Development of the single nucleotide polymorphism markers for *Phytophthora capsici* resistance in *Capsicum annuum* with QTL analysis

Namfon Chomkaeo[1], Darush Struss[2], Chotipa Sakulsingharoj[1], Varaporn Sangtong[1], Saengtong Pongjaroenkit[1]*

[1]*Program in Genetics, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand*

[2]*Department of Biotechnology, East West Seed Company, Chiang Mai 50290, Thailand*

**Corresponding author: saengton@mju.ac.th*

ABSTRACT

Phytophthora capsici Leonian is one of the most important pathogens, which destructs pepper crop (*Capcicum sp*.) worldwide. The inheritance of *Phytophthora* resistant gene is a complex inheritance known as quantitative trait loci (QTL). A major QTL is located on chromosome 5. In this study, bulked segregant analysis (BSA) approach was used for discovery of polymorphic SNP markers in double haploid population derived from highly resistant *Capsicum annuum* accession Criollo de Morelos – 334 and susceptible variety Jindu. The linkage maps were constructed and QTL analysis was performed. The QTL with the highest LOD score, detected on chromosome 5, explained 31% of the phenotypic variance with a LOD score of 11. The second QTL, designated as a minor QTL, which was found on chromosome 8, explained 11.9% of the phenotypic with a LOD score of 3.36. The nearest marker, which was located on the major QTL and the minor QTL were SNP_421 and SNP_694, respectively. Therefore, two SNP markers were the best candidate markers that linked to QTL of *Phytophthora* resistance.

Keywords: single nucleotide polymorphism (SNP) markers; *Capsicum annuum*; *Phytophthora* resistance; quantitative trait loci (QTL); inheritance

INTRODUCTION

Pepper (*Capsicum* spp.) is a member of Solanaceae family, originating from South America, before spreading worldwide (Bosland, 1992). Pepper is an economically important vegetable because it is a good source of antioxidants, nutrients, bioactive compounds, and is also rich in natural colors and aromas. Therefore, there have been continuous efforts to improve pepper cultivars such as yield (Rao *et al*., 2003), fruit color (Huh *et al*., 2001), male sterility (Kim *et al*., 2006), important secondary metabolites (Lee *et al*., 2005) and especially disease resistance.

Root rot disease of the pepper (*Capsicum sp.*), caused by the fungal pathogen *Phytophthora capsici*, is one of the most important diseases of the cultivated pepper. The prevention of this pathogen requires frequent applications of fungicides. This is expensive and undesirable for the environment and leads to the emergence of resistant isolates with increased tolerance. Breeding for resistance to *P. capsici* is the ideal management for this problem.

Several sources of resistance to *P. capsici* have been identified in the cultivated pepper, including Criollo de Morelos 334 (CM334), PI 201232, PI 201234 and AC 2258. Among which CM334 is well-studied and shows high level of resistance (Bartual *et al*., 1994; Thabuis *et al*., 2003;

Glosier *et al*., 2008; Truong *et al*., 2012; Rehrig *et al*., 2014)

The inheritance of *Phytophthora* resistance has been reported in several models depending on breeding lines, environment factors, disease screening conditions, and isolates of *P. capsici* (Oelke *et al*., 2003). Early researches proposed a single gene model of dominant alleles based on complete resistance found in the F_1 and segregation ratios of the F_2 population (Smith *et al*., 1967; Sy *et al*., 2005; Barbosa and Bosland, 2008) as well as a two - three genes model (Ortega *et al*., 1991; Reifschneider *et al.*, 1992; Walker and Bosland, 1999). In contrast, other studies reported a polygenic inheritance (Bartual *et al*., 1991; Bartual *et al.,* 1993; Lefebvre and Palloix, 1996; Thabuis *et al*., 2003; Bonnet *et al.,* 2007; Minamiyama *et al*., 2007) and quantitative trait loci (QTLs) for *Phytophthora* resistance (Thabuis *et al.,* 2003; Ogundiwin *et al.*, 2005; Bonnet *et al*., 2007; Kim *et al*., 2008, Truong *et al*., 2012; Liu *et al*., 2014; Rehrig *et al*., 2014).

