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Research Article

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Inheritance of resistance to phythophtora capsici in capsicum annuum and analysis of relative srap markers

Xiaowan Xu, Li Zeng, Ying Li and Hengming Wang

Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, Guangdong, Peoples Republic of China

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ABSTRACT

Phytophthora capsici causes devastating disease on many crop species, including capsicum. Resistance in capsicum annuum is genetically and physiologically complex. 'Criollo de Morelos 334' (CM334) from America is one of the most promising sources of resistance to Phytophthora capsici in pepper. In this work, using the traditional genetic analysis method, a joint analysis of six-generations (P_1, P_2, F_1, F_2, BC_1 and BC_2) was performed from cross between CM334 and a highly susceptible pepper line 949. The resistance of plants of each population was evaluated by irrigating method. F_2 population was analysed with SRAP molecular markers. Results showed that a single pair of nuclear and dominant gene controlled the resistance in CM334. Among 64 pairs of SRAP primers, 21 pairs of primers produced polymorphic bands in parents. After screening of the F2 population using the 21 pairs of SRAP primers, we obtained a SRAP marker linked with Phytophthora blight resistance, designated SRAP- Me6/Em15.

Key words: Hot pepper; resistance to *phythora* blight; inheritance; SRAP

INTRODUCTION

Pepper is originated from South America [1] and has been an important condiment and an economically important vegetable worldwide as well as in China. *Phytophthora* blight is one of the most destructive pathogens worldwide caused by *Phytophthora capisci* Leon, which attacks pepper plants at all developmental stages [2]. There are over 70 species of *Phytophthora*, many of which live on both dead and live plants, and reproduces both sexually and asexually [3]. *Phytophthora capisci* L. is a soil-borne pathogen that was first identified on pepper (*Capsicum annuum* L.) in New Mexico in 1922 . Since that time, *Phytophthora* blight has been reported throughout the world. Aerial organs may be attacked by splashing water and soil carrying inoculum during heavy rainfalls. *P. capsici* can infect all parts of the pepper plant, causing different disease syndromes, such as damping off, root rot, crown rot, fruit rot, and foliar blight, and resulting in yield reduction even total loss [4]. *Phytophthora* blight has become the major blockade of pepper cultivation. The prevention of *Phytophthora* blight in field depends on chemicals [5]. However, chemical control, such as the use of pesticides, is limited by environmental factors and often ineffective against *Phytophthora* on pepper. Chemical prevention also causes environmental contamination. In addition, many studies indicate that the biological control approach is not effective [5]. With the lack of dependable chemical and biological control measures, the development of adapted *phytophthora*-resistant pepper cultivars is considered to be an essential approach to controlling *phytophthora* disease. While breeding programs have been underway in many locations for at least two decades, none of the resistant varieties currently available show high levels of resistance due to the complex inheritance of resistance [6] and difficulty with reproducible phenotypic screens [5].

Many researchers have studied the genetics of resistance to pepper blight. The resistance displays characteristics of genetic diversity. In different races even different lines, resistance is inherited in different modes, including monogenic, oligogenic and multigenic [4-6]. For example, the genetics of CM334 to *phytophthora capsici* have generated conflicting results. Previously published studies on the resistance of CM334 to *P. capsici* came to various

genetic modes such as two unlinked recessive genes, two dominant genes [7], three genes, and additive gene modes [8]. It was later concluded that polygene with additive or epistatic action controlled *phytophthora* resistance in pepper [9]. Genetic diversity of *phytophthora* resistance in pepper is primarily determined by genetic nature of specific lines [7]. It is also influenced by many environmental factors, including temperature, water stress, soil moisture [8], inoculation concentration, dose [8], pathogenicity [5,10-11], plant age [7], inoculation method and site [12]. A large virulence profile of *P. capsici* was demonstrated when genotypes determined to be resistant in some regions were found to be susceptible in other regions. If reliable markers are available, the breeding process is expedited.

