IJNES
International Journal of Natural and Engineering Sciences 3(3): 161-165, 2009
ISSN: 1307-1149, www.nobel.gen.tr

Screening of Resistance Genes to Fusarium Root Rot and Fusarium wilt Diseases in F_3 family Lines of Tomato (*Lycopersicon esculentum*) using RAPD and CAPs Markers

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Received: July 17, 2009
Accepted: August 30, 2009

ABSTRACT

Fusarium diseases constitute most of the loss in tomato production worldwide, because it spread on all geographic fields that it is so hard to find a place without Fusarium infestation. Thus, the best way to produce tomato is developing resistant cultivars against Fusarium species. In cultivar developing, molecular marker assisted techniques replaced traditional breeding techniques which are high cost and time consuming for breeders.

In this study RAPD and CAPs markers were used to screen tomato (*Lycopersicon esculentum*) lines against resistance genes *Frl* and *I-2* respectively. Results showed that out of 115 plants, 42 were homozygous resistant, 38 were heterozygous resistant and 35 were homozygous susceptible. Under the light of this information, the forthcoming cultivar development studies will be carried out.

Key Words: *Fusarium oxysporum,* Marker Assisted Selection, Tomato Root Rot, CAPs, RAPD

INTRODUCTION

Cultivated tomato (*Lycopersicon esculentum* Mill.) is one of the world's most important crops due to the high value of its fruits both for fresh market consumption and in numerous types of processed products [1]. World volume of production has increased approximately 10 percent since 1985, reflecting a substantial increase in dietary use of the tomato. One of the main constraints to tomato cultivation is damage caused by pathogens, including viruses, bacteria, nematodes and fungi, which cause severe losses in production [2].

The soil-borne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) causes Fusarium crown and root rot of tomato (*Lycopersicon esculentum* Mill.), often referred to as 'crown rot' [3]. *Fusarium oxysporum* f. sp. *lycopersici* inhabits most tomato-growing regions worldwide, causing tomato production yield losses [4] Today, it has an extensive presence in all continents [5][6]. Crown rot develops primarily in cool climates in both field and greenhouse tomatoes. Substantial crop losses in infected fields have given the disease international attention. The host range of this pathogen comprises at least 36 other species [7].

The first symptom of Fusarium wilt in gardens and fields is usually the golden yellowing of a single leaflet or shoot, or a slight wilting and drooping of the lower leaves on a single stem. Yellowed and wilted leaflets drop early. Affected plants turn to bright yellow, wilt, dry up, and usually die before maturity, producing few, if any, fruit.

The control of the pathogen spread mainly involves in three strategies: husbandry practices, application of agrochemicals and use of resistant varieties [2]. Resistant varieties are mostly produced by crossing resistant wild types and existing cultivars developed for their properties like good taste, shape and color. A molecular marker linked to resistance would be useful for tomato improvement program [4].

The virulence profile of *F. oxysporum* f. sp. *lycopersici* isolates affecting tomatoes has been grouped into three races according to their ability to infect a set of differential cultivars carrying distinct resistance loci. Three Fusarium wilt resistance loci has been genetically characterized in *Lycopersicon* species.

The locus *I*, from *L. pimpinel lifolium* (Just) Mill. 'PI 79532' [8], controls resistance to race 1. Isolates capable of infecting cultivars with the locus *I* were shortly after identified [9] and a new disease resistance locus (*I*-2) was characterized in the accession 'PI 126915', which is a natural hybrid between *L. es culentum* and *L. pimpinellifolium* [10]. A third race able to infect cultivars carrying both *I* and *I*-2 loci was reported first in Australia [11] and a new resistance locus (named *I*-3) was identified in the wild species *L. penn ellii* (Corr.) D'Arcy. Races 1 and 2 are distributed throughout the world whereas race 3 has a more limited geographic distribution [12]. Staniazsek et al. [4] developed a marker, $TAO1_{902}$ to identify tomato genotypes possessing the *I*-2 gene, which confers resistance to *F. o. lycop ersici* race 2. Fazio et al. [3] found the marker UBC194, a 10-mer primer, to be closely linked to *Frl* gene, a single dominant gene (Frl) on chromosome 9 confers resistance to Fusarium crown and root rot (crown rot) incited by *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

In this study tomato lines were screened for *I*-2 resistance gene by $TAO1_{902}$ CAPS marker, for *Frl* resistance gene by UBC 194 RAPD marker and those which found to be homozygous resistant were determined to be used as candidate parent in resistant breeding program.