An effective approach for studying complex disease resistances is provided by the use of molecular markers (Young, 1996). However, the QTLs studies have been limited by the number of polymorphisms. Polymorphic molecular markers based on single nucleotide polymorphisms (SNPs) in DNA sequences are extremely sensitive and could identify individuals within a population. Bulk segregant analysis (BSA) has been widely adopted for rapid identification of molecular markers in specific region of a genome. BSA is based on the underlying principle that bulking of individuals from segregating population into pools of alternative phenotypes allows the capture of representative genotypes at a particular locus while a random genetic background is generated at all other unlinked loci (Michelmore *et al*., 1991). Therefore, the combined use of BSA and polymorphism SNP survey could represent a powerful approach for discovery of genetic markers for disease resistance.

The objective of this study is to develop the SNP markers linked to the major QTLs of *Phytophthora* resistant gene in *Capsicum annuum (C. annuum*).

MATERIALS AND METHODS

Plant materials and DNA extraction

The resistant lines used in this study was Criollo de Morelos 334 (CM334), a small-fruited and pungent line from Mexico. Susceptible parent was Jindu, a commercial variety. Double haploid population was designated as “DH” which was developed by shed – microspore culture of F_1 plant derived from a cross between “CM334” and “Jindu” (Yaioon *et al*., 2014). The 158 individuals of DH population were used for detection of QTL linked to resistance. DNA was extracted from young leaves using a modified CTAB method (Nishiguchi *et al*., 2002).

Resistance test

DH plants were inoculated and evaluated for resistance to *P. capsici* with modified method as described by The World Vegetable Center or Asian Vegetable Research and Development Center (AVRDC). The experimental design followed the Randomized Complete Block Design (RCBD). All experiments were conducted with two replications using fifteen plants per DH line per replication. The inoculated plants were grown for a further 21 days in a greenhouse maintained at 25 $^{\circ}$C. Symptoms were scored visually on a scale of 0 – 4 (0 = no symptom; 1 = leaf chlorosis; 2 = leaf chlorosis and slightly necrotic crown; 3 = necrotic crown and severe wilting; 4 = almost dead) (Figure 1). The visual scores were transformed to the Area Under the Disease Progress Curve (AUDPC), using AUDPC values for data analysis.

Figure 1 Scoring rate (score 0-4) of resistance of *Capsicum annuum* to fungal pathogen *Phytophthora capsici*. 0 = no symptom, 1 = leaf chlorosis, 2 = leaf chlorosis and slightly necrotic crown, 3 = necrotic crown and severe wilting, 4 = almost dead.

SNP genotyping assay

The SNP collections were selected from the *C. annuum* genome database (version 1.5) (http://peppergenome.snu.ac.kr/). SNP IDs and their flanking sequences approximately 200 bp were submitted to LGC Company (Beverley, USA) for primer designs to be used in KASPar™ genotyping assays. KASPar™ genotyping system is a competitive allele specific dual FRET based assay for SNP genotyping. Two FRET cassettes were used where a fluorescence dye, VIC or FAM, was conjugated to a primer. Sample DNA was amplified with a thermal cycler using allele specific primers, leading to a separation of the fluorescence dye and the quencher when FRET cassette primer is hybridized to the DNA (Raitio *et al*., 2012).

SNP markers survey and bulked segregant analysis (BSA)

Two parental lines, resistant parent “CM334” and susceptible parent “Jindu”, were screened with 1,036 SNP primers to survey polymorphic SNP markers. Then, 448 polymorphic SNP markers were screened with two DNA pools, which were pooled from 10 individual DNA samples of the extreme resistant and susceptible phenotypes. Finally, the individual samples in DH population were genotyped by using 45 SNP markers that revealed differences between resistant and susceptible pools.

QTL analysis

The phenotypic analysis was conducted using ANOVA for mean and replicate comparison. On the other hand, the linkage groups were performed using 45 SNP markers with the following criteria, a minimum LOD score of 6 and a maximum recombination fraction of 0.35. Then, the QTLs linked to *Phytophthora* resistance were detected by the single marker analysis. All analysis was performed using the R/qtl analysis (Broman and Sen, 2009). Finally, the significant SNP markers were BLAST searched against the *C. annuum* genome database (http://peppergenome.snu.ac.kr/) to identify the corresponding scaffold sequences.

