MK (1999) Genome mapping in capsicum and the evolution of genome structure in the solanaceae. Genetics 152:1183–1202

Matsumoto K, Sawada H, Matsumoto K, Hamada H, Yoshimoto E, Ito T, Takeuchi S, Tsuda S, Suzuki K, Kobayashi K, Kiba A, Okuno T, Hikichi Y (2008) The coat protein gene of tobamovirus P(0) pathotype is a determinant for activation of temperature-insensitive L (1a)-gene-mediated resistance in Capsicum plants. Arch Virol 153:645–650

Mcdowell JM, Woffenden BJ (2003) Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol 21:178–183

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Moscone EA, Scaldaferro MA, Grabiele M, Cecchini NM, Sanchez Garcia Y, Jarret R, Davina JR, Ducasse DA, Barboza GE, Ehrendorfer F (2007) The evolution of chili pepper (*Capsicum*—Solanaceae): a cytogenetic perspective. Acta Hortic 745:137–169

Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R (1997) The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521–532

Pflieger S, Lefebvre V, Caranta C, Blattes A, Goffinet B, Palloix A (1999) Disease resistance gene analogs as candidates for QTLs involved in pepper-pathogen interactions. Genome 42:1100–1110

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sawada H, Takeuchi S, Hamada H, Kiba A, Matsumoto M, Hikichi Y (2004) A New *tobamovirus*-resistance gene, L^{1a}, of sweet pepper (*Capsicum annum* L.). J Jpn Soc Hortic Sci 73:552–557

Simons G, Groenendijk J, Wijbrandi J, Reijans M, Groenen J, Diergaarde P, Van Der Lee T, Bleeker M, Onstenk J, De Both M, Haring M, Mes J, Cornelissen B, Zabeau M, Vos P (1998) Dissection of the fusarium I2 gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055–1068

Sinapidou E, Williams K, Nott L, Bahkt S, Tor M, Crute I, Bittner-Eddy P, Beynon J (2004) Two TIR:NB:LRR genes are required to specify resistance to *Peronospora parasitica* isolate Cala2 in *Arabidopsis*. Plant J 38:898–909

Sugita T, Kinoshita T, Kawano T, Yuji K, Yamaguchi K, Nagata R, Shimizu ALC, Kawasaki S, Todoroki A (2005) Rapid construction of a linkage map using high-efficiency genome scanning/AFLP and RAPD, base on an intraspecific, double haploid population of *Capsicum annuum*. Breed Sci 55:287–295

Tsuda S, Kirita M, Watanabe Y (1998) Characterization of a pepper mild mottle tobamovirus strain capable of overcoming the L3 gene-mediated resistance, distinct from the resistance-breaking Italian isolate. Mol Plant Microbe Interact 11:327–331

Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Walling JG, Pires JC, Jackson SA (2005) Preparation of samples for comparative studies of plant chromosomes using in situ hybridization methods. Methods Enzymol 395:443–460

Yamasaki S, Mori T, Yoshimatsu H, Kubota K, Okuda M, Takeshita M, Takanami Y (2007) Characterization of two PMMoV P1,2,3 strains obtained in Oita, and evaluation of cross-protection ability of an attenuated strain, P1,2 T219 (Abstract). In: Abstract Phytopathol Soc Jpn Ann Meeting 2007, p 104

Yoo EY, Kim S, Kim YH, Lee CJ, Kim BD (2003) Construction of a deep coverage BAC library from *Capsicum annuum*, 'CM334'. Theor Appl Genet 107:540–543