Marker-assisted selection (MAS) has been proposed for many years to offer the means to facilitate the management of complex traits such as resistance to *P.capsici* in pepper [6]. And significant progresses were made on molecular markers associated with *P. capsici* resistance. One consistent major quantitative trait loci (QTL, *Phyto.5.1*) was found with three populations and by two inoculation methods for two strains. The QTLs were very different in their quantitative effect (R^2 values), including major QTLs which explained 41-55% of the phenotypic variance, intermediate QTLs with additive or/and epistatic action (17-28% of the variance explained) and minor QTLs [9]. A dominant SCAR marker (*phyto 5.2*) was developed from the result of bulked segregant analysis (BSA) with randomly amplified polymorphic DNA (RAPD) primers [2], used for rapid identification of *P. capsici* resistance genes. In the double haploid F_1 population between a line susceptible to *Phytophthora capsici* 'K9-11' (*C. annuum* L.) and a line resistant to *P. capsici* 'AC2258' (*C. annuum* L.), three QTLs were detected on LG1, LG6 and LG7,(respectively). It was confirmed that the lines with high resistance could be efficiently selected by using two markers, an AFLP marker, M10E3-6 and a RAPD marker, RP13-1, simultaneously [13]. In other studies, in the F_2 population of a cross between a line resistant to *P. capsici* '93-100-17-1-0' (*C. annuum* L.) and an inbred line susceptible to *Phytophthora capsici* 'Qiemen' (*C. annuum* L.), 18 QTLs were detected on LG1, LG5, LG7 and LG8, which explained over 64% of the phenotypic variance [14]. The F_2 population derived from a sweet pepper 'N1345' with high resistance to *phytophthora* blight and a highly susceptible hot pepper 'N1308' was analyzed with EST-SSR and SSR molecular markers. Two EST-SSR makers including E73 and E318 were closely linked to genes of resistance to *phytophthora* blight [15].

Despite the progresses in the genetic basis of resistance in pepper- *P. capsici* interactions and resistance molecular marker research [16], and some molecular markers linked with *Phytophthora* blight resistance were found, even some molecular markers have been used for molecular breeding in pepper. Due to differences in resistant pepper resources and physiological races of *P. capsici*, molecular markers and mapping information's usability for MAS are very limited. In this study, first, the genetic resistance of CM334 against the main physiological race of *phytophthora* in Guangzhou was analyzed with traditional methods. And Using SRAP molecular marker technology, we analyzed an F_2 segregation population. The objectives of this study were to provide the basis for molecular marker assisted breeding against pepper *Phytophthora* blight, to lay the foundation for further clarifying the genetic of resistance in pepper to the *phytophthora capsici*.

EXPERIMENTAL SECTION

2.1 CONSTRUCTION OF POPULATIONS AND RESISTANCE EVALUATION

The study was performed in the Experiment Base of Guangdong Agriculture Academy Vegetable Institute. Mexican landrace 'Criollo de Morelos-334' (*Capsicum annuum* CM334), resistant to *P. capsici*, and China landrace *C. annuum* 949, susceptible to *Phytophthora*, were used as parents for six basic populations. CM334 was provided by P.W. Bosland (New Mexico State University, America). These parental materials are pure lines. CM334 (P_1) was crossed with 949 (P_2) reciprocally to produce F_1, and F_1 (CM334×949) was self-pollinated by bag isolation to produce an F_2 population. Back crosses were made to obtain BC_1 (F_1×P_1) and BC_2 (F_1×P_2) seeds.

In spring of 2010, The F_1, F_2, BC_1, and BC_2 generation populations, including their reciprocals, were grown together with the parents at Baiyun Experimental Station of Guangdong Academy of Agricultural Sciences. Seeds of the parental lines and cross progeny were planted in 9×9 cm nutrition bowl containing the disinfected nutritive soil by formalin in a plastic shed. Management was carried out according to conventional methods.

2.2 PHYTOPHTHORA CAPSICI

The moderately aggressive *Phytophthora capsici* Leonian was kindly provided by Prof. Liu Aiyuan (College of Horticulture of South China Agricultural University, China). The pathogen (*P. capsici* Leon) was collected from a vegetable farm in the suburban area of Guangzhou. After activation, the pathogen was cultured on carrot agar plate, at 26°C with 12 h light daily for 10 d in an incubator. To prepare inoculum for disease screens, cultures were flooded with ddH_2O, incubated for 1 h at 4℃ and then held at room temperature for 30 min to promote sporulation. The spores were counted and concentration adjusted to 4×10^4 spores/ml in distilled water. At 6-8 leaf stage, roots were

inoculated with swarm spores of *P. capsici* with root wound or root soaking method. Roots were wounded by the insertion of a knife into the soil 1-2 cm off the stem.

