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Screening the Tomato (*Lycopersicum esculentum Mill.*) Lines for the Resistance to Root-knot Nematodes and Verticillium Wilt by DNA Markers

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Abstract

In this study, it was aimed to determine the resistance to root-knot nematodes and Verticillium wilt of 288 tomato (Lycopersicum esculentum Mill.) samples by DNA markers. First, it was established that whether the tomato samples have primer binding site or not by the CAPS primers which are specifically designed for both of the diseases. In the analysis of primer binding site to root-knot nematodes and Verticillium wilt diseases, it is found that in the 288 tomato samples, 247 and 235 tomato samples have specific primer binding site respectively. Then, restriction enzymes (TaqI and HincII) were used to understand whether the individuals were homozygote or heterozygote resistant, or susceptible to these diseases for determination of resistance to root-knot nematodes and Verticillium wilt. It is found that 200 samples were susceptible, 45 samples were heterozygote resistant and 2 samples were homozygote resistant to root-knot nematodes, and 125 samples were susceptible, 110 samples were heterozygote to Verticillium wilt.

Key words: Tomato, root-knot nematode, Verticillium wilt, disease resistance, DNA markers

INTRODUCTION

Tomato (Lycopersicum esculentum Mill.) is considered as one of the most widely grown vegetable crop in the world [1] and constitutes a major agricultural industry. Worldwide, it is the second most consumed vegetable after potato and unquestionably the most popular garden crop. Major tomato producing countries in descending orders include China, USA, India, Turkey, Egypt, and Italy [2].

One of main constraint of tomato cultivation is damage caused by pathogens, including viruses, bacteria, nematodes, fungi, which cause sever losses in production. In order to realize a sustainable agriculture and to get high quality products in terms of health safe, the use of resistant varieties becomes a principal tool to reduce damages caused by pathogens [1]. For tomato, the genetic control of pathogens is a very useful practice and most of used resistance is monogenic and dominant [3].

Soil-borne pathogens belonging to the Verticillium genus, such as V. dahliae and V. albo-atrum jeopardize agricultural production worldwide. In fact, a severe infection of Verticillium spp. may dramatically decrease the yield and quality of many important cultivated crops [4]. Often no symptoms are seen until the plant is bearing heavily or a dry period occurs. The bottom leaves become pale, then tips and edges die and leaves finally die and drop off. V-shaped lesions at leaf tips are typical of Verticillium wilt of tomato [5].

Verticillium wilt resistance is conferred by the Ve gene. Kawchuk et al. (2001) found that two V. dahliae resistance genes, Ve1 and Ve2, independently confer resistance to the same pathogen [3]. Two closely linked genes (Ve1 and Ve2) are encoded within the locus on the short arm of chromosome 9 and are likely the result of a recent duplication event [6]. Both Ve genes encode proteins with extracellular leucine-rich repeats and intracellular motifs that indicate a role in receptor-mediated endocytosis and protein-protein interactions [7].

Genus Meloidogyne, especially Meloidogyne incognita Kofoit and White, is in an economical view an important pathogen, and is considered as the most dangerous pest of in greenhouse-grown plants. M. incognita is spread out in all five live continents and makes large economical losses in many countries. Quantity of over- and underground part of tomato plants is smaller in consequence of attack by the pest. Females of M. incognita make on the roots of host plants 4–5 cm galls that form on the base of fusion of few smaller galls [8].

Species Lycopersicon peruvianum L. is considered as the donor of Mi resistance gene against Meloidogyne incognita. Later other Mi genes were identified (Mi1, Mi2, Mi3) [8, 9]. Gene Mi1 has been exploited extensively in the last two decades for modern tomato

cultivar development. Mi1 confers resistance to three species of root-knot nematodes, Meloidogyne arenaria, Meloidogyne incognita and Meloidogyne javanica at < 280 C [10, 11, 12, 13], as well as to the potato aphid (Macrosiphum euphorbiae)[14]. Genetic and physical mapping localized Mi1 in the introgressed region on the short arm of chromosome 6 [9, 12, 15].

DNA marker technology has been used in commercial plant breeding programs since the early 1990s, and has proved helpful for rapid and efficient transfer of useful traits into agronomically desirable varieties and hybrids. Markers linked to disease resistance loci can now be used for marker-assisted selection programs [1]. Tomato is very rich in the number of available molecular markers. Currently, there are >1000 RFLP markers, most of which have been mapped onto the 12 tomato chromosomes, and 214000 ESTs of which only a small portion has been mapped onto tomato chromosomes. In addition to RFLPs and ESTs, several other molecular marker types, including SSRs, CAPS, RAPDs, SCARs, RGAs, and AFLPs have been developed and mapped in tomato. Recently, the development and use of PCR-based markers have increased in tomato as these markers are generally more user friendly, cheaper, faster, and less labor intensive to develop compared with conventional DNA markers such as RFLPs and AFLPs [2].