RESULTS AND DISCUSSION

Resistance test and phenotypic analysis

The scores for resistance to *P. capsici* were used for the Area Under the Disease Progress Curve (AUDPC). The resistant parent “CM334” showed a low value of AUDPC. In contrast, the susceptible parent “Jindu” showed a high score of AUDPC. The distribution of AUDPC in “DH population” was continuous and similar to bi-modal

distribution (Figure 2). Therefore, the inheritance of *Phytophthora* resistance was controlled by quantitative trait loci (QTL) and consistent with many previous studies (Thabuis, *et al.*, 2003; Ogundiwin *et al.*, 2005; Bonnet, *et al.*, 2007; Kim, *et al.*, 2008; Liu *et al.*, 2014; Rehrig *et al.*, 2014). The phenotype evaluation was done by ANOVA in two replicates and the results from two replicates were not different. Therefore, the average of AUDPC values was used for QTLs analysis (Figure 3).

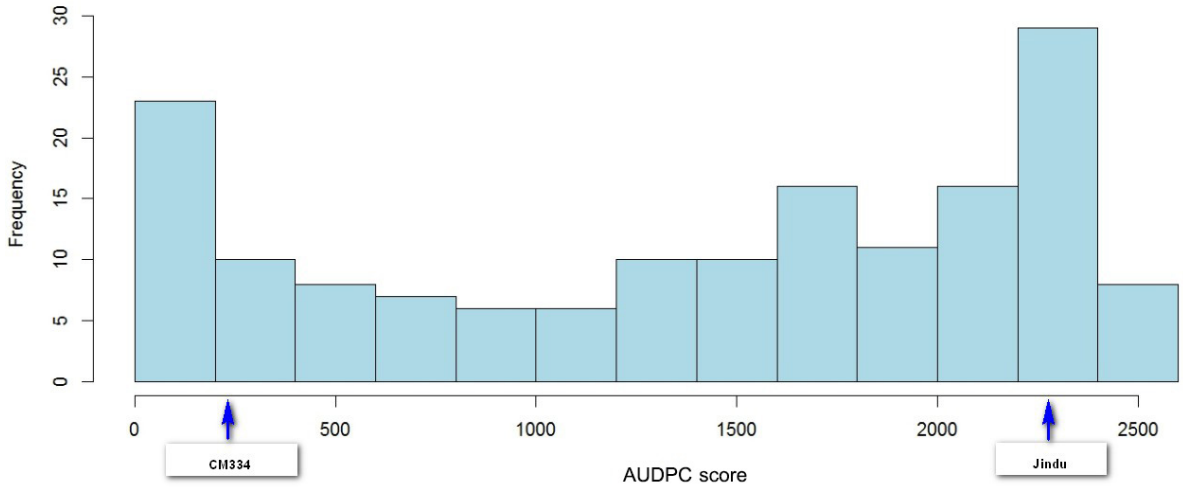

Figure 2 The AUDPC distribution of DH population for *Phytophthora* resistance. The vertical axis indicated the number of lines, and the horizontal axis indicated the AUDPC score. Blue arrow indicated resistant parent "CM334" (left) and susceptible parent "Jindu" (right) with average AUDPC values of 226 and 2275, respectively.