2.3 DISEASE SCORNING

Once susceptible check genotype 949 began to exhibit severe disease symptoms, all other five populations (P_1, P_2, F_1, F_2, B_1 and B_2) were scored for disease severity and the disease symptoms were measured every 2 days until 949 were basically dead. Each plant of the six populations was scored on a scale of 0-5 based on the following criteria: grade 0, no symptoms/healthy plant; grade 1, root and stem blacking, no leaf wilting or recoverability of wilting; grade 2, the necrosis of the stem 1-2 cm in length, leaf unrecoverable wilting or lower leaf occasionally falling off; grade 3, the necrosis of the stem more than 2 cm in length, leaf wilting obviously or falling off significantly; grade 4, stem necrosis and constriction, in addition to the growing point, all other leaves falling off or plant wilting; grade 5, dead plant. For each population, the resistance scores were averaged over all the replicates. Plants that received an average score of 0, 1, 2, or 3 were classified as resistant, while those that received an average score of 4 or 5 were classified as susceptible.

2.4 GENOMIC DNA EXTRACTION AND SRAP ANALYSIS

Sequence-related amplified polymorphism (SRAP) is a novel PCR-based marker system which detects polymorphisms preferentially in coding sequences, making it a more efficient technique due to its capacity to reveal more informative bands. 64 pairs of SRAP primers were used to study the F_2 population. Leafs were crushed into powder with the aid of liquid nitrogen, and the genomic DNA of individual plant was extracted from the sixth or seventh healthy leaf using modified CTAB method. Integrity and quality of DNA were evaluated by electrophoresis on 1% agarose gel. The genomic DNA was stored at -20°C. Resistant ad susceptible bulks comprising equal amounts of DNA from ten resistant and ten susceptible F_2 plants, respectively, were used for bulked segregant analysis. Primers with polymorphism in the parents were used for screening of the samples. SRAP-PCR reactions were performed in a total volume of 20μl as follows: 2.0μl of 10×PCR buffer (200 mM Tris-HCl (ph8.8); 100 mM KCl; 100 mM $(NH_4)_2SO_4$; 20 mM $MgSO_4$; 10×), 0.2μl of dNTPs (10 mM), 1.0μl of forward primer (100nM μl^{-1}, Sangon Biotech (Shanghai) Co., Ltd., China), 1.0μl of reverse primer (100nM μl^{-1}, Sangon Biotech (Shanghai) Co., Ltd., China) (Table 1), 0.2μl of *Taq* DNA polymerase (5 u μl^{-1}, Sangon Biotech (Shanghai) Co., Ltd., China), 20 ng of DNA template. DNA amplifications were performed with an initial step at 94°C for 5 min, and five cycles of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C. The following 35 cycles consisted of 94°C for 1 min, 50°C for 1 min and 72°C for 10 min. The amount of DNA template, brand of dNTPs, *Taq* DNA polymerase and primers were standardized and kept constant throughout the study. Amplified products were analyzed on 6% polyacrylamide gels in 1×TBE buffer running at 120 V constant voltage for 1.0 h and the sliver stained with $AgNO_3$.

Table 1. Sequence of forward and reverse SRAP primers used in this study

Forward primers (5'to 3')		Reverse primers (5'to 3')	
Me_1	TGA GTC CAA ACC GGA TA	Em_1	GAC TGC GTA CGA ATT AAT
Me_2	TGA GTC CAA ACC GGA GC	Em_2	GAC TGC GTA CGA ATT TGC
Me_3	TGA GTC CAA ACC GGA AT	Em_3	GAC TGC GTA CGA ATT GAC
Me_4	TGA GTC CAA ACC GGA CC	Em_4	GAC TGC GTA CGA ATT TGA
Me_5	TGA GTC CAA ACC GGA AG	Em_5	GAC TGC GTA CGA ATT AAC
Me_6	TGA GTC CAA ACC GGT AG	Em_6	GAC TGC GTA CGA ATT GCA
Me_7	TGA GTC CAA ACC GGT TG	Em_7	GAC TGC GTA CGA ATT ATG
Me_8	TGA GTC CAA ACC GGT GT	Em_{15}	GAC TGC GTA CGA ATT CTG

