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ORIGINAL PAPER

BAC-derived markers converted from RFLP linked to *Phytophthora capsici* resistance in pepper (*Capsicum annuum* L.)

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Abstract *Phytophthora capsici* Leonian, an oomycete pathogen, is a serious problem in pepper worldwide. Its resistance in pepper is controlled by quantitative trait loci (QTL). To detect QTL associated with *P. capsici* resistance, a molecular linkage map was constructed using 100 F_2 individuals from a cross between *Capsicum annuum* 'CM334' and *C. annuum* 'Chilsungcho'. This linkage map consisted of 202 restriction fragment length polymorphisms (RFLPs), 6 WRKYs and 1 simple sequence repeat (SSR) covering 1482.3 cM, with an average interval marker distance of 7.09 cM. QTL mapping of *Phytophthora* root rot and damping-off resistance was performed in $F_{2:3}$ originated from a cross between resistant Mexican landrace *C. annuum* 'CM334' and susceptible Korean landrace *C. annuum* 'Chilsungcho' using composite interval mapping (CIM) analysis. Four QTL explained 66.3% of the total phenotypic variations for root rot resistance and three 44.9% for damping-off resistance. Of these QTL loci, two were located close to RFLP markers CDI25 on chromosome 5 (P5) and CT211A on P9. A bacterial artificial chromosome (BAC) library from *C. annuum* 'CM334' was screened with these two RFLP probes to obtain sequence information around the RFLP marker loci for development of PCR-based markers. CDI25 and CT211 probes identified seven and eight BAC clones, respectively. Nine positive BAC clones containing probe regions were sequenced and used for cytogenetic analysis. One single-nucleotide amplified polymorphism (SNAP) for the CDI25 locus, and two SSRs and cleaved amplified polymorphic sequence (CAPS) for CT211 were developed using sequences of the positive BAC clones. These markers will be valuable for rapid selection of genotypes and map-based cloning for resistance genes against *P. capsici.*

H.-J. Kim and S.-H. Nahm contributed equally to this work.

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Introduction

Pepper is originated from South America (Lee et al. 2005) and has been an important condiment and an economically important vegetable worldwide (Bosland 1992) as well as in Korea (Hong et al. 1998). There have been continuous efforts to improve pepper traits related to disease resistance (Minamiyama et al. 2007), male sterility (Kim et al. 2006), yield (Rao et al. 2003), fruit color (Huh et al. 2001), and important secondary metabolites (Lee et al. 2005). Among these efforts of pepper breeding, disease resistance has been a major target trait.

One of the most destructive pepper pathogens worldwide (Barksdale et al. 1984) is *Phytophthora capsici* Leonian (Leonian 1922), which attacks pepper plants at all developmental stages and all tissues (Quirin et al. 2005). It causes several plant symptoms depending on the infected tissue, such as root rot, crown rot, foliar blight, stem lesion, fruit rot, and the damping-off of seedlings (Ristaino 1990). This soil-borne pathogen (Walker and Bosland 1999) is a multicyclic disease, living on both dead (necrotroph) and live (biotroph) plants, and reproduces both sexually and asexually (Bonnet et al. 2007), causing a persistent problem especially in the regions of repeated chili cultivation.

Resistant pepper resources such as 'CM334' (Guerrero-Moreno and Laborde 1980; Lefebvre and Palloix 1996; Thabuis et al. 2003) from Mexico (Guerrero-Moreno and Laborde 1980), 'AC2258' (Smith et al. 1967; Sugita et al. 2006), 'PI201232' (Smith et al. 1967; Ortega et al. 1995), and 'PI201234' (Ogundiwin et al. 2005) from Central America (Kimble and Grogan 1960) have been reported. Among these resistant varieties, 'CM334' has mainly been used for disease resistance studies because it has the highest resistance level (Bosland and Lindsey 1991; Ortega et al. 1992; Quirin et al. 2005).

The expression of resistance to *P. capsici* in pepper is affected by many environmental factors such as plant cultivar and age (Reifschneider et al. 1992; Kim and Hwang 1989), inoculum dose, temperature, soil moisture (Ortega et al. 1995), fungal isolate (Reifschneider et al. 1992), inoculation method (Kim and Hwang 1989), and the pathogenicity of isolate (Ogundiwin et al. 2005). Due to these factors, *P. capsici* is a complicated pathogen to control (Ogundiwin et al. 2005), and developing resistant cultivars by conventional breeding approach alone has not been successful (Reifschneider et al. 1992; Quirin et al. 2005). If reliable markers are available, the breeding process will be expedited.

Many studies have been reported on the genetic basis of resistance in pepper–*P. capsici* interactions and the development of molecular markers for MAS (Bonnet et al. 2007; Minamiyama et al. 2007; Thabuis et al. 2003). Previously published studies on the resistance of 'CM334' to *P. capsici* came to various genetic models such as two recessive genes (Guerrero-Moreno and Laborde 1980), two dominant genes (Reifschneider et al. 1992; Walker and Bosland 1999), three genes, and additive gene models (Ortega et al. 1995). It was later concluded that polygene with additive or epistatic action controlled *Phytophthora* resistance in pepper (Lefebvre and Palloix 1996).

Furthermore, one consistent major quantitative trait loci (QTL, *Phyto.5.1*) was found with three populations and by two inoculation methods (one component for root inoculation test and three for stem) for two strains. In the F_2 population of a cross between 'Yolo Wonder' and 'CM334' (YCM), five common QTL regions (regions involving more than two components) on P4, P5, P6, P11, and P12, and four specific QTL regions (regions involving one component; Lefebvre and Palloix 1996) on P5, P6, P12, and the linkage group 1 (LG 1) were detected (Thabuis et al. 2003). In other studies, one common QTL (*Phyto.U*) on P5 was found among five QTL from a root rot test in an F_2 population of a cross between 'Joe E. Parker' and 'CM334' inoculated with 'M' isolate, and among 16 QTL from root rot and foliar blight tests in RIL_7 from 'PSP-11' and 'PI201234', which had been inoculated with seven isolates (Ogundiwin et al. 2005). In the double haploid F_1 population between 'Manganji' and 'CM334', two QTL on LG3 and P5 were frequently found (Minamiyama et al. 2007), and eight QTL in RIL_5 of YC on P1, P4, P5, P6, and P11 were detected (Bonnet et al. 2007).

Moreover, a successful transfer of four resistant QTL was done by MAS on two backcross populations using ten markers (three on P5, three on P10, and four on P2; Thabuis et al. 2004). Loss and conservation of QTL alleles on P4, P5, P6, P11, P12 and five other chromosomes (without resistant QTL) from 'CM334' into bell pepper were observed from the results of a recurrent selection program using 34 amplification fragment length polymorphisms (AFLP) and two PCR markers (Thabuis et al. 2004). Some of these resistant polygene, as well as favorable epistatic effects, would be easily lost in the process of several crossbreeds and phenotypic selections in conventional breeding for genetically resistant cultivars (Bartual et al. 1994; Thabuis et al. 2004). To conserve QTL alleles during breeding process, developing and application of QTL markers are necessary. However, previously developed AFLP markers could be difficult to be applied on other mapping populations, because of low reproducibility and multi-bands in a polyacrylamide gel. A dominant SCAR marker (*phyto 5.2*) was developed from the result of bulked segregant analysis (BSA) with randomly amplified polymorphic DNA (RAPD) primers (Quirin et al. 2005). However, because of the dominant nature of the marker, its usability for MAS is very limited. To overcome these limitations, more informative and highly reproducible co-dominant QTL markers are needed. Moreover, these co-dominant QTL markers should be more easily mapped on different mapping populations.

