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SCIENCE DIRECT
Agricultural Sciences in China
2006, 5(7): 517-521
July 2006

Inheritance Analysis and Identification of SSR Markers Linked to Late Blight Resistant Gene in Tomato

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Abstract

Late blight caused by *Phytophthora infestans* is the most serious disease of tomato production in China. Studies on the genetics of resistance and identification of molecular markers are very useful for breeding late blight resistant varieties. The objective of this paper was to study the inheritance of late blight resistance and identify simple sequence repeat (SSR) markers associated with resistance allele in tomato (*Lycopersicon esculentum* Mill). The results came from an F_2 progeny of 241 plants derived from a cross between 5# inbred line that is susceptible to late blight and a resistant accession CLN2037E. The late blight responses of F_2 plants were tested by artificially inoculation of detached-leaflets in plate and natural infection assayed under greenhouse conditions. Both methods showed that the resistance is dominant and inherited as monogenic trait. Genetic mapping and linkage analysis showed that the late blight resistance gene *Ph-ROL* was located on chromosome 9 with a genetic distance of 5.7 cM to the SSR marker TOM236.

Key words: disease resistance, late blight, *Phytophthora infestans*, SSR, tomato

INTRODUCTION

Late blight, caused by the fungal pathogen *Phytophthora infestans* is an increasingly significant problem in tomato production worldwide. In many parts of the world, the control of late blight is heavily relied on the frequent application of protectant fungicides, which are applied every 5-14 days (Wang 2003). The use of resistant tomato cultivars could sustainally reduce reliance on chemical sprays for the control of this disease. Two types of resistance to late blight on tomato have been reported. First, race-specific or vertical resistance is controlled by a dominant gene and is very effective against a specific race (strain) of the fungus. Plants with race-specific resistance react to infection by forming small, dead, non-spreading spots. Three dominant genes have been identified: *Ph-1* on chromosome 7 (Clayberg *et al.* 1965; Perirce 1971), *ph-2* on chromosome 10 (Morear *et al.* 1998), *Ph-3* on chromosome 9 (Chunwongse *et al.* 1998). The second type of resistance, horizontal resistance, is controlled by several genes. Plants with horizontal resistance form small, atypical leaf spots that produce relatively few spores. The second type of resistance has an advantage of being effective against many races of the late blight fungus. Fray *et al.* (1998) identified two QTLs associated with horizontal resistance on chromosomes 6 and 8. In addition, Brouwer and St. Clair (2004) identified three other QTLs, on chromosomes 4, 5, and 11.

Wild tomato species have been used as sources of resistance to late blight and were reported to be presented in *L. pimpinellifolium* and *L. hirsutum* (Lobo and Navarro 1987; Kim and Mutschler 2000). An in-

Received 17 February, 2006 Accepted 27 April, 2006
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terspecific F_1 progeny from *L. esculentum* × *L. hirsutum* LA2099-MDI exhibited resistance in detached-leaflet assays to numerous *Phytophthora infestans* isolates and was highly resistant in the field in North Carolina (Brouwer *et al.* 2004). Breeding for late blight resistance at AVRDC began in 1993.

Two resistant lines CLN2037B and CLN2037E, derived from crosses to *L. pimpinellifolium* L3708 were developed and selected for international distribution and testing (Wang 2003).

The use of molecular markers can facilitate tomato breeding through marker assisted selection (MAS) for improvement of agronomic traits such as disease resistance, yield and fruit quality. Simple sequence repeats (SSR) or microsatellites are not only very common but also hypervariable among the types of tandem repetitive DNA in the genome of eukaryotes (He *et al.* 2003). SSR markers are becoming the preferred molecular markers in crop breeding and genetic studies because of their properties of genetic co-dominance, high reproducibility and multiallelic variation. Thus, microsatellite loci can be useful in distinguishing cultivars of tomato, which are genetically very closely related to each other (Smulders *et al.* 1997). However, the number of polymorphic SSR markers is still low so that resistance genes in tomato were mapped primarily using RFLP or AFLP markers. In this study, SSR markers from the map developed by Suliman-Pollatsche *et al.* (2002) and the SOL Genomics Network (SGN) of Cornell University were used. This is the first report using the SSR markers to analyze the inheritance of late blight resistance and mark the resistance gene in tomato.

MATERIALS AND METHODS

Plant materials The tomato population was developed by crossing a susceptible cultivar 5# inbred line and the resistant genotype CLN2037E to generate F_1, F_2 populations. All the parents and F_1, F_2 plants were planted in the greenhouse in Kunming of Yunnan Province, China, during the summer of 2004. Leaves collected from each plant were divided into two parts, one for evaluation of late blight resistance by detached-leaflet assay and the other one for DNA extraction.