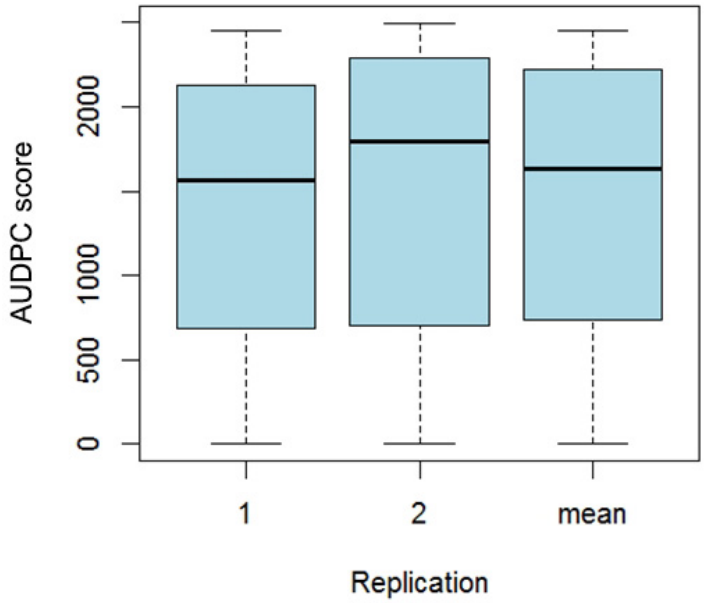

Figure 3 The AUDPC values. Box plot 1, 2, and 3 represented replication 1, 2, and an average, respectively. The black lines in each box plot represented median value in each replication.

SNP markers survey and bulked segregant analysis (BSA)

The screening of two parental lines revealed 448 polymorphic SNP markers out of 1,036 SNP markers. These 448 SNP markers were screened with pooled DNA of resistant and susceptible phenotype. Forty-five SNP markers showed a difference between resistant and susceptible pools (Figure 4A). The 158 individuals of DH population were genotyped by 45 SNP markers (Figure 4B). All the SNP markers were confirmed to correspond to a homozygous genotype at each locus in all the DH lines.

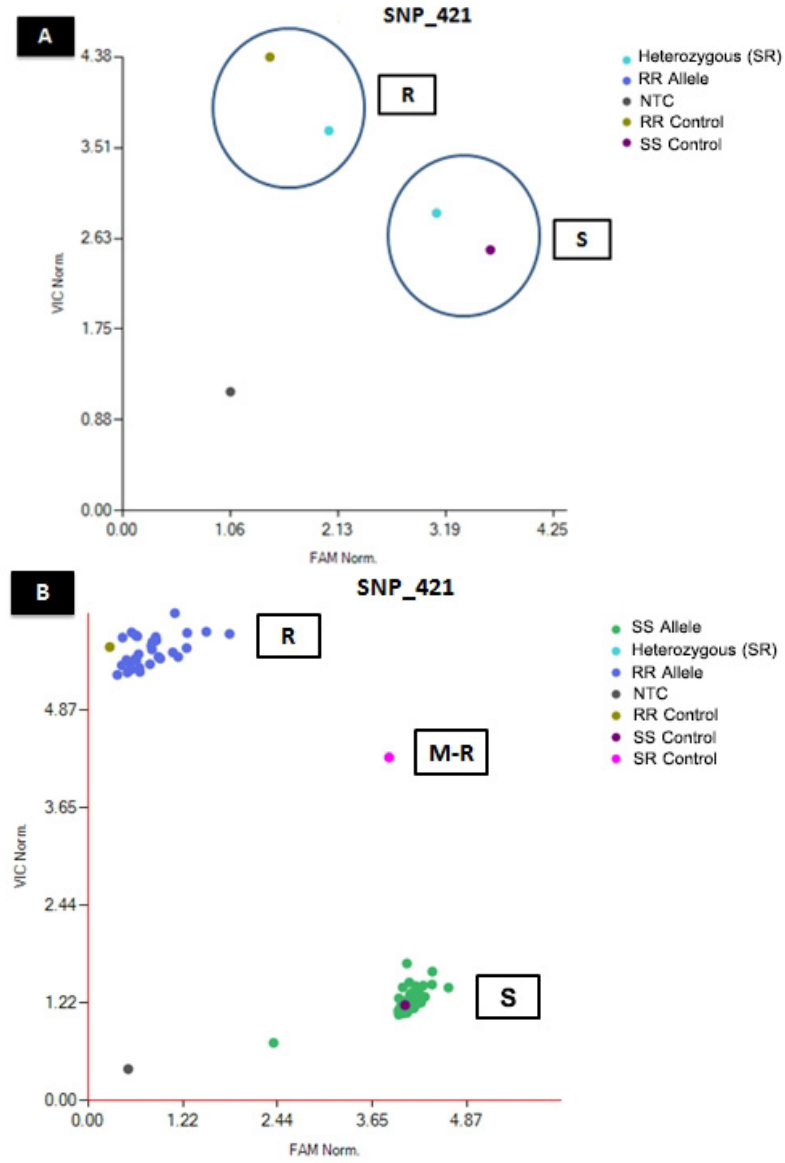

Figure 4 Scatter plot patterns. R = Resistance; M-R = Moderate resistance; S = Susceptible. A represented criteria pattern used for selecting polymorphism SNP primers in bulked segregant analysis. B showed some scatter plot chart of individuals of DH population screening.