The objectives of this study were, first, to investigate the mode of inheritance and to select markers near QTL defining resistance against *Phytophthora* root rot and damping-off disease. To meet the objectives sequence information of QTL using bacterial artificial chromosome (BAC) clones and BAC sequence derived markers were developed, and were applied to 13 resistant commercial cultivars.

Materials and methods

Plant materials and genomic DNA extraction

Mapping population

Mexican landrace 'Criollo de Morelos-334' (*Capsicum annuum* 'CM334'), resistant to *P. capsici*, and Korean landrace *C. annuum* 'Chilsungcho', susceptible to *Phytophthora*, were used as parents for a mapping population. 'CM334' was provided by A. Palloix (INRA-Avignon, France) and 'Chilsungcho' by B. S. Kim (Kyungpook National University, Korea). An F_2 mapping population consisting of 100 plants was developed from a cross between parental lines to construct a genetic map. $F_{2:3}$ families were used for resistance evaluation. Genomic DNA extraction was performed as described earlier (Kang et al. 2001).

Molecular markers

RFLPs

Restriction fragment length polymorphisms were developed as described earlier (Kang et al. 2001). Disease-related EST clones of tobacco and pepper were provided by D. Choi (KRIBB, Korea), and markers from the probes were designated as Tob and CDI, respectively. Potato clones adjacent to *Phytophthora* resistance loci in potatoes were isolated with previously reported primers for GP and StPto markers (Oberhagemann et al. 1999; Collins et al. 1999). Thirty-three NBS-LRR analogous clones were isolated from 'CM334' using degenerate PCR with primers designed from conserved sequences of NBS and LRR regions (pR markers; Kanazin et al. 1996; Wenkai et al. 2006). Of the 510 probes, 207 produced polymorphisms between the parental lines and were used for F_2 screening.

WRKYs and SSRs

WRKYs were amplified using PCR with primer sets designed from conserved nucleotides of the WRKY domain in WRKY group II (Kim et al. 2008). These PCR products were separated in 6% polyacrylamide gels for 2.5 h at 100 W. Gels were dried for 40 min using a Hoefer Slab Gel Dryer GD2000 (Amersham Pharmacia Biotech, USA) and exposed to the X-ray film. PCR bands were scored as dominant markers. Thirty-one WRKY markers polymorphic between the parental lines were further screened in the F_2 population. Three simple sequence repeats (SSR) markers (HpmsE04, HpmsE015, and HpmsE027) that were developed in this laboratory were also used as a reference (Yi et al. 2006).

Map construction

An intraspecific map was constructed using 207 RFLP, 31 WRKY, and 3 SSR markers. The segregation ratio of all markers was tested for goodness of fit to 3:1. Markers selected by the chi-square test ($P < 0.01$) were arranged on linkage groups using CARTHAGENE (Schiex and Gaspin 1997). Recombination frequencies were converted into mapping distances in centiMorgan (cM) using the Kosambi function (Kosambi 1994) using MAPMAKER (Lander et al. 1987). The minimum LOD value and maximum distance were 4.0 and 40, respectively.

Preparation for inoculation with *P. capsici*

Moderately aggressive *P. capsici* isolate 'Pa23' from Korea was provided by H. J. Jee (Rural Development Administration, Korea). Pa23 was grown on V8 juice agar media in petri dishes (80 mm in diameter) at 28°C for 4 days, and then its mycelial plugs (8 mm in diameter) were cut out at the periphery and cultured on new V8 juice agar media for 3 days. The mycelia were scraped with cotton swabs and incubated under continuous light for 3 days to stimulate zoosporangia formation. Sterile water was added to petri plates, which were incubated at 4°C for 1 h. Consecutively, plates were put back at 28°C for 30 min. Suspensions of released zoospores were used for root rot and damping-off screening.

Root rot resistance assay

To assess the resistance level of 100 individuals in the F_2 population, F_3 plants derived from each F_2 plant (F_2:$_3$) as well as both parents were screened. Twenty-five plants for each parent and 40–50 F_3 plants per F_2 line (F_2:$_3$) were tested per replicate, and the experimental design was a randomized complete block, with three replicates. Parents and F_3 plants were grown in trays of 50 cells (cell size: 4.7 cm × 4.7 cm × 5 cm) in a glasshouse and used for inoculation at the first flowering stage (10 week old). Ten milliliter of inoculum (10^4 zoospores/ml) was introduced into the soil of each cell. The second inoculation was performed with the same concentration 7 days post-inoculation (dpi) to minimize experimental errors. The glass-

house temperature was controlled at 30°C/18°C (day/night time), and the plants were kept in a flooded condition for 3 dpi. Four weeks after the first inoculation, roots were washed with tap water, and root rot severity was scored on a scale of 1–5 (resistant 1–susceptible 5) based on the ratio of the necrosis region extended to the entire root.

Damping-off resistance assay

Both parents and 25 F_3 plants from each 100 F_2 individual ($F_{2:3}$) per replication in three replications were sown into sterilized soil in Magenta boxes (size: 7.5 cm × 7.5 cm × 10 cm) and cultured in the growth chamber at 25°C under 50–60% relative humidity. Seedlings with four leaves at 3 weeks after germination were inoculated with 10 ml of inoculum (10^4 zoospores/ml). The damping-off of seedlings appeared on susceptible plants starting from 3 days after inoculation. The number of newly dead seedlings was counted daily cumulatively until 3 weeks after inoculation when near normal distribution was achieved for the fraction of dead plants.

Statistical analysis and QTL detection

The statistical analysis of the data was performed using SAS 9.1.3 service pack 3 software (SAS Institute 1989). Normality was checked using PROC UNIVARIATE and Wilk and Shapiro's test (Thabuis et al. 2003). The mean of the disease index of each $F_{2:3}$ set was calculated as the disease index of each F_2 plant and the data of root rot assay were transformed by calculation of the inverse hyperbolic sine prior to QTL analysis. One-way analysis of variance (ANOVA) using a PROC GLM procedure of the SAS software was used to determine the associations between the marker and phenotype of *Phytophthora* resistance. A significance level of $P < 0.01$ was retained. Two-way analysis was tested to survey digenic interactions between markers at a significance level of $P < 2 \times 10^{-4}$. Correlations among the results obtained from repeated experiments were calculated by Pearson phenotypic correlation coefficients.

Resistance associated QTL were detected by composite interval mapping (CIM) with a forward and backward stepwise regression method using Windows QTL Cartographer version 2.0 (Basten et al. 2002). CIM was performed with a 10 cM window and a maximum of five marker cofactors per model at 2 cM intervals. When two QTL were detected by CIM within less than 20 cM, only the most significant was retained (Thabuis et al. 2003). For each resistance component, significance thresholds were computed by permutation tests (1,000 permutations, a genome-wide $P < 0.05$). For QTL detection of root rot and damping-off resistance, the calculated thresholds were LOD scores of 1.48 and 1.44, respectively.