MATERIALS AND METHODS

DNA Extraction

Total genomic DNA of the 115 F3 plants, derived from 12 F2 populations, shown in Table 1, used for DNA extraction. Fresh young leaves of the lines were subjected to extraction, using 200 mg of fresh tissue, the procedure described by Doyle & Doyle [13]. Was applied.

PCR Conditions

Extracted DNA was diluted as 5ng per 1 µl. RAPD analysis was performed according to Fazio et al. [3]. The nucleotide sequence for UBC 194 primer is: 5'- AGG ACG TGC C -3'. The marker was amplified in 15 µl reaction volume containing 0.8 mM dNTPs, 0,067 mM of primer, 2.67 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris HCl (pH 8.75), 1% Triton X-100, 1 mg/ml BSA and 0.2 units of Taq polymerase with 40 ng template DNA. The PCR parameters were: 94° C for 30 s followed by 36 cycles of 94°C for 25 s, 35° C for 45 s, 72° C for 60 s and a final extension time of 5 min at 72° C, using an MJ THERMAL CYCLER PTC-225 (Gradient).

Forward and reverse primers for TAO1 [4] were:

f: 5'-GGGCTCCTAATCCGTGCTTCA-3';

r: 5'-GGTGGAGGATCGGGTTTGTTTC- 3'

The marker $TAO1_{902}$ was amplified in 15 µl reaction volume of 2 mM dNTPs, 0,67 mM of each primer, 2.67 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris HCl (pH 8.75), 1% Triton X-100, 1 mg/ml BSA and 0.2 units of Taq polymerase with 40 ng template DNA. The PCR parameters were: 94° C for 60 s followed by 40 cycles of 93° C for 15 s, 62° C for 20 s, 72° C for 60 s and a final extension time of 5 min at 72° C, using an MJ THERMAL CYCLER PTC-225 (Gradient). Two parallel amplifications were performed for each sample in order to determine the marker (901 kb) and restriction reaction.

PCR products were digested with 5 units of *Fok*I restriction endonuclease (New England BioLabs Inc ®) in a 20 µl reaction volume containing recommended buffer (NEBuffer 4) for 1 h at 37° C and an enzyme inactivation step was performed for 20 min at 70°C.

Gel Electrophoresis and Imaging

Amplification products were resolved using 2% agarose gel under 3 V/cm potential and 100 mA currency in TAE buffer for 3h. Gels were stained with ethidium bromide for 30 min and visualized under UV light using Kodak 1D UV Imaging System.

RESULTS

Amplification with RAPD Primer

According to Fazio et al. [3], amplification with UBC 194 primer gives a marker (590 bp) linked to *Frl* gene. In this study none of the 115 samples revealed the 590 bp marker (Fig. 1) so they were determined to be *Frl* negative.

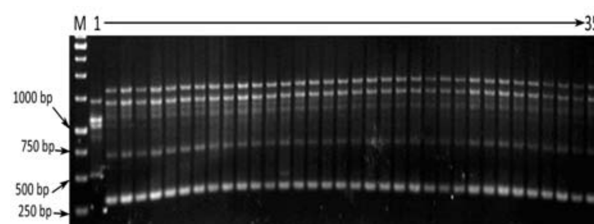

Figure 1: PCR products amplified with UBC 194 primer. None of the samples show the *Frl* linked marker 590 bp fragment.

Amplification with CAPS Primers

In contrast to RAPD analysis, amplification with TAO1 primers revealed the 902 bp fragment for 80 out of 115 plants (Fig. 2). A size of 902-bp-long fragment of the TAO1 marker was found to be polymorphic in resistant tomato lines [4]. This shows that these plants have the resistance gene (*I*-2). But to understand better the genotypic structure if they are homozygous or heterozygous, further analysis carried out by digestion the PCR products with *Fok*I restriction endonuclease.

