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Screening of F_3 Segregation Population Lines Revealed by *Ty-1* Markers Linked to Resistance Locus of Tomato Yellow Leaf Curl Disease (TYLCD) in Tomato (*Lycopersicum. esculentum*)

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ABSTRACT

Tomato yellow leaf curl virus (TYLCV) is one of the most devastating viral pathogens of cultivated tomatoes, causes severe losses in tomato production in tropical and subtropical regions. In this study, F_3 plants originated from 11 F_2 populations (individual numbers varied from 10 to 14 for each population, a total of 131 individuals) (*Lycopersicum esculentum*) were screened for resistance to Tomato yellow leaf curl virus (TYLCV) using Random Amplified Polymorphic DNA (RAPD) and Cleaved Amplified Polymorphic Sequence (CAPS) marker techniques. After DNA extraction from plants, CAPS primers were applied and screened for primer annealing of gene locus. Out of 131 plants, 120 plants were detected containing gene locus. After that, the amplicons, obtained from PCR with CAPS primers (REX-F1 and REX-R3), were digested with *Taq*I restriction endonuclease enzyme to identify whether the lines carrying resistance gene is homozygous or heterozygous. Hundred and five plants were found to be susceptible and 15 out of 131 were heterozygous for the resistance gene. Rest of the plants did not have primer annealing sites and no homozygous resistant lines were detected.

Key Words: Disease resistance, Marker assisted selection, RAPD, CAPS, Tomato yellow leaf curl disease

INTRODUCTION

Tomato, *Lycopersicon esculentum*, is one of most important crops in the world, with an annual production of more than 115 million tons [1], and it is currently the most highly consumed vegetable in the world. Tomatoes and tomato-based products are considered as healthy foods for several reasons. They have very low in fat and calories, as well as being a good source of fibre. In addition, tomatoes are rich in carotenoids such as lycopene and β-carotene, vitamin C and other antioxidants and including total phenols [2].

Tomato yellow leaf curl disease (TYLCD) causes important yield losses in tomato (*Solanum lycopersicum*) crops all over the world [3]. Symptoms of the disease consist of a more or less prominent upward curling of leaflet margins, reduction of leaflet area and yellowing of young leaves, together with stunting and flower abortion [4]. This disease is induced by a number of begomoviruses, the type member being TYLCV, transmitted by the whitefly *Bemisia tabaci* (Gennadius), whose severe population outbreaks are usually associated with high incidence of the disease [5].

Begomoviruses are small, circular, single-stranded DNA plant viruses [6], that affects tomatoes in greenhouses and open fields, causing up to 100% crop losses in many countries [7, 8]. Control measures in infected areas usually rely on seclusion of the whitefly vector, mainly through multiple applications of insecticides or physical barriers [8, 9, 10, 11, 12]. Due to the large populations of whiteflies, and their ability to develop pesticide resistance, vector seclusion is not an ideal way of fighting the spread and damage induced by TYLCV. Hence, increasing of genetic resistance in the tomato host is the best solution for any virus problem, and especially for whitefly-transmitted viruses such as TYLCV, since it requires no chemical input and/or plant seclusion and may be stable and long lasting [7, 13, 14].

Breeding of resistant or tolerant tomatoes is one of the most promising ways to reduce TYLCV damage. Wild tomato species have been screened for their response to the virus and a number of TYLCV-resistant accessions identified, because no resistance has been found in the domesticated tomato (*Solanum lycopersicum*) [7, 14 15].

Resistance to TYLCV has been observed in several wild-type species such as *Solanum chilense, S. pimpinellifolium, S. peruvianum, S. habrochaites,* and *S. cheesmaniae* [16]. Thus, breeding programs have been based on the transfer of resistance genes from accessions of wild origin into the cultivated tomato. Progress in the breeding for TYLCV resistance has been slow, due in part to the complex genetics of the resistance and the presence of interspecific barriers between the wild and domesticated tomato species. The lack of an accurate and reliable mass inoculation and selection system has also slowed the tomato breeding programs [5].