QTL analysis

Seventeen linkage groups were generated based on genotyping results of 45 SNP markers (Figure 5). The total of linkage groups (LG) was higher than the number of pepper chromosomes (x=12) because some chromosomes have more than one linkage group. For example, chromosome 3 has two linkage groups (LG2 and LG6). The 45 SNP markers were distributed on almost all pepper chromosomes except chromosome 6 and 10 because the density of SNP primers was low. The QTLs analysis was conducted by using the single marker analysis. The result of QTLs was presented in Figure 6. Two out of 17 linkage groups gave a LOD score higher than 3. The first QTL was detected on linkage group 1 (LG1) with a LOD score of 3.5 and the phenotypic variance explained about 11%. The other QTL was detected on linkage group 3 (LG3) with a LOD score of 11, and the phenotypic variance explained about 31%. The SNP markers named SNP_694 and SNP_421 were linked to *Phytophthora* resistance and located on LG1 and LG3, respectively. BLAST searching against the pepper genome database (The pepper Genome database (release 2.0)) was performed. The result showed that LG3 was on chromosome 5 and LG1 was on chromosome 8. The QTL on chromosome 5 was designated as a major QTL and the QTL on chromosome 8 was designated as a minor QTL. The major QTL of this study was consistent with other studies (Quirin *et al*. 2005; Minamiyama *et al*. 2007; Truong *et al*, 2012; Liu *et al*, 2014; Rehrig *et al*, 2014). In all studies, the major QTL was located on chromosome 5, even though those experiments were performed under different populations and environment conditions because of the origin of resistance derived from CM334.