BAC libraries screening and BAC clone size determination

The pepper BAC library has been described by Yoo et al. (2001, 2003). Probes of RFLP markers related to *Phytophthora* resistance were used for BAC library screening. Library screening and plasmid DNA isolation were performed as described earlier (Yoo et al. 2003). To determine the size of screened BAC insertions, BAC clones were digested with *Not*I and separated by pulsed-field gel electrophoresis on a CHEF DRIII (Bio-Rad, USA) for 8 h at 16°C with an initial pulse time of 5 s and a final pulse time of 10 s, at a 120° angle and 6 V cm ± 1.

BAC contig construction

Screened BAC clones were hybridized with the RFLP probes to exclude pseudo-signals as described earlier (Kang et al. 2001). These BAC insertion ends were sequenced using Sp6 or T7 primers (NICEM, Korea). Two BAC insertions from CDI25 and CT211 probes were fully sequenced (KRIBB, Korea). The sequence information from the BAC insertions was used for the BAC-specific PCR primer design and the alignment of BAC clone sequences. Overlapped regions among BAC insertions were confirmed by fingerprinting assembly (Macrogen, Korea) using the fingerprinted contig program (FPC; Luo et al. 2003) and cytogenetic analysis using fluorescence in situ hybridization (FISH; Park et al. 1999). The direction of contig extension was verified by genetic mapping of BAC clones, BAC-PCR with BAC-derived primer sets, and BAC sequence alignment.

Genomic sequence data analysis

Repeat sequences were filtered by online RepeatMasker (http://www.repeatmasker.org) and JDotter (http://athena.bioc.uvic.ca). Candidates for gene coding sequences were identified in the genomic regions by FGENESH (http://linux1.softberry.com) of the dicot plant database (*Arabidopsis*, tomato, and tobacco), GENSCAN (http://genes.mit.edu) and BLASTX searches (E value $< 3 \times 10^{-15}$) to differentiate between intron and exon regions. Primers were designed for regions containing introns to capture a high level of polymorphism.

Results

Map construction

A total of 207 markers polymorphic between *C. annuum* 'CM334' and *C. annuum* 'Chilsungcho' were detected by 172 probes of the 510 probes used for the RFLP analysis.

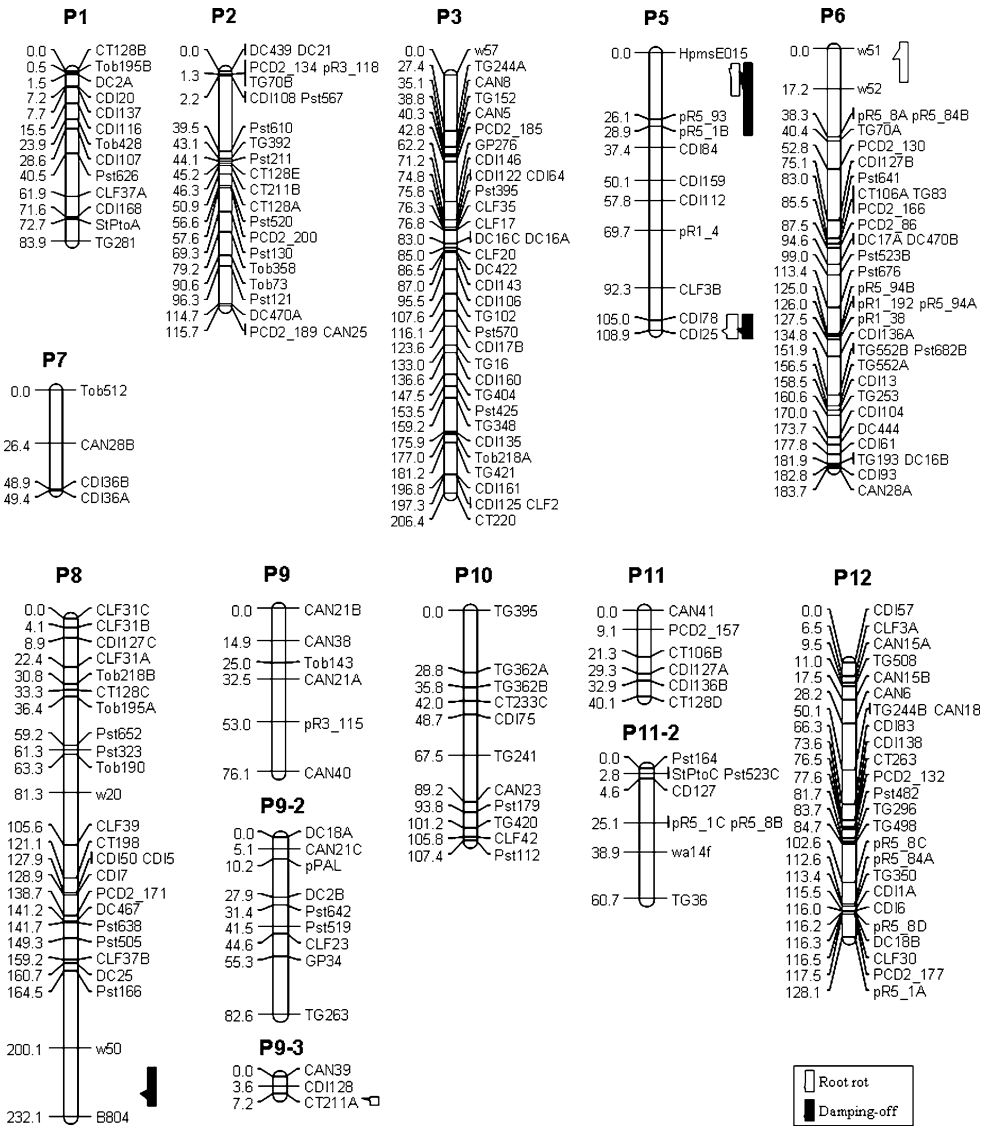

Fig. 1 The positions of QTL that affect resistance to root rot and damping-off caused by *Phytophthora capsici* 'Pa23' on an SNU 4 chili pepper linkage map of 'CM334' × 'Chilsungcho' F_2 population. On the *left* of the vertical double lanes are map distances in cM calculated by the Kosambi function and on the *right* are DNA marker names. *W* indicates WRKY marker, and Hpms SSR. Chromosome number (P) is at the *top*; genetic markers are on the *right* of each linkage group. *Bars* on the *right* of marker names indicate the locations of four and three QTL affecting *Phytophthora* root rot and damping-off resistance, respectively

A total of 202 RFLPs, 6 WRKYs, and 1 SSR were placed on all chromosomes except chromosome 4 (Fig. 1). This intraspecific map designated as an SNU 4 chili pepper map was composed of 209 markers and covered 1,482.3 cM with an average map distance of 7.09 cM between markers.

QTL mapping

The $F_{2:3}$ families and both parents were evaluated for resistance against *Phytophthora* root rot and damping-off. The distribution of root rot scores was clearly biased toward resistance (Fig. 2a), while that of damping-off scores showed a more symmetrical distribution (Fig. 2b). Pearson's correlation coefficient between the two independent resistance experiments showed a weak correlation ($r = 0.50$, $P < 10^{-4}$), which means that genetic factors of the two experiments are not completely independent.