The genetic bases of the resistance vary from a single dominant gene to a polygenic recessive pattern [17]. With the availability of PCR-based markers for the three mapped TYLCV resistance genes including *Ty-1*, *Ty-2*, and *Ty-3*, it is promising and relatively facile to bring these genes together in a single genotype to reach the maximum level of resistance [18]. DNA marker technology has been used in commercial plant breeding programs since the early 1990s and has proved helpful for the rapid and efficient transfer of useful traits into agronomically desirable varieties and hybrids [19]. Markers linked to disease resistance loci can now be used for marker-assisted selection (MAS) programs, thus also allowing several resistance genes to be accumulated in the same genotype.

Objective of this study was to screen F_3 lines with RAPD and CAPS markers controlling TYLCD locus in Tomato.

MATERIALS AND METHODS

DNA Extraction

Total genomic DNA of the F_3 plants originated from 11 F_2 populations (individual numbers varied from 10 to 14 for each population, a total of 131 individuals) was extracted from 200 mg of fresh tissue. The procedure was followed described by Doyle & Doyle [20].

Markers

All markers used in this study were PCR-based, including RAPD markers and CAPS markers. RAPD markers, UBC264 and UBC697 [21], used to amplify genomic DNA fragments of the breeding lines are listed in Table 1.

Table 1. RAPD markers.

Marker	Primer Sequence	Amplicon Size
UBC264	5'-TCC ACC GAG C-3'	750 kb
UBC697	5'-CGC AGG TCA C-3'	1165 kb

Table 2. Markers of the region of gene *Ty-1* screened

Marker	Primer Sequence	Design basis	Restriction enzyme
REX-1	REX-F1: 5'-TCGGAGCCTTGGTCTGAATT-3'	[22] Williamson et al. (1994)	*Taq*I
	REX-R3: 5'-ATGCCAGAGATGATTCGTGA-3'		

RAPD and PCR analysis

The PCR amplification reaction contained 0.8 mM dNTPs, 0,067 mM primer, 1 unit (U) of *Taq* polymerase, 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 and 1 mg/ml BSA and 40 ng template in a reaction volume of 15 µl. RAPD reactions were performed in an MJ Thermal Cycler PTC-225 (Gradient) following the procedures described previously [21]. Thermal Cycler for 45 cycles of 60 s at 94° C, 60 s at 35° C and 90 s at 72° C. The amplified products were resolved via electrophoresis on 2% agarose gels in TAE buffer and then agarose gel stained with ethidium bromide, visualized under ultraviolet (UV) light and transferred images of gels to computer using KODAK 1D imaging system.

Amplification with CAPS marker and restriction conditions

The PCR reaction was carried out in a total volume of 25 µl containing: 10x buffer recommended by suppliers, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 0.4 mM dNTPs, 1 U of *Taq* DNA polymerase and 40 ng of template DNA. The amplification was carried out in a MJ Thermal Cycler PTC-225 (Gradient) with the following conditions: 30 cycles of 94° C for 30s, 55° C for 30 s and 72° C for 1 min, followed by an extension step of 10 min at 72° C. The amplified products were resolved via electrophoresis on 2% agarose gels in TAE buffer and then agarose gel stained with ethidium bromide, visualized under ultraviolet (UV) light and transferred images of gels to computer using KODAK 1D imaging system. Restrictions of 10 µl of the amplified products were performed, in a total volume of 25 µl with 5 U of the *Taq*I (New England BioLabs, Inc.) enzyme, using NEBuffer 4 recommended by the suppliers at 65° C for 1 h and enzyme inactivation accomplished at 80° C for 20 min. Digestion products were analyzed by agarose gel electrophoresis (2% agarose w/v with TAE buffer) and stained with ethidium bromide, visualized under ultraviolet (UV) light and transferred images of gels to computer using KODAK 1D imaging system.

RESULTS

Screening the F_3 lines for RAPD Markers

Two RAPD markers amplified by random oligomer primers UBC264 and UBC697 were previously shown to be associated with a begomovirus resistance locus [21]. The PCR fragment sizes for the markers UBC264 and UBC697 were ca. 750 kb and 1165 bp respectively. Figure 1 shows the fragments generated after the amplification of the genomic DNA of the tomato plants using the UBC264 marker. Primer UBC264 were applied and screened for primer annealing of gene locus. Out of 131 plants, 30 plants were detected containing gene locus.