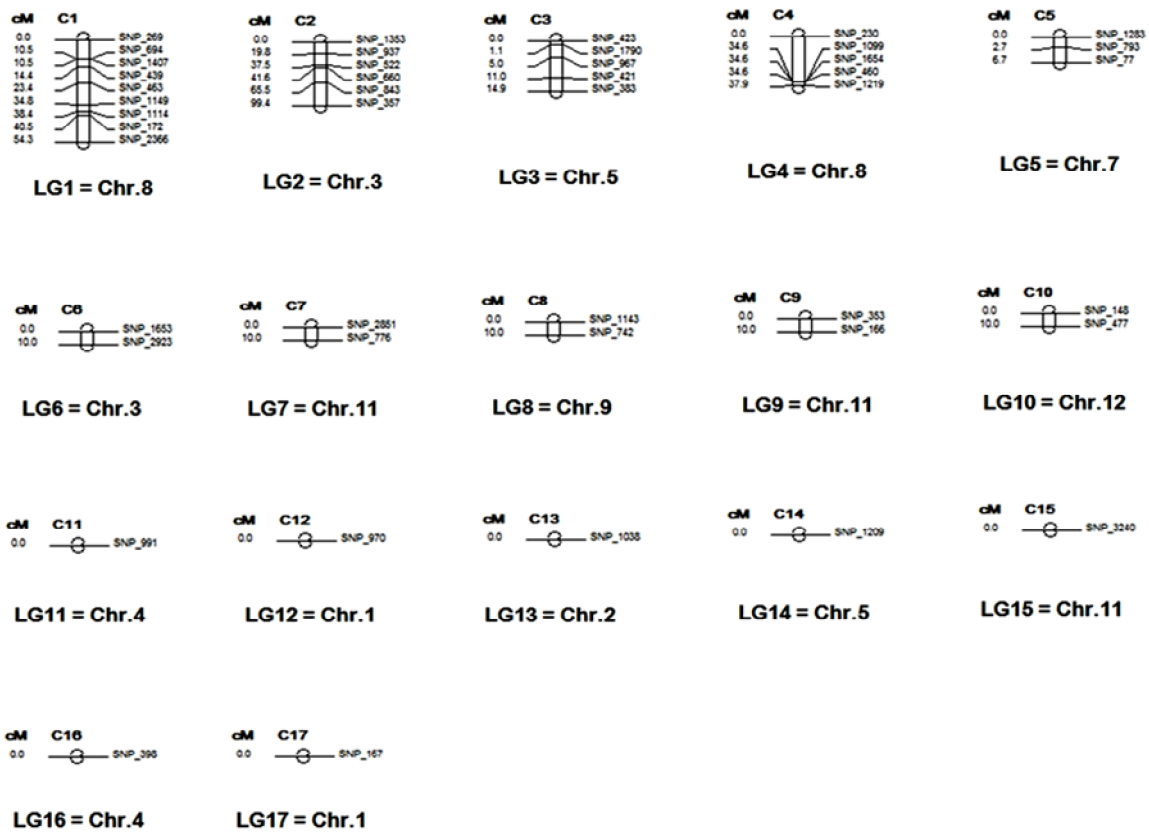

Figure 5 Seventeen linkage groups. LG or C:Linkage group , Chr. :Chromosome number of pepper.

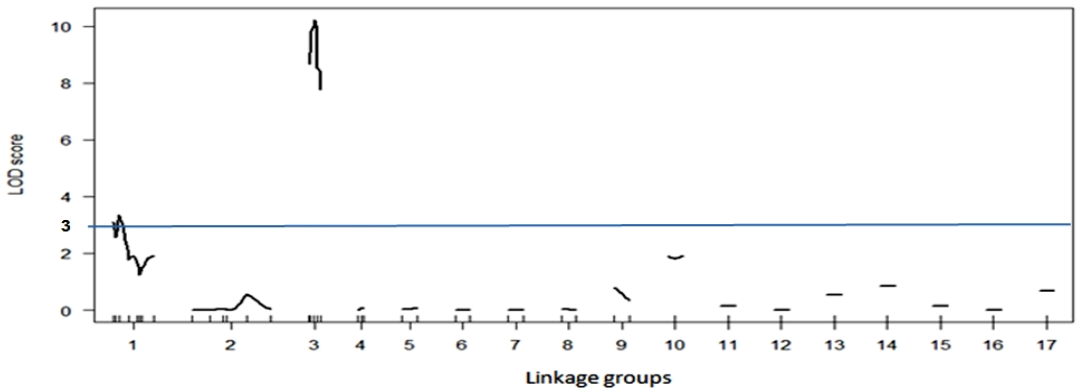

Figure 6 Plot of LOD scores for *Phytophthora* resistance. Linkage group 1 represented chromosome 8 with a LOD score of 3.3. Linkage group 3 represented chromosome 5 with a LOD score of 11.

SNP markers linked to *Phytophthora* resistance

The primer sequences of the SNP markers named SNP_421 and SNP_694 were presented in Table 1. The SNP_421 matched the sequences of PGA v.1.5 scaffold 74, and the SNP_694 matched the sequence of PGA v.1.5 scaffold 282 (Table 1). The effect of these SNPs to DH population and their positions were presented in Table 2. In this study, two SNP markers could be identified for QTLs that are suitable for high-throughput analysis and can be applied for marker-assisted selection in breeding programs.

Table 1 Primer sequences and their matched scaffold from *C. annuum* genome database (The pepper genome database platform).

SNP name	Scaffold	Primer sets	
		Primers	**Sequences (5′- 3′)**
SNP_694	Scaffold282	F1	GTG CCT CGG TGA GAG TTG TAT TTA
		F2	GTG CCT CGG TGA GAG TTG TAT TTG
		R	ATT GAG CCT GCT GAA AAC TGA TAA G
SNP_421	Scaffold74	F1	GTT CCT GTT GAT CAC TTG CCC CTC
		F2	GTT CCT GTT GAT CAC TTG CCC CTT
		R	CAA ACT TCT GCT TGA AAG AAT CTT G

Table 2 Effect of QTLs for *Phytophthora* resistance detected in the DH population derived from a cross between “CM334” and “Jindu”.

LG[a]	SNP markers[b]	LOD[c]	R^2 (%)[d]	Positions[e]		
				Chr.	**Physical map**	**SNP**
1	SNP_694	3.36	11.9	8	1170904	A/G
3	SNP_421	11	31	5	34810892	C/T

[a] The linkage groups, [b] The closest markers to the QTL, [c] LOD of the QTL, [d] Percentage of phenotypic variance , and [e] The positions of SNPs located (The Pepper Genome database (release 2.0)). Chr. : Chromosome number of pepper.