In this research, our aim was to screen the resistance to Verticillium wilt and root knot nematode infection, which highly effect tomato yield, by CAPS DNA marker techniques that provide us to understand genotypic structure and thus it is realized rapid and precise selection of resistant tomato lines.

MATERIALS AND METHODS

Plant material and DNA extraction

288 F3 tomato samples originated from 30 F2 generations (8-10 individuals from each F2). Total genomic DNA was extracted from 200 mg of fresh tissue according to the procedure described by Doyle & Doyle [16]. Isolated DNA was diluted in 100 µl of sterile, distilled water and kept at -800 C.

CAPS marker analysis

To determine whether the samples have genes related to the resistance to Verticillium wilt, according to Acciarri et al (2007) [3], CAPS primers V2Le03F (CAAACATAGCTGGAAGAATC) and V2Le03R (TAGGAGGAAAAGAATTGG) were used. PCR conditions were set as follow for Verticillium wilt resistance analysis: 4 min at 940 C as initial denaturation step, 45 s at 940 C, 45 s at 470 C, 120 s at 720 C (35 times), 180 s at 720 C, last cycle was at 40 C for unlimited time by using an MJ THERMAL CYCLER PTC-225 (Gradient).

PCR amplifications for CAPS were performed in a volume of 15 µl containing 20 ng of genomic DNA, 1.5 µl of 10 X buffer (50 mM of KCl, 10 mM of Tris-Cl at pH 8.3, 0.001 % gelatin), 1.5 mM MgCl2, 0.2 mM each dNTPs, 10 pM forward primer, 10 pM reverse primer and 1 unit of Taq DNA polymerase enzyme (MBI Fermentas).

To determine whether the samples have genes related to the resistance to Meloidogyne incognita, according to Williamson et al (1994) [17], CAPS primers REX-F1 (5'-TCGGAGCCTTGGTCTGAATT-3') and REX-R2 (5'- GCCAGAGATGATTCGTGAGA-3') were used. PCR conditions were set as follow for Meloidogyne incognita resistance analysis: 180 s at 940 C as initial denaturation step, 60 s at 940 C, 120 s at 550 C, 120 s at 720 C (30 times), 480 s at 720 C, last cycle was at 40 C for unlimited time. PCR amplifications for CAPS were performed same as Verticillium wilt resistance analysis.

CAPS amplification products were resolved in a 2% agarose gel (in 1xTAE) and visualized by staining with EtBr. The lambda DNA (digested EcoRI/HindIII) size marker was used to estimate amplification products. KODAK 1D gel imaging system was used to transfer the gel image into computer.

Restriction enzyme digestion analysis

The amplified tomato sample DNA's by CAPS primers were digested with HincII restriction endonuclease enzyme for Verticillium wilt resistance analysis, and TaqI restriction endonuclease enzyme for Meloidogyne incognita resistance analysis. Restriction enzyme digestion analysis for Verticillium wilt resistance were performed in a 25 µl volume containing of 15 µl of amplified DNA from CAPS amplification, 2,5 µl 10x Buffer for HincII, 2 U HincII and 100µg/ml Bovine Serum Albumin.

Restriction enzyme digestion analysis for Meloidogyne incognita resistance were performed in a volume 20 µl containing of 15 µl of amplified DNA from CAPS amplification, 2 µl 10x Buffer for TaqI, 5 U TaqI. After digestion, for both restriction enzyme analyses, digestion fragments were resolved in a 2% agarose gel and visualized same as CAPS amplification products.

RESULTS

CAPS Marker Analysis

The tomato DNA's which were amplified with REX-F1 and REX-R2 CAPS markers for root knot nematode resistance analysis and with V2Le03F and V2Le03R for Verticillium wilt resistance analysis were separated with agarose gel electrophoresis and they were analyzed whether if they have primer binding site or not.

The DNA's which have primer binding site for root-knot nematode resistance analysis were observed with a 750 kb single band whereas the DNA's which have primer binding site for Verticillium wilt were observed with a 1029 kb single band.

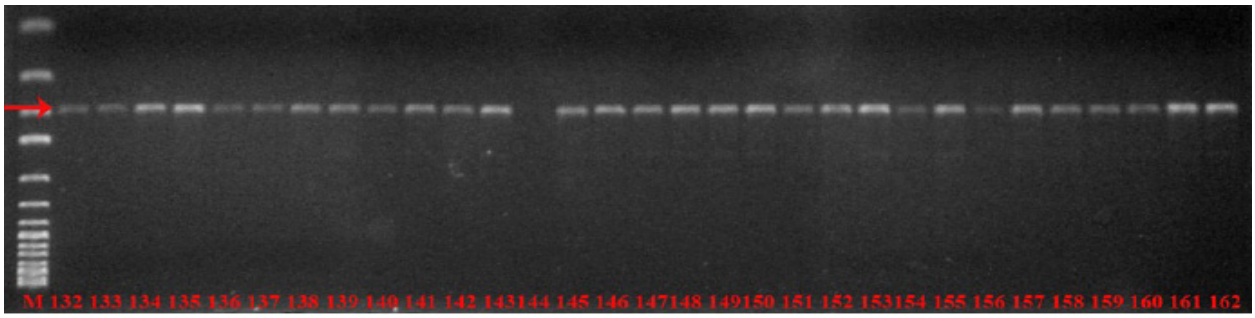

Figure 1. DNA's which have primer binding site for root knot nematode analysis.