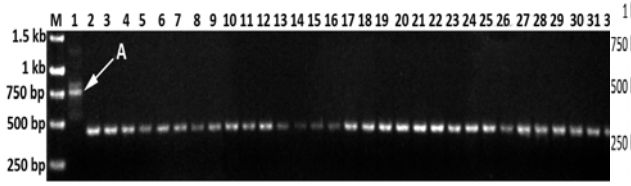

Figure 1. RAPD marker UBC264 tested on tomato plants (1-35). M: 1 kb DNA ladder, Letter "A" represent the band linked to the resistance (750 bp).

Figure 2 shows the fragments generated after the amplification of the genomic DNA of the tomato plants using the UBC697 marker. Out of 131 plants, 120 plants were detected containing gene locus.

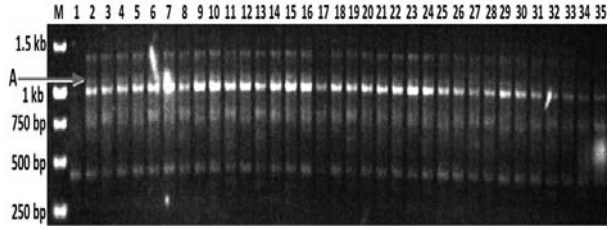

Figure 2. RAPD marker UBC697 tested on tomato plants (1-35). M: 1 kb DNA ladder, Letter "A" represent the band linked to the resistance (1165 bp).

Screening the F_3 lines for CAPS Markers

PCR amplification of 131 tomato plants DNA and subsequent digestions, when possible, were carried out using the primers and enzymes listed in Table 2. Clear amplification products were obtained for markers tested.

In this study, out of 131 plants, 120 plants were detected containing gene locus.

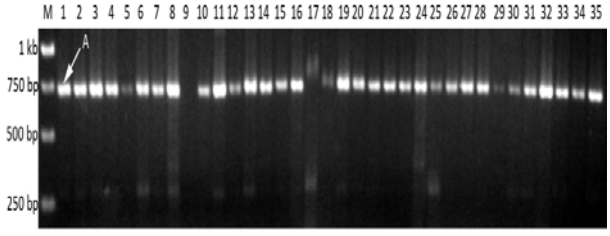

Figure 3. CAPS marker tested on tomato plants (1-35). M: 1 kb DNA ladder, Letter "A" represent the marker linked to the resistance (750 bp).

After that, the amplicons, obtained from PCR with CAPS primers (REX-F1 and REX-R3), were digested with *Taq*I restriction endonuclease enzyme to identify whether the plants carrying resistance gene is as homozygous or heterozygous.

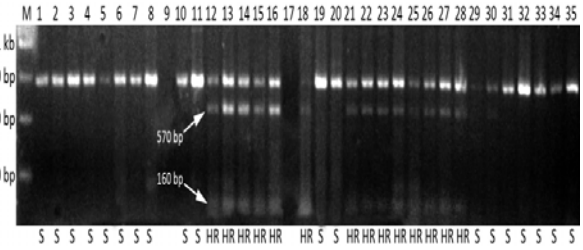

Figure 4. *Taq*I digestion of the PCR fragments. S: susceptible, HR: Heterozygous resistance

Hundred and five plants were found to be susceptible and 15 out of 131 were heterozygous for the resistance gene. In the heterozygous there were three fragments (750 bp, 570 bp and 160 bp) obtained. For the homozygous resistant plant there were two characteristic fragments (570 bp and 160 bp). Rest of the plants did not have primer annealing sites and no homozygous resistant plants were found.

In this study F_3 segregation populations including 131 individuals were screened for TYLCV. All of the plants in lines D1, H1 and M1 were susceptible. We found that none of the lines were homozygous resistant. In lines A1, C1, and E1 most of the plants were susceptible while the remaining were heterozygous resistant. In line B1, 7 of the samples are heterozygous resistant 2 of them are susceptible and 1 plant does not have primer annealing sites.

Table 3. Scoring results for 131 samples.