Detached-leaflet and natural infection assay Side lateral healthy leaflets removed from greenhouse-grown plants (approximately 12 weeks old, around fruiting stage) were collected and transported into the laboratory. After washing with sterile water, leaflets were blotted dry with sterile tissue paper and placed on 1% agar medium. Three leaflets were placed in a plate. The lower surface of leaflet was inoculated with 20 μL of sporangial suspension of tomato physiological race T1, 2, 3 of *Phytophthora infestans* at 2000 spores mL^{-1}. After inoculation, the leaflets were incubated for the first 24 hours at 20°C without light, thereafter, maintained at 20°C with a 12 hours light (70 μE M^{-2} S^{-1}) period per day for 6 days. There were three replications and disease severity ratings (DSR) were done at the 5th day after inoculation by scoring each plate individually. Severity rating scale were from 0 to 6 (Wang 2003), with 0 being no symptoms, and 6 being 91-100% leaflet area affected. The late blight reactions of tomato plants were placed into one of the following categories based on their DSR of the 5th day after inoculation, resistant being a DSR of 0-4.5 and susceptible being a DSR of 4.6-6.0, according to Wang (2003). Besides the detached-leaflet assay, all plant materials (5# inbred line, CLN2037E, F_1 and F_2) were also evaluated under natural conditions of a severe epiphytotic in the greenhouse. DSR was made at the fastigium of late blight.

DNA extraction and SSR analysis Genomic DNA was extracted from young leaves following the method modified from He *et al.* (2003). A total of 41 SSR primers previously mapped on tomato [Suliman-Pollatsche *et al.* 2002 and SOL Genomics Network (SGN) of Cornell University] were assayed for polymorphism between 5# inbred line and CLN2037E. Primers were excluded from the study if band patterns were difficult to score or if the primers failed to amplify specific band patterns in three templates. A final set of 8 SSR primers (SSR104, SSR66, SSR320, SSR306, SSR43, TOM59, TOM184, TOM236) were chosen for further analysis. The PCR reaction mixture (25 μL total volume) consisted of 30 ng template DNA, 1 μM primer, 1.5 mM $MgCl_2$, 400 μM dNTP, 2.5 μL 10× PCR buffer containing, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, and 1.5 units of *Taq* DNA polymerase [TaKaRa Biotechnology (Dalian) Co., Ltd.]. PCR cycles started at 96°C (2 min) in an Eppendorf mastercycler AG, followed by 30 cycles of 94°C (10

s), 45-49°C (1 min), 72°C (1 min), and ended with 10 min 72°C. PCR products were size-separated on a 2.5% agarose gel in 1×TBE buffer at 4 V cm^{-1}, stained with ethidium bromide (1 μg mL^{-1}) for 20 min, and visualized on a GelDoc-It imaging system (UVP, USA) with a 3 UV transilluminator LMS-26E (UVP). Bands were scored as '0' for homozygote from susceptible parent 5# inbred line, '1' for homozygote from resistant parent CLN2037E, '2' for heterozygote, and '-' for missing data.

Data analysis The data of late blight reaction of detached-leaflet and natural infection assays were tested for significant deviation from the expected Mendelian ratio of 3:1 using chi-square (χ^2) test. Linkage analysis was undertaken using MapMaker/EXP (3.0b), and genetic distances were measured in centimorgan (cM).

RESULTS

Inheritance analysis The plants of 5# inbred line shriveled and dead at the final period of natural infection, while the plants of CLN2037E still had green and healthy appearance. The detached-leaflets of 5# inbred line became brown and are covered with a gray to white moldy growth, and the CLN2037E developed small, atypical spots (Fig.1).

The response of four populations in natural infection and detached-leaflet assays showed that the resistance is dominant and suggested that it is inherited as monogenic dominant trait in CLN2037E resistant accession (Table).

Detection of the positive marker Among 41 SSR primers used in this study, 8 SSR primers were able to detect polymorphism between 5# inbred line and CLN2037E. Only marker TOM236, located on tomato chromosome 9, was shown linkage to the resistant gene. Five susceptible DNA templates chosen from F_2 population had a specific DNA fragment, 185 bp in size, the same as the susceptible parent, while five DNA ones of resistant plants from F_2 population had another specific DNA fragment, 155 bp in size, the same as the resistant parent (Fig.2).

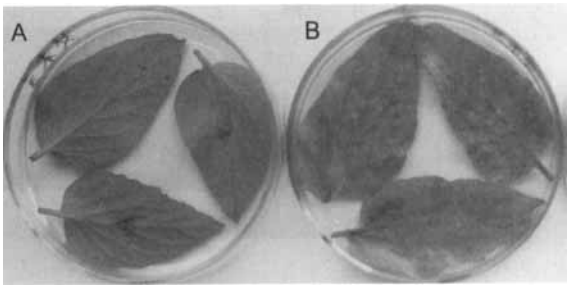

Fig. 1 Compatible interactions at 5th day after inoculation on A, CLN2037E; and B, 5# inbred line detached-leaflets inoculated with tomato physiological race T1, 2, 3 of *Phytophthora infestans*.