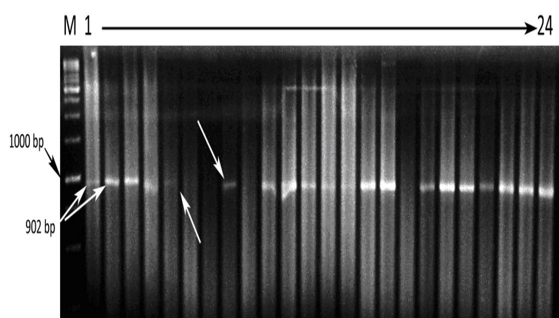

Figure 2. PCR products resolved in 2% agarose gel of samples coded as 1-24. White arrows show the 902 bp pair product and black one is size marker of 1000bp..

Restriction with *Fok*I

Restriction fragments of 390 and 410 bp with *Fok*I digestion of $TAO1_{902}$ were revealed that in the homozygous-resistant parent lines were A241 and A238 [4]. After digestion with *Fok*I, some of the samples revealed 390 and 410 bp fragment as reported by Staniazsek et al. [4]. These fragments shows that both alleles from parents related to the *I*-2 gene are present in the sample, thus the lines considered to be a homozygous resistant plant. Forty-two plants showed these restriction fragments. Full list of the lines was given in Table 1 showing the positive and negative results.

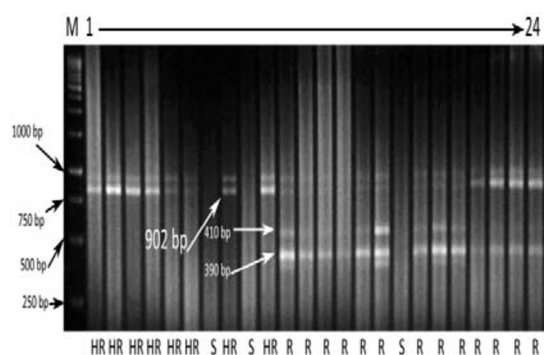

Figure 3. PCR products digested with *Fok*I restriction endonuclease of samples coded as 1-24. White arrows show the restriction products of the amplified fragments indicating the homozygous resistant samples. S: Susceptible HR: Heterozygous resistant R: Homozygous resistant.

Table 1. Scoring results for 115 samples.

*Positive (+): Samples which revealed 902 bp marker.

**Positive (+): Samples which revealed restriction fragments.

Code	Line	CAPS*	*Fok*I**	Code	Line	CAPS*	*Fok*I**
1	1-1	+	-	**31**	4-1	-	-
2	1-2	+	-	**32**	4-2	-	-
3	1-3	+	-	**33**	4-3	-	-
4	1-4	+	-	**34**	4-4	-	-
5	1-5	+	-	**35**	4-5	-	-
6	1-6	+	-	**36**	4-6	-	-
7	1-7	-	-	**37**	4-7	-	-
8	1-8	+	-	**38**	4-8	-	-
9	1-9	-	-	**39**	4-9	-	-
10	1-10	+	-	**40**	4-10	-	-
11	2-1	+	+	**41**	5-1	+	-
12	2-2	+	+	**42**	5-2	+	-
13	2-3	+	+	**43**	5-3	+	-
14	2-4	+	+	**44**	5-4	+	-
15	2-5	+	+	**45**	5-5	+	-
16	2-6	+	+	**46**	5-6	+	-
17	2-7	-	-	**47**	5-7	+	-
18	2-8	+	+	**48**	5-8	+	-
19	2-9	+	+	**49**	5-9	+	-
20	2-10	+	+	**50**	5-10	+	-
21	3-1	+	+	**51**	6-1	-	-
22	3-2	+	+	**52**	6-2	-	-
23	3-3	+	+	**53**	6-3	-	-
24	3-4	+	+	**54**	6-4	-	-
25	3-5	-	-	**55**	6-5	-	-
26	3-6	+	+	**56**	6-6	-	-
27	3-7	+	+	**57**	6-7	-	-
28	3-8	+	+	**58**	6-8	-	-
29	3-9	+	+	**59**	7-1	-	-
30	3-10	+	+	**60**	7-2	+	+