Code	Line	UBC264[a]	UBC697[b]	REX-[c]	*Taq*I	Code	Line	UBC264[a]	UBC697[b]	REX-[c]	*Taq*I
1	A1-1	+	-	+	S	34	C1-11	-	+	+	S
2	A1-2	-	+	+	S	35	D1-1	-	+	+	S
3	A1-3	-	+	+	S	36	D1-2	-	+	+	S
4	A1-4	-	+	+	S	37	D1-3	-	+	+	S
5	A1-5	-	+	+	S	38	D1-4	-	+	+	S
6	A1-6	-	+	+	S	39	D1-5	-	+	+	S
7	A1-7	-	+	+	S	40	D1-6	-	+	+	S
8	A1-8	-	+	+	S	41	D1-7	+	+	+	S
9	A1-9	-	+	-	-	42	D1-8	+	+	+	S
10	A1-10	-	+	+	S	43	D1-9	+	+	+	S
11	A1-11	-	+	+	S	44	D1-10	+	+	+	S
12	A1-12	-	+	+	HR	45	D1-11	+	+	+	S
13	A1-13	-	+	+	HR	46	E1-1	+	+	+	S
14	B1-1	-	+	+	HR	47	E1-2	+	-	+	S
15	B1-2	-	+	+	HR	48	E1-3	+	-	+	S
16	B1-3	-	+	+	HR	49	E1-4	+	-	+	S
17	B1-4	-	+	-	-	50	E1-5	+	-	+	S
18	B1-5	-	+	+	HR	51	E1-6	+	+	+	S
19	B1-6	-	+	+	S	52	E1-7	+	+	+	S
20	B1-7	-	+	+	S	53	E1-8	+	+	+	HR
21	B1-8	-	+	+	HR	54	E1-9	+	+	+	S
22	B1-9	-	+	+	HR	55	E1-10	-	-	-	-
23	B1-10	-	+	+	HR	56	E1-11	+	-	-	-
24	C1-1	-	+	+	HR	57	E1-12	-	+	+	S
25	C1-2	-	+	+	HR	58	E1-13	-	+	+	S
26	C1-3	-	+	+	HR	59	E1-14	-	+	+	S
27	C1-4	-	+	+	HR	60	F1-1	-	+	+	S
28	C1-5	-	+	+	HR	61	F1-2	-	+	+	S
29	C1-6	-	+	+	S	62	F1-3	-	+	+	S
30	C1-7	-	+	+	S	63	F1-4	-	+	+	S
31	C1-8	-	+	+	S	64	F1-5	-	+	+	S
32	C1-9	-	+	+	S	65	F1-6	-	+	-	-
33	C1-10	-	+	+	S	66	F1-7	-	+	-	-

Table 3 continued.

Code	Line	UBC264[a]	UBC697[b]	REX-[c]	*TaqI*	Code	Line	UBC264[a]	UBC697[b]	REX-[c]	*TaqI*
67	F1-8	-	+	+	S	100	K1-1	+	+	+	S
68	F1-9	-	+	+	S	101	K1-2	+	+	+	S
69	F1-10	-	+	+	S	102	K1-3	+	+	+	S
70	F1-11	-	+	+	S	103	K1-4	+	+	+	S
71	F1-12	-		+	S	104	K1-5	+	-	+	S
72	G1-1	-	+	-	-	105	K1-6	+	+	+	S
73	G1-2	-	+	+	S	106	K1-7	+	+	+	S
74	G1-3	-	+	+	S	107	K1-8	+	+	+	S
75	G1-4	-	+	+	S	108	K1-9	+	+	+	S
76	G1-5	-	+	+	S	109	K1-10	-	+	-	-
77	G1-6	-	+	+	S	110	L1-1	+	+	+	S
78	G1-7	-	+	+	S	111	L1-2	-	+	+	S
79	G1-8	-	+	+	S	112	L1-3	-	+	+	S
80	G1-9	-	+	+	S	113	L1-4	-	+	+	S
81	G1-10	-	+	-	-	114	L1-5	-	+	-	-
82	G1-11	-	+	+	S	115	L1-6	-	+	-	-
83	G1-12	-	+	+	S	116	L1-7	-	+	+	S
84	G1-13	-	+	+	S	117	L1-8	-	+	+	S
85	G1-14	+	+	+	S	188	L1-9	-	+	+	S
86	H1-1	-	+	+	S	199	L1-10	-	+	+	S
87	H1-2	-	+	+	S	120	L1-11	-	+	+	S
88	H1-3	-	+	+	S	121	M1-1	-	+	+	S
89	H1-4	-	+	+	S	122	M1-2	-	+	+	S
90	H1-5	-	+	+	S	123	M1-3	-	+	+	S
91	H1-6	-	+	+	S	124	M1-4	-	+	+	S
92	H1-7	-	+	+	S	125	M1-5	-	+	+	S
93	H1-8	-	+	+	S	126	M1-6	-	+	+	S
94	H1-9	-	+	+	S	127	M1-7	-	+	+	S
95	H1-10	-	+	+	S	128	M1-8	-	+	+	S
96	H1-11	-	-	+	S	129	M1-9	-	+	+	S
97	H1-12	+	+	+	S	130	M1-10	-	+	+	S
98	H1-13	+	+	+	S	131	M1-11	-	+	+	S
99	H1-14	+		+	S						