By comparative interval mapping (CIM, Fig. 1), four QTL in the root rot resistance test explained 66.3% of the total phenotypic variation with an LOD threshold of 1.48, and three QTL in the damping-off resistance test explained 44.9% of the total variations with an LOD threshold of 1.44 (Table 1). Among the QTL, two loci on P5 were detected for both traits and were closely located with each other. One QTL on P8 was damping-off resistance-specific and two QTL on P6 and P9 were root rot resistance-specific in this population. Of the five resistance alleles, two QTL on P5 and one QTL on P9 were originated from 'CM334', whereas one each QTL on P6 and P8 were originated from 'Chilsungcho'. Of the detected QTL, one QTL was positioned close to CDI25 on P5 and the other was near CT211A on P9, both with a distance of about 0.01 cM. The two RFLP markers were detected by CIM and by single-factor ANOVA analysis with a significance level of $P < 5 \times 10^{-3}$.

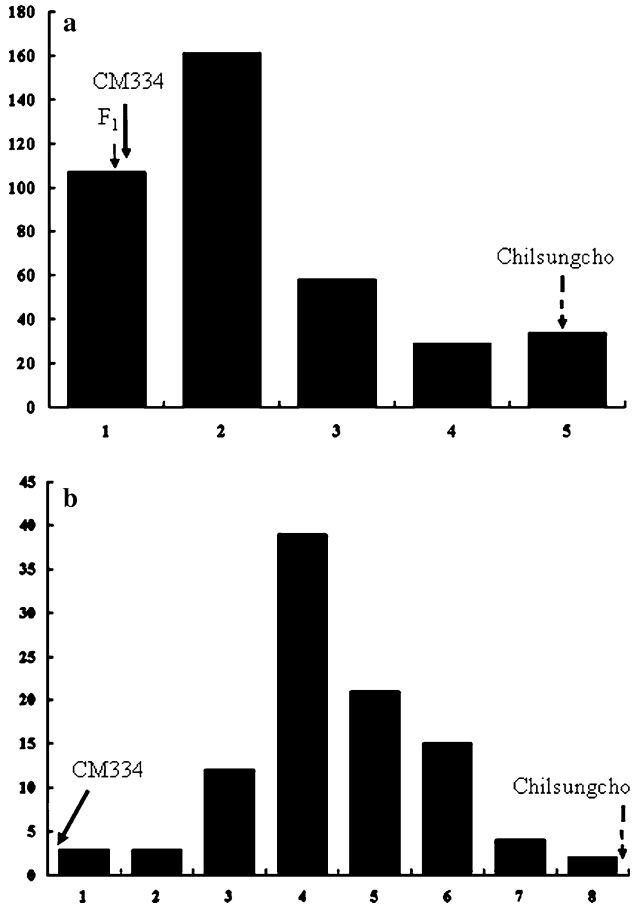

Fig. 2 Frequency distributions in the F_2 population for resistance to *Phytophthora capsici* isolate 'Pa23'. *Arrows* indicate the approximate positions of the parental and F_1 values. **a** Root rot scores (mean of three replicates) as root rot tissue in degree, on *1* (resistant) to *5* (susceptible) scale, **b** damping-off scores (mean of three replicates) on a scale from *1* (resistant) to *8* (susceptible) based on the number of break down seedlings

Digenic interactions

Epistatic interactions were investigated using two-factor ANOVA analysis at a significance level of $P < 2.0 \times 10^{-4}$. Significant digenic interactions were shown between QTL on P9 and CAN15B on P12 ($P = 7.6 \times 10^{-5}$, $R^2 = 19.19\%$), QTL on P5 and HpmsE04 ($P = 1.2 \times 10^{-4}$, $R^2 = 26.46\%$), and HpmsE04 and CDI17A ($P = 1.5 \times 10^{-4}$, $R^2 = 26.03\%$) in the damping-off trait, and between QTL on P5 and DC17A ($P = 5.8 \times 10^{-5}$, $R^2 = 19.68\%$), QTL on P5 and DC470B ($P = 5.8 \times 10^{-5}$, $R^2 = 19.68\%$), QTL on P9 and TG508 ($P = 1.3 \times 10^{-4}$, $R^2 = 22.91\%$), and QTL on P9 and CLF3A ($P = 8.1 \times 10^{-5}$, $R^2 = 23.62\%$) in the root rot trait. The direct interaction between two QTL on P5 and P9 was not detected. The QTL on P5 was capable of interacting with CDI17A in the damping-off trait. More interactions between undefined markers in the root rot trait were observed, but the others were not located on the map.

BAC screening with the RFLP probes

A BAC library of *C. annuum* 'CM334' was used for hybridization-based screening. Using the CDI25 and CT211 probes tightly linked to *Phytophthora* resistance QTL, seven and eight BAC clones were found, respectively. Average insert sizes of total BAC clones screened by CDI25 and CT211 probes were 107.1 and 106.6 Kb, respectively, and they ranged from 90 to 130 Kb (Fig. 3).

BAC clone confirmation and contig construction

To exclude pseudo-clones, BAC clones were placed on a nitrocellulose membrane together with parent genomic DNAs and pIndigo536 vector, which was used to construct the BAC library. The membrane was hybridized with CDI25 and CT211 probes (Fig. 3). The CDI25 probe detected four of the seven BAC clones and the CT211 probe detected six of the eight BAC clones. All 15 clones were also fingerprinted to construct BAC contigs for the two probes. The fingerprinting was based on band patterns cut by five enzymes (*Eco*RI, *Bam*HI, *Xba*I, *Xho*I, and *Hae*III). Four (475, 642, 708, and 770) and five (216b, 216c, 464, 499, and 502) BAC clones were assembled into each contig for CDI25 and CT211 marker loci, respectively. The results of hybridization and fingerprinting of BACs associated with the CDI25 probe were perfectively matched. On the other hand, the BAC 216a clone selected from BAC hybridization with the CT211 probe was discarded from further study.

Four BAC clones confirmed from CDI25 probe and five from CT211 were used as probes for FISH to reaffirm their positions on metaphase chromosomes of *C. annuum* 'CM334' (Fig. 4). Three (642, 216c, and 502) of nine clones with corresponding RFLP markers presented clear and strong signals, while the others produced background or no main signals. The BAC 642 clone (red signal) from CDI25 hybridized on the middle of a chromosome (Fig. 4a), which could be considered as P5 (Fig. 4c). Using a dual color FISH, the signals of BACs 216c (green) and 502 (red) resided near the telomere (the second euchromatic region from the end) of a chromosome (Fig. 4b), which could be P9 (Fig. 4d). The FISH demonstrated that two BAC probes screened by the same RFLP marker hybridized to the same chromosomal region.

All BAC clones were end-sequenced, and aligned with full sequences of two BACs using the CodonCode Aligner program (http://www.codoncode.com/aligner) to design PCR primer sets. These primers were used for PCR amplification of the 15 BAC clones to detect overlapping regions. From the results of hybridization, fingerprinting, FISH, and BAC-PCR, BAC contigs from two corresponding probes were constructed and confirmed (Fig. 5).