Code	Line	CAPS*	*Fok*I**	Code	Line	CAPS*	*Fok*I**
61	7-3	-	-	**91**	10-5	-	-
62	7-4	+	+	**92**	10-6	+	-
63	7-5	-	-	**93**	10-7	+	-
64	7-6	+	+	**94**	10-8	+	-
65	7-7	+	+	**95**	10-9	+	-
66	7-8	-	-	**96**	10-10	+	-
67	7-9	+	+	**97**	11-1	+	+
68	7-10	+	+	**98**	11-2	-	-
69	8-1	+	+	**99**	11-3	+	+
70	8-2	+	+	**100**	11-4	+	+
71	8-3	+	+	**101**	11-5	+	+
72	8-4	-	-	**102**	11-6	+	+
73	8-5	-	-	**103**	11-7	-	-
74	8-6	+	+	**104**	11-8	+	+
75	8-7	+	-	**105**	11-9	-	-
76	8-8	+	-	**106**	12-1	+	+
77	9-1	+	-	**107**	12-2	+	+
78	9-2	+	-	**108**	12-3	+	+
79	9-3	+	-	**109**	12-4	+	+
80	9-4	+	-	**110**	12-5	+	+
81	9-5	-	-	**111**	12-6	-	-
82	9-6	+	-	**112**	12-7	-	-
83	9-7	+	-	**113**	12-8	+	+
84	9-8	+	-	**114**	12-9	+	+
85	9-9	+	-	**115**	12-10	+	+
86	9-10	+	-				
87	10-1	+	-				
88	10-2	+	-				
89	10-3	+	-				
90	10-4	+	-				

DISCUSSION

Tomato (*Solanum lycop ersicum*, formerly *Lycopersicon esculentum*) is one of the most widely grown vegetable crops in the world. It is used as a fresh vegetable and can also be processed and canned as a paste, juice sauce, powder or as a whole [2]. One of the devastating disease, is Fusarium wilt, caused by three races of *Fusarium oxysporum* f. sp. *lycopersici*, is one of the most important diseases of tomato (*Lycopersicon esculentum*). Races 1 and 2 are distributed worldwide, whereas race 3 has a more limited geographic distribution [12]. *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) cause Fusarium crown and root rot of tomato often referred to as 'crown rot' [3] which also gives a substantial damage to crops.

A phenotypic selection for *F. o. lycopersici* resistance is a complex and time-consuming process in tomato [14]. DNA marker technology has been used in commercial plant breeding program since the early 1990s, and has proved helpful for the rapid and efficient transfer of useful agronomically important traits into desirable varieties and hybrids [15; 16]. Rapid death of the infected plants means that each F_2 tomato seedling is a unique genotype for any resistance bioassay. Identification of homozygote's for the *I*-2 gene is especially important when F_2 plants are to be used in further crossing. Knowledge of the genetic state for *I*-2 in F_2 individuals via testing of F_3 seedlings involves much more extensive disease screening [4].

In this study F_3 segregation populations were screened for *I*-2 resistance gene by $TAO1_{902}$ CAPs marker and for *Frl* resistance gene by UBC 194 RAPD marker. These analyses revealed that none of the lines screened were resistant to the soil-borne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) while most of them had the *I*-2 gene which confers resistance to *F. o. lycopersici* race 2. We found that not all of the lines which had the resistance gene were homozygous. Even there came out polymorphisms among the population of lines. In lines 2, 3 and 12 most of the plants were homozygous resistant while the remaining were homozygous susceptible. In lines 4 and 6 all the plants were homozygous susceptible. Most of the plants in lines 1, 5, 9 and 10 were heterozygous resistant. These information show that, susceptible or resistant, these lines have homogenous genotypes among the plants. But lines 7, 8 and 11 show a more mixed structure. In line 7 and 11, 6 of the plants are homozygous resistant and remaining are susceptible. In line 8, 4 of the samples are homozygous resistant, 2 heterozygous resistant and 2 susceptible.

According to Table 1, homozygous resistant lines which show a homogenous structure, can be selected and used in further studies to combine various resistance genes against different pathogens like nematodes, viruses and bacteria in the same thus obtaining an optimum line for cultivating.

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