ACKNOWLEDGEMENTS

SNP primers and assay, resistance test, and double haploid plants of pepper were provided by East West Seed Company. We thank Eliot M. Cline for advice on statistical analysis and critical reading of the manuscript.

REFERENCES

Barbosa AM, Bosland PW (2008) Genetic analysis of phytophthor*a* root rot race-specific resistance in Chile pepper. J Am Soc Hortic Sci 133: 825–829.

Bartual R, Carbonell EA, Marsal JI, Tello JC, Campos T (1991) Gene action in the resistance of peppers (*Capsicum annuum*) to *Phytophthora* stem blight (*Phytophthora capsici* L.). Euphytic 54: 195–200.

Bartual R, Lacasa A, Marsal JI, Tello JC (1993) Epistasis in the resistance of pepper to phytophthora stem blight (*Phytophthora capsici* L.) and its significance in the prediction of double cross performances. Euphytica 72: 149–152.

Bartual R, Lacasa A, Marsal JI, Tello JC (1994) Epistasis in the resistance of pepper to phytophthora stem blight (*P. capsici* L.) and its significance in the prediction of double cross performances. Euphytica 72: 149–152.

Bonnet J, Danan S, Boudet C, Barchi L, Sage-Palloix A-M, Caromel B, Palloix A, Lefebvre V (2007) Are the polygenic architectures of resistance to *Phytophthora capsici* and *P. parasitica* independent in pepper? Theor Appl Genet 115: 253–264.

Bosland PW (1992) Chiles: a diverse crop. Hort Technol 2: 6–10.

Broman KW, Sen S (2009) A guide to QTL mapping with R/qtl. Springer, pp 1–389.

Glosier BR, Ogundiwin EA, Sidhu GS, Sischo DR, Prince JP (2008) A differential series of pepper (*Capsicum annuum*) lines delineates fourteen physiological races of *Phytophthora capsici*. Euphytica 162: 23–30.

Huh JH, Kang BC, Nahm SH, Kim S, Ha KS, Lee MH, Kim BD (2001) A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum spp*.). Theor Appl Genet 102: 524–530.

Kim DS, Kim DH, Yoo JH, Kim BD (2006) Cleaved amplified polymorphic sequence and amplified fragment length polymorphism markers linked to the fertility restorer gene in chili pepper (*Capsicum annuum* L.). Mol Cells 21: 135–140.

Kim HJ, Nahm SH, Lee HR, Yoon GB, Kim KT, Kang BC, Choi D, Kweon OY, Cho MC, Kwon JK, *et al.* (2008) BAC–derived markers converted from RFLP linked to *Phytophthora capsici* resistance in pepper (*Capsicum annuum* L.). Theor Appl Genet 118: 15–27.

Lee CJ, Yoo EY, Shin JH, Lee JM, Hwang HS, Kim BD (2005) Nonpungent *Capsicum* contains a deletion in the capsaicinoid synthetase gene, which allows early detection of pungency with SCAR markers. Mol Cells 19: 262–267.

Lefebvre V, Palloix A (1996) Both epistatic and additive effects of QTLs are involved in polygenic induced resistance to disease: a case study, the interaction pepper *Phytophthora capsici* Leonian. Theor Appl Genet 93: 503–511.

Liu WY, Kang JH, Jeong HS, Choi HJ, Yang HB, Kim KT, Choi D, Choi GJ, Jahn M, Kang BC (2014) Combined use of segregant analysis and microarrays reveals SNP markers pinpointing a major QTL for resistance to *Phytophthora capsici* in pepper. Theor Appl Genet 127: 2503–2513.

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis : a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88: 9828–9832.

Minamiyama Y, Tsuro M, Kubo T, Hirai M (2007) QTL analysis for resistance to *Phytophthora capsici* in pepper using a high density SSR-based map. Breed Sci 57: 129–134.