Table 1 Features of QTL for resistance to *Phytophthora capsici* root rot and damping-off as detected by composite interval mapping in the F_2 ('CM334' × 'Chilsungcho') population

Trait[a]	Chr no.	Marker interval[b]	Resistance allele[c]	Composite interval mapping results			
				LOD[d]	R^2 (%)[e]	Additive effect[f]	2 Id/aI[g]
Root rot	5	CDI25–CDI78	CM	10.48	41.97	−0.38	2.90
	5	pR5_93–HpmsE015	CM	2.40	8.01	−0.05	0.25
	6	W51–W52	Chil	2.03	8.56	0.13	1.48
	9	CT211A–CDI128	CM	2.87	7.73	−0.03	0.16
Damping-off	5	CDI25–CDI78	CM	4.65	18.72	−3.33	0.75
	5	pR5_93–HpmsE015	CM	2.25	9.00	−1.51	0.33
	8	W50–B804	Chil	2.87	17.14	1.63	2.42

[a] Scored pepper symptom from *Phytophthora capsici* for QTL mapping. Root rot for adult chili plant, and damping-off for seedling

[b] Markers flanking the peak log of odds ratio (LOD) of the QTL

[c] Resistance alleles were estimated by the phenotypic means of each allele. CM; 'CM334', Chil;'Chilsungcho'

[d] LOD of the QTL

[e] Percentage of phenotypic variation explained by the QTL under consideration; R^2 of marker linked to the QTL was calculated with multiple regression analysis

[f] Additive estimate by CIM

[g] Dominance ratio; its significance is DR < 0.2 (additive), 0.2 < DR < 0.8 (partially dominant), 0.8 < DR < 1.2 (dominant), DR > 1.2 (overdominant)

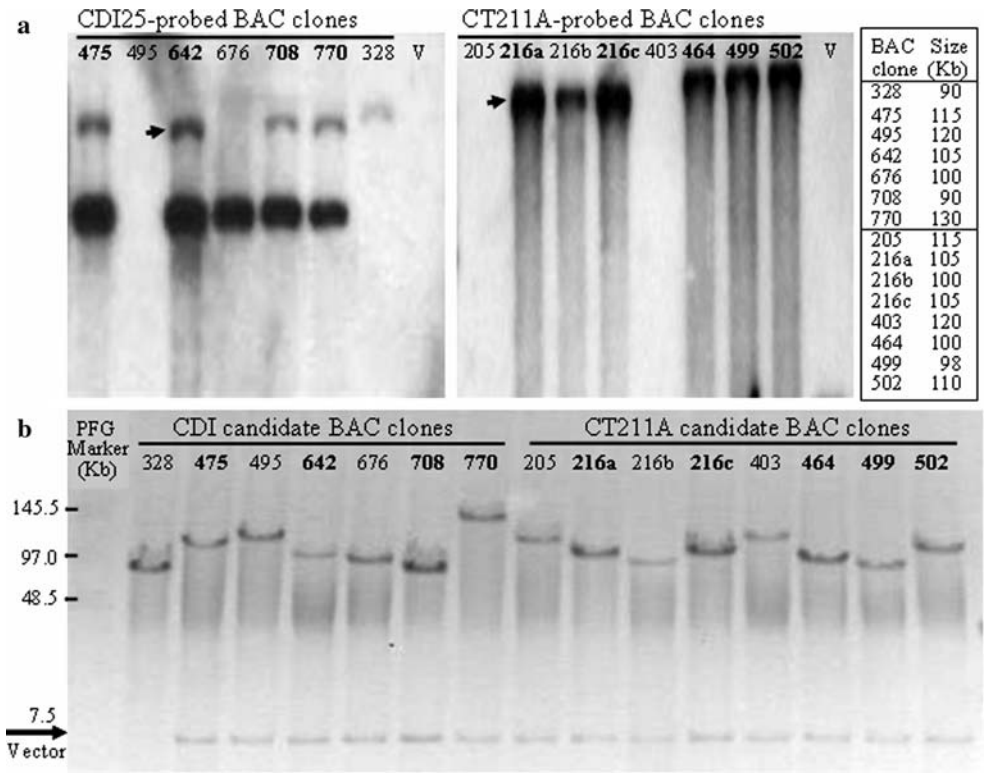

Fig. 3 Analysis of BAC clones. **a** An autoradiogram showing Southern blot analysis. The gel was transferred to a nylon membrane and hybridized with either the CDI25 or the CT211 probe. All DNA were digested with *Hin*dIII and *Eco*RI for CDI25 and CT211 probes, respectively. The *arrow* indicates the band confirmed by fingerprinting. **b** Ethidium bromide-stained agarose gel showing 15 random BAC clones (*lanes 2–16*) digested with *Not*I and separated by pulsed-field gel electrophoresis (PFGE). The sizes of insertion were determined on the basis of lambda concatamer size markers (*lane 1*) and the 7.3 kb pIndigo536 vector (*lane 17*). The sizes of BAC insertions ranged from 90 to 130 kb