In the analysis of primer binding site to root-knot nematodes and Verticillium wilt diseases, it is found that in the 288 tomato samples, 247 (85,8% of all) and 235 (81,6% of all) tomato samples have specific primer binding site, respectively.

Restriction enzyme digestion analysis

After primer binding site analysis, the samples which were observed with a single band for both resistance analyses by agarose gel electrophoresis were digested with restriction endonuclease enzymes. For root-knot nematode and Verticillium wilt resistance analysis, the DNA's were digested with TaqI and HincII restriction enzymes, respectively.

The samples, which were analyzed for root-knot nematode resistance analysis, were digested with TaqI restriction enzyme and they were analyzed if the gene region is heterozygote resistant, homozygote resistant or susceptible. The DNA's were digested with HincII restriction enzyme and analyzed for Verticillium wilt resistance. After digestion of the DNA's, digested DNA samples were separated with agarose gel electrophoresis and analyzed if they are susceptible or resistant to the diseases.

In the root-knot nematode resistance analysis, the susceptible DNA samples were observed with an undigested single 750 kb band, the heterozygote resistant DNA samples were observed bands with 750, 570, 160 kb in length and also the homozygote resistant DNA's were observed bands with 570 and 160 kb in length (Table 1). As a result of the analysis of root-knot nematode resistance, it was found that in the 288 tomato samples, homozygote resistant sample score was 2 (0,69% of all), heterozygote resistant sample score was 45 (15,6% of all) and susceptible sample score was 200(69,4% of all). It was observed that 45 samples had no primer binding site and they were 14,24% of all samples.

In the Verticillium wilt resistance analysis, the susceptible DNA samples were observed with an undigested single 1029 kb band, the heterozygote resistant DNA samples were observed with 1029, 601, 428 kb in length digested three bands and also the homozygote DNA samples were expected to observe with 601 and 428 kb in length digested two bands. It is found that in the 288 tomato samples, none of the samples were homozygote resistant. The heterozygote resistant sample score was found 110 (38,2% of all) and susceptible sample score was found 125 (43,4% of all).

Table 1. Dominancy and expected band pattern after TaqI digestion for root knot nematode resistance analysis.

Genotype	Primer Combination	Expected Band Pattern after *Taq*I Digestion
MiMi	REX-F1 & REX-R2	2 bands (570, 160 kb)
mimi	REX-F1 & REX-R2	1 band (750 kb)
Mimi	REX-F1 & REX-R2	3 bands (750, 570, 160 kb)

Table 2. Dominancy and expected band pattern after HincII digestion for Verticillium wilt resistance analysis.

Genotype	Primer Combination	Expected Band Pattern after *Hinc*II Digestion
Ve2 / Ve2	V2Le03F & V2Le03R	2 bands (601 kb, 428 kb)
ve2 / ve2	V2Le03F & V2Le03R	1 band (1029 kb)
Ve2 / ve2	V2Le03F & V2Le03R	3 bands (1029, 601, 428 kb)

It was observed that 53 samples had no primer binding site and they were 18,4% of all samples.

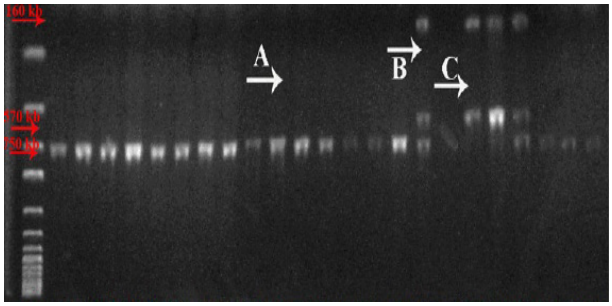

Figure 2. The analyses of root-knot nematode resistance on agarose gel. In the gel, sample number 223 which is shown as A is susceptible, sample number 232 which is shown as B is heterozygote resistant, sample number 238 sample which is shown as C is homozygote resistant.