Nishiguchi MK, Doukakis P, Egan M, Kizirian D, Phillips A, Prendini L, Rosenbaum HC, Torres E, Wyner Y, DeSalle R, *et al*. (2002) DNA isolation procedures. In: DeSalle R, Giribet G, Wheeler W (eds) Techniques in molecular systematics and evolution. Birkhäuser Basel, Switzerland, pp 249–287.

Oelke LM, Bosland PW, Steiner R (2003) Differentiation of race specific resistance to *Phytophthora* root rot and foliar blight in *Capsicum annuum*. J Am Soc Hortic Sci 128: 213–218.

Ogundiwin EA, Berke TF, Massoudi M, Black LL, Huestis G, Choi D, Lee S, Prince JP (2005) Construction of 2 intraspecific linkage maps and identification of resistance QTLs fpr *Phytophthora capsici* root-rot and foliar-blight diseases of pepper (*Capsicum annuum* L.). Genome 48: 698–711.

Ortega RG, Espanol CP, Zueco JC (1991) Genetics of resistance to *Phytophthora capsici* in the pepper line 'SCM-334'. Plant Breed 107: 50–55.

Quirin EA, Ogundiwin EA, Prince JP, Mazourek M, Briggs MO, Chlanda TS, Kim KT, Falise M, Kang BC, Jahn MM (2005) Development of sequence characterized amplified region (SCAR) primers for the detection of *Phyto. 5.2*, a major QTL for resistance to *Phytophthora capsici* Leon. in pepper. Theor Appl Genet 110: 605–612.

Raitio M, Perala A, Harinen RR, Lampinen J (2012) Validation of KASPar™ dual FRET based SNP genotyping assay. Thermo Fisher Scientific, Finland, pp 1–4.

Rao GU, Ben Chaim A, Borovsky Y, Paran I (2003) Mapping of yield-related QTLs in pepper in an interspecific cross of *Capsicum annuum* and *C. frutescens*. Theor Appl Genet 106: 1457–1466.

Rehrig WZ, Ashrafi H, Hill T, Prince J, Deynz AV (2014) *CaDMR1* co-segregates with QTL *Pc5.1* for resistance to *Phytophthora capsici* in pepper (*Capsicum annuum*). Plant Genome doi: 10.3835/plantgenome2014.03.0011.

Reifschneider FJB, Boiteux LX, Della Bechia PT, Poulos JM, Kurada N (1992) Inheritance of adult-plant resistance to *Phytophthora capsici* in pepper. Euphytica 62: 45–49.

Smith PG, Kimble KA, Grogan RG, Millett AH (1967) Inheritance of resistance in peppers to *Phytophthora* root rot. Phytopathology 57: 377–379.

Sy O, Bosland PW, Steiner R (2005) Inheritance of *Phytophthora* stem blight resistance as compared to *Phytophthora* root rot and *Phytophthora* foliar blight resistance in *Capsicum annuum* L. J Am Soc Hortic Sci 130: 75–78.

Thabuis A, Palloix A, Pflieger S, Daubeze AM, Caranta C, Lefebvre V (2003) Comparative mapping of *Phytophthora* resistance loci in pepper germplasm: evidence for conserved resistance loci across Solanaceae and for a large genetic diversity. Theor Appl Genet 106: 1473–1485.

Truong HTH, Kim KT, Kim DW, Kim S, Chae Y, Park JH, Oh DG, Cho MC (2012) Identification of isolate-specific resistance QTLs to phytophthora root rot using an intraspecific recombinant inbred line population of pepper (*Capsicum annuum*). Plant Pathol 61: 48–56.

Walker SJ, Bosland PW (1999) Inheritance of phytophthora root rot and foliar blight resistance in pepper. J Am Soc Hort Sci 124: 14–18.

Yaioon S, Teerawatsakul Y, Struss D (2014) Double haploid production in pepper (*Capsicum annuum* L.) by shed microspore culture. Khon Kaen Agr J 42 Suppl 3: 136.

Young ND (1996) QTL mapping and quantitative disease resistance in plants. Annu Rev Phytopathol 34: 479–501.

Online the pepper genome database (release 2.0), http://peppersequence.genomics.cn/page/species/blast.jsp (October 2014).

Online the pepper genome database platform, http://peppergenome.snu.ac.kr/blast (October 2014).