Identification of the resistant gene DNA templates of F_2 population consisted of 241 individuals were assayed using marker TOM236. Among the 241 individuals, 63 5# inbred line homozygotes, 104 CLN2037E homozygotes, 62 heterozygotes were detected and there were 12 individuals failed to generate visual band pattern. The phenotypic performance of resistant trait in detached-leaflet assay was designated *Ph-ROL*, and the one in natural infection assay was designated *Ph-ROF*. After linkage analysis by MapMaker, *Ph-ROL*, *Ph-ROF* and 8 SSR markers grouped into 2 linkage groups, markers SSR320 and TOM184 were unlinked, and *Ph-ROL*, *Ph-ROF* and TOM236 were linked in one group. The results showed the genetic linkage distance was d5.7 cM between marker TOM236 and resistant trait Ph-ROL, and 30.4

Table Late blight reactions of CLN2037E, 5# inbred line and progenies

Population	Assay[1)]	Nos. of plant	Nos. of plant		Expected ratio	$\chi^{2\,2)}$
			R	S		
CLN2037E (R)	NI	40	40	0		
	DL	40	39	1		
5# inbred line (S)	NI	40	0	40		
	DL	40	0	40		
F_1	NI	40	38	2		
	DL	40	36	4		
F_2	NI	241	180	61	3:1	0.001[ns]
	DL	241	191	50	3:1	2.325[ns]

[1)] NI, natural infection under field conditions; DL, detached-leaflet assay in growth chamber.
[2)] [ns] denotes not significant at $P=0.05$.

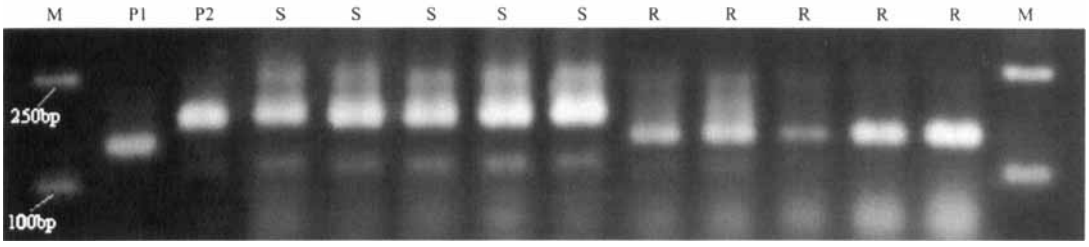

Fig. 2 The amplified profile obtained among genomic DNA in male, female parents, resistant and susceptible plant with the primer of TOM236. M, marker; P1, male parent, CLN2037E; P2, female parent, 5* inbred line; S, susceptible plant; R, resistant plant.

cM between *Ph-ROL* and *Ph-ROF*. The results suggested that the resistant gene *Ph-ROL* is located on the chromosome 9 (Fig.3), similar to the findings of Suliman-Pollatsche *et al.* (2002).

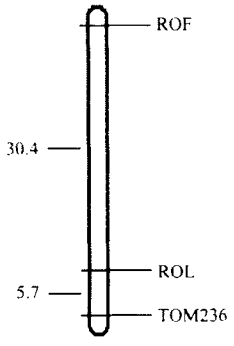

Fig. 3 The genetic linkage map between the resistant gene to late blight *Ph-ROL*, *Ph-ROF* and the SSR marker TOM236 of chromosome 9 in tomato.

DISCUSSION

The disease test on the F_2 population demonstrated that resistance to late blight of tomato in CLN2037E is the dominant monogenic trait. *Ph-ROL/Ph-ROF* was located on tomato chromosome 9, which is similar to the results of *Ph-3* (Chunwongse *et al.* 1998), but it is not known with certainty whether the resistant gene is *Ph-3*. Since no polymorphism between 5* inbred line and CLN2037E was detected for other SSR markers on the chromosome 9, further work is required to verify the location of *Ph-ROL/Ph-ROF*.

There are several methods for resistance assay to *Phytophthora infestans*, including whole plant, detached leaf, detached leaflet, leaf disc, stem segment and field assay. The former five methods can also be called laboratory assay. Although all the methods can be used in resistance assay, the most effective and reliable methods are generally accepted to be natural infections or inoculated test plots under field conditions (Guzmàn 1964). The detached-leaflet assay has proven effective for separating germplasm into distinct resistant or susceptible categories (Dorrance and Inglis 1997). In this study, there was a certain divergence between natural infection and detached-leaflet assay due to that the resistance response in field was influenced by many variables. Natural infection required a site where late blight epidemics occur reliably year after year, which need appropriate temperature, humidity and representative of fungal populations. Detached-leaflet assay was accomplished in a growth chamber where the environmental conditions were controlled. Our data suggested that the genetic distance is 5.7 cM between marker TOM236 and resistance *Ph-ROL*. The result can be used in MAS of tomato.

Acknowledgements

This study was sponsored by Key Project of 10th Five-Year Plan of Yunnan Provincial Government (2001NG21). We are grateful to Professor Yang Yanli for offering pathogen identification; Professor/Dr. Wang Tiancheng from Asia Vegetable Research and Development Center (AVRDC) for providing the resistant accession CLN2037E and reviewing this manuscript.

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(Edited by WANG Lu-han)