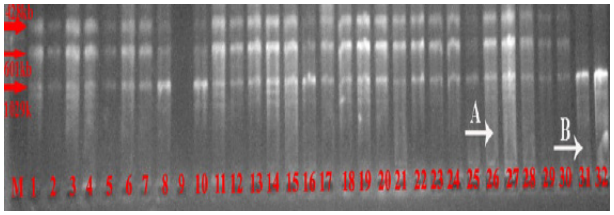

Figure 3. The analyses of Verticillium wilt resistance. In the gel, sample number 26 which is shown as A is heterozygote resistant, sample number 31 which is shown as B is susceptible.

DISCUSSION

The cultivated tomato, Lycopersicon esculentum Mill., a fruit that is often consumed as a vegetable, is widely grown around the world and constitutes a major agricultural industry [2]. Tomato is susceptible to over 200 diseases caused by pathogenic fungi, bacteria, viruses, or nematodes [1]. Verticillium wilt and root-knot nematodes are one of the most important pathogens which cause sever losses in production. The greatest contribution of modern plant breeding to tomato improvement has been through development of cultivars with improved disease resistance [2]. The control of pathogen spread mainly involves three strategies, which are husbandry techniques, application of agrochemicals, use of resistant varieties. Although conventional plant breeding had a significant impact on improving tomato breeding for resistance to important diseases, the time-consuming process of making crosses and backcrosses, and the selection of the desired resistant progeny make it difficult to react adequately to the evolution of new virulent pathogens [1]. Because of that, 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 [1, 18]. Markers tightly linked to resistance genes can greatly aid disease resistance programs, by allowing to follow the gene under selection through generations rather than waiting for phenotypic expression of the resistance gene. And also several resistance genes could be cumulated in the same genotype by pyramiding resistance genes in marker-assisted selection programs [1].

In this study, in order to determine whether the samples have genes related to the resistance to Verticillium wilt and Meloidogyne incognita, CAPS primers V2Le03F, V2Le03R and REX-F1, REX-R2 were used, respectively and the DNA's which have primer binding site for root-knot nematode resistance analysis were observed with a 750 kb single band whereas the DNA's which have primer binding site for Verticillium wilt were observed with a 1029 kb single band in the agarose gel electrophoresis. Then, for determination of resistance to root-knot nematodes and Verticillium wilt, restriction enzymes, TaqI and HincII, respectively, were used to understand whether the samples were homozygote or heterozygote resistant, or susceptible to these diseases.

As a result of the restriction enzyme digestion, it is found that the susceptible DNA samples were separated as an undigested single 750 kb band, the heterozygote resistant DNA samples were separated as 750, 570, 160 kb in length digested three bands and also the homozygote resistant DNA's were separated as 570 and 160 kb in length digested two bands in root-knot nematode resistance analysis in agarose gel electrophoresis. Like root-knot nematode resistance analysis, the DNA's were applied to agarose gel electrophoresis in order to determine the dominancy for Verticillium wilt resistance. It was observed that the susceptible DNA samples were separated as an undigested single 1029 kb band, the heterozygote resistant DNA samples were separated as 1029, 601, 428 kb in length digested three bands and also the homozygote DNA samples were expected to observe with 601 and 428 kb in length digested two bands. Acciarri et al (2007) [3] also found similar results in Verticillium wilt resistance analysis of their tomato lines. They observed two digested bands, 428 kb and 601 kb in length, for homozygote resistant tomato DNA's, an undigested band, 1029 kb in length, for homozygote susceptible tomato DNA's and also undigested band, 1029 kb in length, and two digested bands, 428 and 601 kb in length, were observed for heterozygote resistant tomato DNA's.

The CAPS markers, that we used according to Williamson et al (1994) [17], have been also used by many researchers for many years in tomato root knot nematode resistance analysis [8, 12, 17, 19, 20]. They also found similar results as REX-1 amplified product (750 kb) digested with TaqI from homozygote resistant plants resulted in two bands of approximate 570 kb and 160 kb in length. Heterozygote resistant plants were shown three bands of approximate 750, 570 and 160 kb in length, and susceptible plants were shown only one band of 750 kb

in length. Also Devran et al (2004) were used different Mi gene specific primers (1/2 and C2S4 primers) in their root knot nematode resistance analysis of their tomato lines. With no enzymatic digestion, they found that resistant plants and susceptible plants were distinguished from each other whereas resistant heterozygote and homozygote individuals were not distinguishable.

The main purpose of this study was to determine whether if the tomato lines have homogeneity inside themselves and to minimize the fault ratio in crossbreeding by early determination of susceptible and resistant individuals. By this way, it will be reduced pesticide utilization for pathogen control against the disease factors and it will be achieved maximum healthy individuals in minimum area. And also the expenses like labor, cost and water consumption during breeding program will be became more inefficient in production. More importantly, the usage of DNA markers specific to the diseases make shortened the breeding time. When it is considered these advantages, it is thought that the usage of DNA markers will be of great importance to breed healthy individuals now and in future.

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