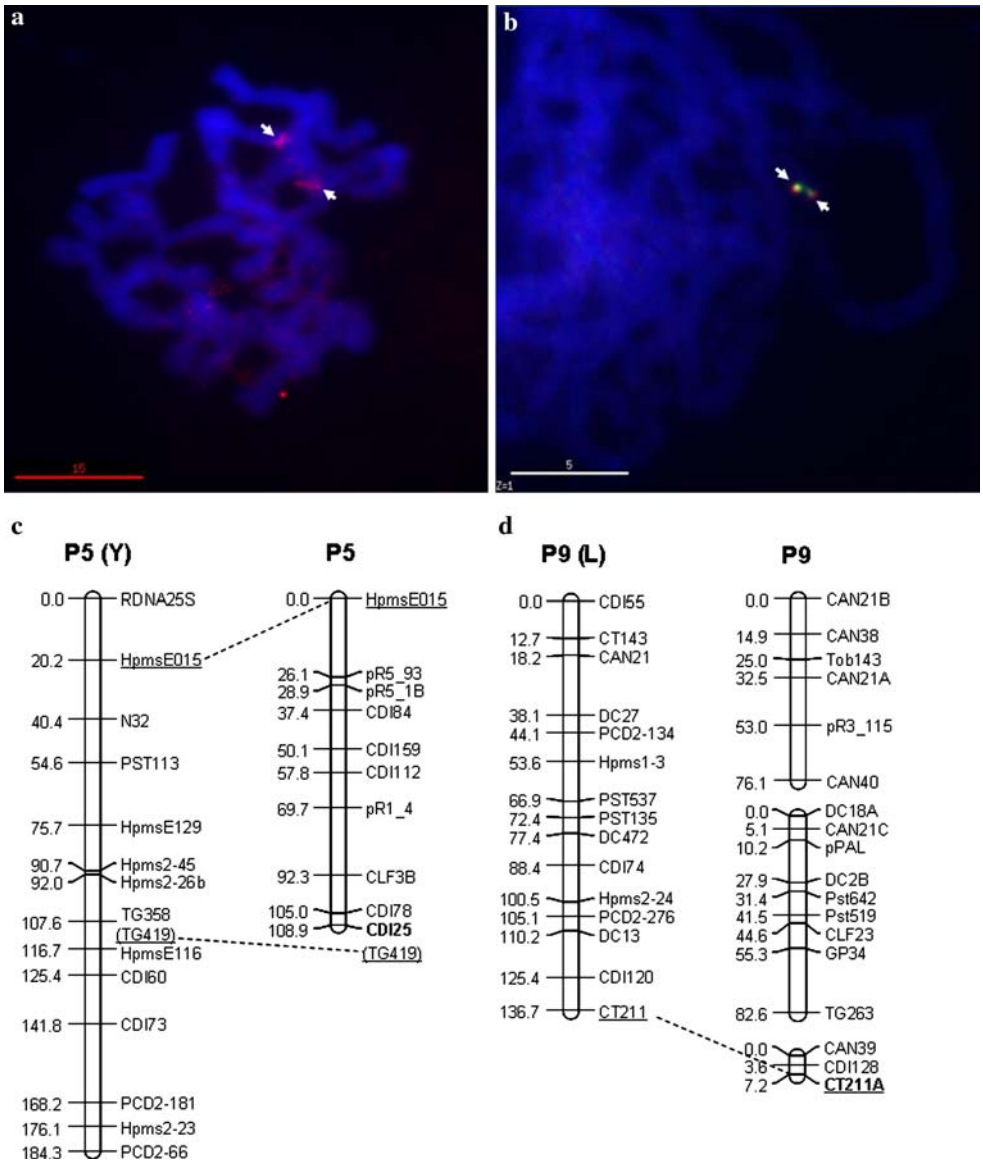

Fig. 4 In situ localization of BAC clones on pepper chromosomes. *Arrowheads* point to BAC clone hybridization. **a** BAC 642 clone was used as the FISH probe (*red*) to determine its genomic location on somatic chromosome spreads. Strong hybridization is observed in the middle of the chromosome. *Scale bar* equals 15 μm. **b** Hybridization of BAC 216c (*green*) and 502 (*red*) clones is found only at the second block of euchromatin in the telomere of the chromosome and exhibits the same pattern. *Scale bar* equals 5 μm. **c** Comparison of P5 with one, P5 (Y) of SNU3 (Yi et al. 2006). **d** Comparison of P9 with one, P9(L) of SNU2 (Lee et al. 2004)

Development of BAC-derived markers

For the development of BAC-derived markers, full and end-sequences of 15 BAC clones were used for primer design. Four primer sets out of 117 primer sets produced polymorphic bands between the two parents of the F_2 population (Fig. 7). The used primers were designed from full-length sequences of BAC 642 and BAC 502, and from end-sequences of BAC 216c and BAC 464, as described in Table 2. One single-nucleotide polymorphism (SNAP, A/G) marker (P5-SNAP) derived from the H1 region in the sequence of BAC 642 (Fig. 5) was scored codominant, and labeled as P5-SNAP-CM and P5-SNAP-Chil to be specific to each parent. One cleaved amplified polymorphic sequence (CAPS) from BAC 216c clone, and two SSR markers from BAC 464 and 502 clones were also developed (Fig. 6). All four developed codominant markers (SNAP from CDI25 on P5, and one CAPS and two SSRs from CT211A on P9) were closely mapped to the genetic positions of markers used for the BAC library screening.

QTL mapping and application of BAC-derived markers

The results of QTL mapping on an F_2 population together with BAC-derived markers showed that the QTL of *Phytophthora* resistance on P5 was located closer to the BAC-derived marker (P5-SNAP on P5) than the CDI25 RFLP marker. Using single-factor ANOVA analysis, P5-SNAP ($P = 5.4 \times 10^{-9}$, $R^2 = 35.78\%$ in root rot trait; $P = 3.0 \times 10^{-4}$, $R^2 = 16.65\%$ in damping-off trait) was identified at a higher significance than CDI25 ($P = 8.7 \times 10^{-5}$, $R^2 = 20\%$ in root rot trait; $P = 1.8 \times 10^{-3}$, $R^2 = 13.33\%$ in damping-off trait). CT211A was closer to the QTL than the BAC-derived markers (CAPS, SSR-3,

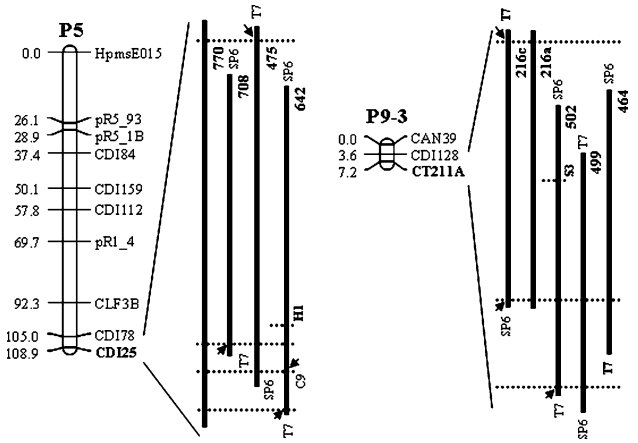

Fig. 5 Contig construction of BAC clones screened by CDI25 and CT211 probes. The result was deduced from BAC-PCR, Southern analysis, fingerprinting, BAC sequence alignment, and BAC-FISH. The *dotted line* indicates the overlapping region of BAC clones detected by BAC-PCR with the arrowed primer set. The *bold Arabic figure* is the BAC clone number, and *P* indicates the chromosome number. The following regions were converted into the respective markers: H1 of BAC 642 clone into P5-SNAP, S3 of BAC 502 into SSR-3, T7 of BAC 216c into CAPS, and T7 of BAC 464 clone into SSR-9

and SSR-9), although the BAC markers were closely located near CT211A on the map.

Genotypes of parents and F_1 for mapping population (Fig. 7), and 13 resistant commercial cultivars (Table 3) were determined by these markers and compared with phenotype data. P5-SNAP marker identified 11 resistant cultivars (11/13 = 85%) and SSR marker identified 8 cultivars (8/13 = 62%). Marker and phenotype comparison showed that 11 cultivars have resistant allele of P5-SNAP and 8 cultivars have that of SSR-9 allele.

Discussion

Intraspecific map

To position the resistance, we constructed an intraspecific map of 100 F_2 peppers derived from a cross between *C. annuum* 'CM334' and *C. annuum* 'Chilsungcho'. This intraspecific map designated as an SNU 4 chili pepper linkage map consisted of 14 linkage groups using 202 codominant RFLPs, and 6 WRKYs, and 1 SSR. The 14 linkage groups were assigned to 11 chromosomes (the exception was P4) by comparing the RFLP markers with the 12 published pepper chromosomes (Livingstone et al. 1999; Lee et al. 2004; Yi et al. 2006). This intraspecific map can serve as a good reference for comparative pepper genome studies. The results of this QTL analysis will provide a source for studying quantitative genetic variations in *Phytophthora* root rot and damping-off resistance in chili pepper 'CM334'. P1 on the interspecific pepper map is made up of P1 and P8 on the intraspecific map because of translocation within species (Livingstone et al. 1999; Lee et al. 2004). That could confuse the assignment of P1 and P8 on an intraspecific map. RFLP markers on the intraspecific map in this study helped with this assignment, 13 for P1 and 25 for P8. Furthermore, our RFLP-based intraspecific map was found useful in detecting a mis-assignment of one QTL to P1 which should have been assigned to P8 (Sugita et al. 2006).