[a]Positive (+): Samples which revealed 750 bp marker.
HR denotes a heterozygous resistant plant.

[b]Positive (+): Samples which revealed 1165 bp marker.
S denotes a susceptible plant..

[c]Positive (+): Samples which revealed 750 bp marker.

DISCUSSION

Tomato is one of the world's largest vegetable crops and known as protective food both because of its important nutritive value and also because of its wide spread production. The principal limitation to tomato production is damage caused by fungi, nematode, bacteria and virus. TYLCV infection provokes rigorous yield losses via flower drop, reduction of leaflet area, yellowing of young leaves and impedes fruit set in the tomato crop. If the infection occurs at the early stage of the growth, yield loss rises up to 100%.

The availability of PCR-based markers for many resistance genes allows the MAS for begomovirus resistance in tomato to be successfully applied without the need for highly sophisticated techniques. In addition, the rapid development of new molecular techniques, combined with the increasing knowledge on structure and function of resistance genes [23]. Indeed, once a marker has been set up, its use on large populations for resistance screening is then routine. Technical facilities are today available for screening many samples simultaneously and also costs for equipment are decreasing.

In addition, the rapid development of new molecular techniques, combined with the ever-increasing knowledge about the structure and function of resistance genes [23], will help to identify new molecular markers for MAS.

The RAPD method, which was applied with UBC264 and UCB697, was used to screen the tomato lines for resistance to TYLCV. Ji et al. [21] reported that UBC697, which is closely located near *Ty-1* gene, and UBC264 markers showed tight linkage to the TYLCV resistant genes. They stated RAPD markers UBC697 and UBC264 can be used in plant breeding selection for TYLCV and ToMoV resistance. In this study the 750 bp fragment (Figure 1) was obtained in 30 out of 131 F_3 plants derived from 11 F_2 populations using UBC264 marker. Amplification with UBC697 marker revealed 1165 bp fragment for 120 out of the 131 plants.

The CAPS method, applied with REX-1 marker, was used to determine genotypic constitution of *Ty-1* gene in 131 F_3 plants originated from 11 F_2 populations (individual numbers varied from 10 to 14 for each population, a total of 131 individuals). The CAPS method can be recommended for fast and precise determination of resistance or susceptibility of tomato plants against the TYLCV. The goal of the method was the possibility of detection of genotypic constitution of *Ty-1* gene that can significantly speed up the process of creation of new resistant varieties. For the REX-1 marker, three different alleles appeared in *S. lycopersicum* plant material [24]. Two of these alleles were previously described by Williamson et al. [22]. Allele 1 consisted of a band of 750 bp and allele 2 of two bands of approximately 570 and 160 bp. These two alleles were co-dominant. Castro et al. [24] found one more allele, allele 3, which presented three bands of 350, 220 and 160 bp. In this study 107 plants were found to be susceptible and 15 out of 131 were heterozygous for the resistance gene. Rest of the plants did not have primer annealing sites and no homozygous resistant plants were found. Allele 2, which is present in *S. chilense* LA1969, the source of this gene, is not frequently introgressed along with *Ty-1*. Therefore, REX-1 marker and *Ty-1* are not tightly linked to TYLCD. In other words, marker REX-1 is not useful in marker-assisted selection for *Ty-1* [24]. Furthermore, it has been reported that begomovirus-resistant lines derived from *S. habrochaites* that are susceptible to M. incognita give false positive results for the REX-1 marker [24].

It should be noted that failure of the enzyme *TaqI* to cleave the amplicon from the plants derived allele of REX-1 would result in incorrectly scoring a homozygous resistant plant as susceptible.

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