CONCLUSION

3.1 RESISTANCE EVALUATION AND GENETIC ANALYSIS

At 6-8 leaf stage, CM334 showed highly resistant (36 plants in grade 0), whereas, 949 were susceptible (31 plants in grade 5, and 5 plants in grade 4). All the F_1 progeny plants in forward cross $P_1 \times P_2$ and reverse cross $P_2 \times P_1$ were resistant (in grade 0), suggesting that the nuclear and dominant gene controlled the resistance in CM334. The 154 F_2 plants segregated into 118 resistant (118 plants in grade 0-1) and 36 susceptible (36 plants in grade 4-5), which conformed to 3R:1S segregation ratio (χ^2=0.216, 1 *df*, P≥0.05). Moreover, in the BC_1 population (backcross with CM334), all plants (154) were resistant (Table 2). The BC_2 population (backcross with 949) segregated into 74 resistant (66 plants in grade 0 and 8 plants in grade 1-2) and 80 susceptible (32 plants in grade 4 and 48 plants in grade 5), which is shown to 1R:1S segregation ration (χ^2=0.234, 1 *df*, P≥0.05). Comprehensive analyzes above data showed that a single pair of nuclear and dominant gene controlled the resistance in CM334.

Table 2. Segregation data for disease reaction of parental (p), F_1, backcrosses (BC) and F_2 populations of *Capsicum annuum* after inoculation with *phytophthora capsici*

Crosses	Generation	Number of plants						Expected ratio	(R:S)	χ^2 ($\chi^2_{0.05}$=3.84)
		Resistant				Susceptible				
		0	1	2	3	4	5			
CM334	P_1	36	0	0	0	0	0	1:0		
949	P_2	0	0	0	0	5	31	0:1		
CM334×949	F_1	36	0	0	0	0	0	1:0		
949×CM334	F_1	36	0	0	0	0	0	1:0		
(CM334×949)□	F_2	117	1	0	0	18	18	3:1		0.216
(CM334×949) ×CM334	BC_1	136	0	0	0	0	0	1:0		
(CM334×949) ×949	BC_2	66	3	5	0	32	48	1:1		0.234

3.2 SRAP MOLECULAR MARKER

Screening for polymorphic markers in the parents: DNA of the P_1, P_2 and F_1 plants were screened with the 64 SRAP primer combinations (Fig. 1). The majority of primer combinations could generate bands, and the average fragments generated per primer was 17-50. Fifty seven primers generated uniform, clear, stable and reproducible bands. Five primers produced smear bands and 2 primers did not produced bands. Twenty one primer combinations produced polymorphic bands through comparative study of the amplification profiles.

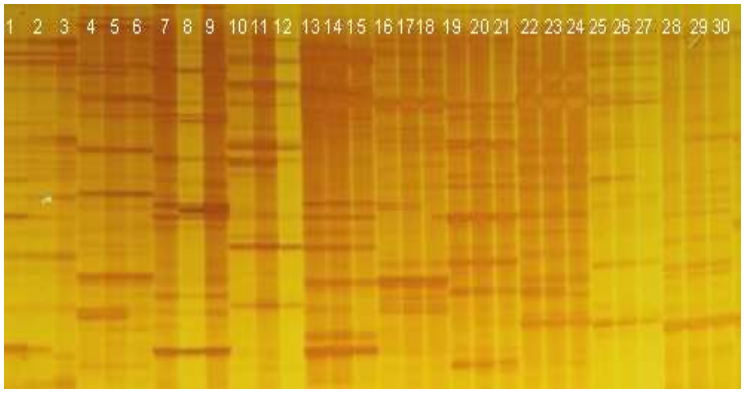

Fig.1: Banding profile in parents and F_1 from different primer combinations

(lanes 1,4,7,10,13,16,19,22,25,28 were P_1;lanes 2,5,8,11,14,17,20,23,26,29 were F_1;Lanes 3,6,9,12,15,18,21,24,27,30 were P_2)

SRAP Markers amplified in mixed pool and F_2 segregation populations: A total of 21 SRAP primer pairs combinations were used for the amplification of DNA samples of parents, F_1 and F_2 populations. A primer combination Me_6/Em_{15} (TGA GTC CAA ACC GGT AG/ GAC TGC GTA CGA ATT CTG) produced a specific band from CM334, F_1, and F_2 resistant pool (Fig. 2).

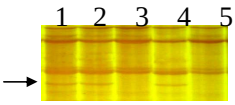

Fig.2. Resistant and susceptible F_2 bulks screened with the Me6/Em15 SRAP primer

Arrowhead indicates specific band. Lane 1: CM334 (R); Lane 2: F_1 (R); Lane 3: 949; Lanes 4: Resistant pool; Lanes 5: Susceptible pool.

Individual plants of F_2 population were further analyzed with primer combination Me_6/Em_{15}. Among the 92 resistant plants, eighty seven plants showed this specific band and 5 plants did not. Among 28 susceptible plants, 20 plants did not show the band and 8 plants showed this characteristic band (Fig. 3). The marker is now termed SRAP-Me6/Em15.