QTL detection

Quantitative trait loci mapping of root rot and damping-off resistance was performed in $F_{2:3}$ families originating from a cross between 'CM334' and 'Chilsungcho', and a total of five QTL were identified using moderately aggressive *P. capsici* 'Pa23'. In previous studies, different numbers of QTL on populations derived from CM334 were identified for different *P. capsici* strains: nine QTL from four experimental components to the very aggressive *P. capsici* 'S197' (from France) in an F_2 population of YC (Thabuis et al. 2003) and eight QTL from four experimental components to '*Pc*197' (France) in RIL_5 of YC (Bonnet et al. 2007). Five QTL from a root rot test of 'M' (from New Mexico) in an F_2 population between 'Joe E. Parker' and 'CM334' (Ogundiwin et al. 2005) and two QTL to 'P-5' (Japan) in the double-haploid population of 'Manganji' and 'CM334' were found (Minamiyama et al. 2007). These results suggested that the aggressiveness of isolates is related to the number of detected QTL (Reifschneider et al. 1992; Ogundiwin et al. 2005). The aggressiveness of 'Pa23' may be considered to be comparable with that of 'M'. In the double-haploid population of 'K9-11' and 'AC2258' with the inoculation of 'Keihoku' (Japan), three QTL were reported (Sugita et al. 2006).

In all cases the major QTL was detected on P5, even though those experiments were performed under different environmental conditions. Therefore, this major QTL was considered stable across several populations and *P. capsici* isolates, similar to the *RB* gene that was cloned by a map-based approach in potato (Song et al. 2003; Staples 2004). The gene in potato controls race-nonspecific and broad-spectrum resistance to *Phytophthora infestans*. We also detected two QTL on P5 for both resistance traits by CIM and one-way ANOVA analysis, which were in accordance with a previous report (Thabuis et al. 2003). To assign the two QTL, a SCAR marker (Quirin et al. 2005) and nine SSR markers on P5 (Minamiyama et al. 2007) were tried to map on the linkage map, but they did not produce polymorphism between the two parents, with the exception of CAMS211 which was mapped near CDI25 on P5, but this was not enough to assign the two QTL on the intraspecific map.

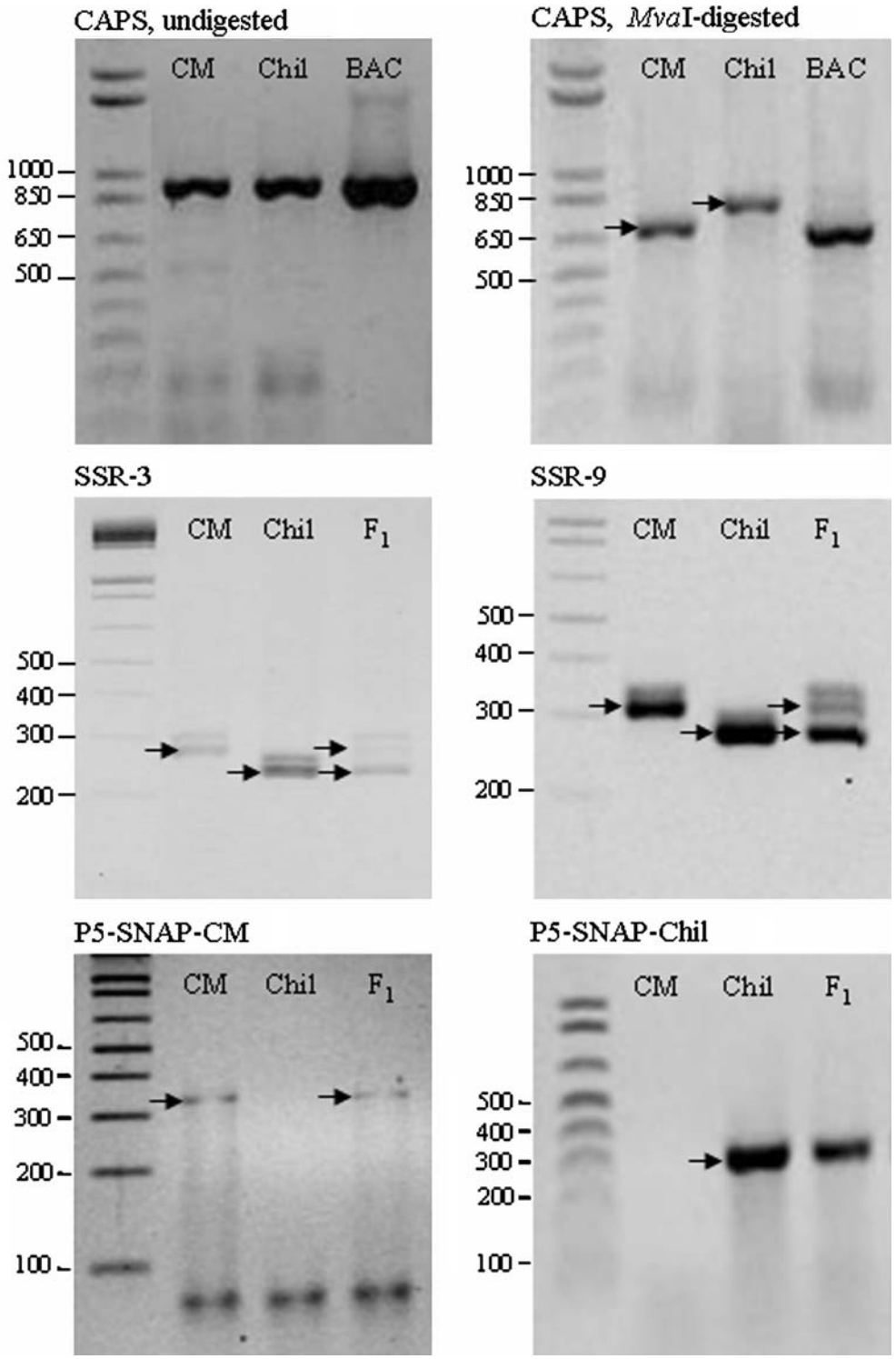

Fig. 6 PCR products showing co-dominant nature of the BAC-derived markers. Marker sequences were developed from BAC 216c for CAPS, BAC464 for SSR9, BAC502 for SSR3 and BAC642 for both P5-SNAP-CM and P5-SNAP-Chil. *CM* 'CM334', *Chil* 'Chilsungcho', *BAC* BAC clone where the primer was derived from. On the *left* of the gel are band sizes in bp. The *left lane* is the size marker, 1 Kb plus DNA ladder (Invitrogen, USA). The *arrowed band* is polymorphic between the parents

Two QTL on P6 and P9 were identified for root rot resistance, and one QTL on P8 was found for damping-off resistance. Three QTL on P8 (Ogundiwin et al. 2005; Sugita et al. 2006), P6 (Thabuis et al. 2003), and P9 (Ogundiwin et al. 2005) were reported, which is consistent with our results.

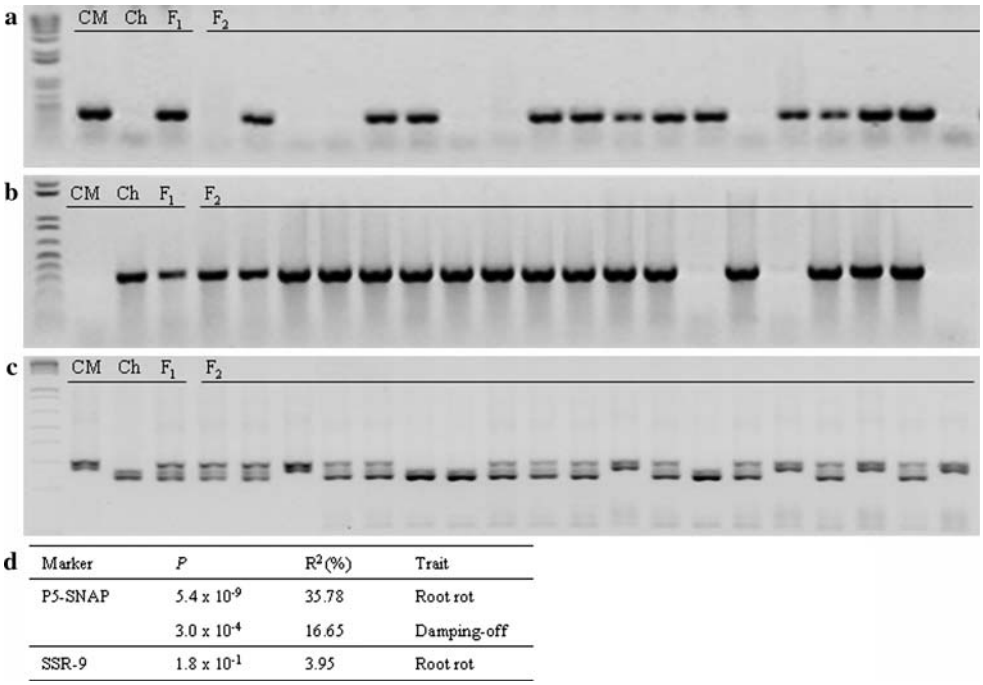

Fig. 7 PCR screening for polymorphism in the mapping population including 'CM334', and 'Chilsungcho' parents, F_1 and F_2 progeny using the BAC-derived primers, **a** P5-SANP-CM, **b** P5-SANP-Chil, and **c** SSR-9. The *left lane* is the size marker of 1 Kb plus DNA ladder (Invitrogen, USA). **d** A single-factor ANOVA analysis of the BAC markers

Table 2 PCR primers used to generate BAC-derived markers on a chili intraspecific (*Capsicum annuum* × *C. annuum*) cross population

Chr no.	Primer	Primer sequence 5′–3′	Tm (°C)	Size (bp)	Type	Enzyme
5	P5-SNAP-CM-F[a]	TCATGAGGTTGCTATTAAGATTGGTCCTGTTATATA	65.1	335	SNP	–
	P5-SNAP-Chil-F	GAGGTTGCTATTAAGATTGGTCCTGTTATCCG	66.8	331	SNP	–
	P5-SNAP-R	CATAGAAAGGGATATCATCTGGTACATGCAGAAA	67.6			
9	SSR-9-F	CAAGCACCTACAAATGCAAAAT	59.2	268	SSR	–
	SSR-9-R	CCGGATGAGAAAACTTGCTACT	59.8			
	SSR-3-F	TTTCCAAGCACCTACAAATGC	60.2	272	SSR	–
	SSR-3-R	CCGGATGAGAAAACTTGCTACT	59.8			
	CAPS-F	CCAACCCTATTGAACGTCTT	56.7	900	CAPS	*Mva*I
	CAPS-R	CTGATTCTTGATGCCTCTTG	55.6			

[a] F, sequence of the forward primer; R, sequence of the reverse primer

Quantitative trait loci detected by CIM explained phenotypic variations of 66.3 and 44.9% in root rot and damping-off resistance, respectively, at a minimum LOD of 2.0 (Table 2). From single-factor ANOVA analysis, two additional markers were identified at a significance level of $P < 0.005$. CDI17A ($P = 0.00035$, $R^2 = 17.23\%$ in root rot trait; $P = 0.0047$, $R^2 = 11.45\%$ in damping-off trait) in both traits and W16 ($P = 2.6 \times 10^{-6}$, $R^2 = 29.01\%$) in the root rot trait were not mapped and could not be converted into PCR markers. Epistatic interactions could have an important effect on the resistance gene expression (Bartual et al. 1994; Palloix et al. 1990; Lefebvre and Palloix 1996). From the digenic interaction results, CDI25 and CT211 interacted with two each marker, although direct interaction between CDI25 and CT211 was not detected. Interactions of CDI25 to HpmsE04 and HpmsE04 to CDI17A in the damping-off trait seemed to be similar to a resistance signal pathway. These epistatic effects might support complicated gene actions involved in the resistance to *P. capsici* in pepper plants and motivate more dedicated research design focused on digenic interactions.

Screening and confirmation of BAC clones

Two RFLP markers, CDI25 on P5 and CT211A on P9, were located close to QTL and supported by one-way ANOVA. These two RFLP probes were used for screening of a 'CM334' BAC library. The library contains approximately 12 genome equivalents (about 2,702 Mbp/C; Arumuganathan and Earle 1991) and consists of 235,000 clones with an average insert size of 130 kb (Yoo et al. 2003). Because the RFLP probes were multi-copy in the pepper genome, we verified positive BAC clones using several methods such as Southern analysis, fingerprinting, BAC-

Table 3 Alleilism survey of *Phytophthora* QTL in resistant commercial cultivars

Cultivar	Company	P5-SNAP	SSR-9
Gangryukjosaenggun	Nongwoo	R	R
Geummedal	Coregon	R	R
Dogyachungchung	Syngenta	R	S
Boeungun	Nongwoo	R	S
Sintongyil	Coregon	R	R
Yeukganghongjanggoon	Coregon	R	R
Hot	Coregon	R	R
PR-Bulsacho	Daenong	R	R
PR-Bulsae	Daenong	R	S
PR-Topgun	Daenong	R	S
PR-Gangja	Nongwoo	R	S
PR-Datta	Nongwoo	S	R
PR-Bulmyeol	Nongwoo	S	R

PCR, and BAC-FISH. The BAC-FISH technology has provided a cytogenetic approach to correlate molecular maps with cytological maps (Jiang et al. 1995; Wang et al. 2007). To assay their cytological locations, FISH was performed on pachytene chromosomes of 'CM334' pepper using screened BAC clones as probes. The cytogenetic positions matched the real locations of CDI25 and CT211A markers on the genetic map. The CDI25 and CT211A markers have been reported to be near the middle of P5 and the end of chromosome 9, respectively (Livingstone et al. 1999; Kang et al. 2001; Lee et al. 2004; Yi et al. 2006). The BAC probes (642, 216c, and 502) could be valuable as chromosome-specific cytogenetic markers in pepper genome research.

Development and application of BAC-derived markers

Of the nine BAC clones that were screened by two RFLP markers close to the *P. capsici*-resistance QTL, two end-sequenced BAC clones (216c and 464) and two full-sequenced BAC clones (502 and 642) finally generated four PCR markers such as one CAPS, two SSR, and one SNAP. The BAC markers were closely located near the original RFLP markers on the map. One-way analysis of closely located RFLP and PCR markers with QTL was performed, and their relative distances from QTL were considered. To determine the usefulness of these markers for selection of plants, SSR-9 and P5-SNAP markers were applied to 13 resistant commercial cultivars. From the application result of P5-SNAP-CM, 11 cultivars may be originated from a same resistant source, CM334. Our results demonstrate that P5-SNAP and SSR-9 will be useful for marker assisted breeding of phytophthora resistance especially with CM334 originated resistance. In addition BAC sequence information will be useful to develop additional marker and QTL cloning. These results also suggest that the QTL on chromosome 5 are essential for phytophthora resistance and well conserved in resistant cultivars during breeding.

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