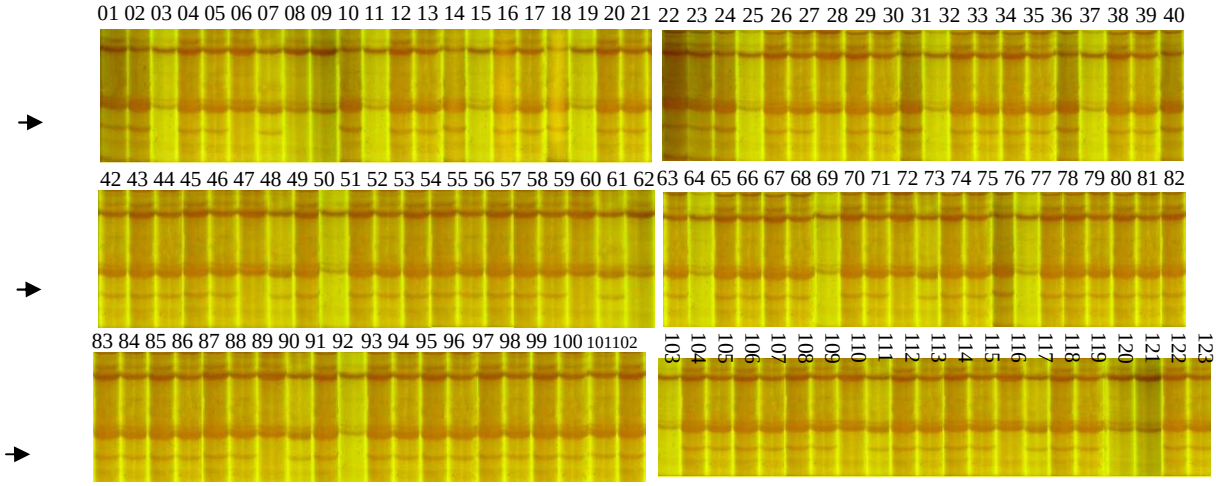

Fig.3: Banding profile of primer combination Me_6/Em_{15} in F_2 population.
Lane 1: CM334 (R); Lane 2: F_1 (R); Lane 3: "949"; Lanes 4-123: F_2 individual plants.

DISCUSSION

One of the most economically destructive pathogens to Chile (*Capsicum annuum* L.) worldwide is *Phytophthora capsici* Leonian, has drawn attention in many countries. Scholars have made significant progress in biological characterization of the pathogen, host resistance, and developed evaluation methods. *Phytophthora* resistance is inherited with diverse mechanisms. For example, resistance in CM334 has been debated for many years. In the current study, the high *phytophthora* resistance resource CM334 was analyzed for resistance to isolates of *Phytophthora* from the Guangzhou suburban area. Results of our study indicate that blight resistance in CM334 was controlled by 1 dominant gene. This is consistent with the reports by Walker and Bosland [17], but different from those reported by Ortega *et al.* [8], who concluded that three genes or multiple alleles are responsible. These seemly conflicting results indicate that the pepper has plenty resistance genes as well as complex resistance mechanism. Due to differences in pathogens, experimental conditions, resistance evaluation criteria and susceptible parents, the conclusion can be different.

Researchers have conducted many studies to map QTL or to link molecular markers. Quirin *et al.* obtained a single RAPD marker OpD04 from resistant parent and progeny [2] . Linkage analysis indicates that OpD04 is closely linked to *phyto 5.2* disease resistant genes on chromosome 5. Sugita *et al.* [13] mapped disease resistanculation, demonstrated that *phyt-1* is on linkage group 7. AFLP is the closet marker to M10E3-6. Re QTLs in a DH popesistance gene *Phyt*-2 is on the first linkage group, and RAPD marker RP13-1 is closest to *phyt-2*.

Due to the complexity of blight resistance inheritance in the pepper, it is a "must" to analyze the genetics and molecular marker for resistance against specific physiological races of the pathogen. SRAP markers display high polymorphism, reproducibility, thus have been widely used in genetic diversity analysis, comparative genetics and map constructions. The current study used SRAP markers and successfully identified a marker SRAP- Me6/Em15 in CM334 for blight resistance. The discovery opened the ground for molecular marker assisted breeding. Further study is needed to develop molecular marker SRAP- Me6/Em15, including specific fragment recovery, purification, cloning and transform into SCAR marker, for application in breeding practice.

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