Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 129-134
DOI: 10.29136/mediterranean.1101666

Molecular analysis of resistance gene locus to bacterial canker and wilting disease in tomato mutants

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ARTICLE INFO

Received: April 11, 2022
Received in revised form: May 13, 2022
Accepted: July 1, 2022

***Keywords*:**

Polymerase Chain Reaction
SSR markers
Maping
Clavibacter michiganensis subsp. *michiganensis*

ABSTRACT

The tomato plant is one of the most widely produced vegetables in the world. However, there are several disease factors which limit tomato production. The *Clavibacter michiganensis* subsp. *michiganensis (Cmm)* pathogen causes wilting and canker symptoms on the leaves of plants and bird's eye symptoms on the tomato and then the plants completely die. Development of resistant tomato varieties is prerequisite due to absence of an effective control methods against the bacterial disease. The resistant M3-9 and M3-15 tomato plants have been developed because of mutation from susceptible NCEBR3 tomato seeds with ethyl methanosulfonate (EMS). For each chromosome of the tomato genome, 24 SSR markers were selected from each end of the haploid, 12 tomato chromosomes, and polymorphic differences between susceptible and resistant tomato plants were studied. Polymorphisms were found with SSR13 and SSRB18031 markers located on chromosome 5 with resistant mutants, M3-9, M3-15 and susceptible original NCEBR3 plants. It is envisaged that a resistance gene is located on the 5th chromosome of resistant M3-9 and M3-15 plants. Further fine mapping studies will reveal the location of the resistance gene(s) for controlling bacterial canker and wilting pathogen.

1. Introduction

Tomatoes are among the most important vegetables for agricultural production worldwide. According to data from the United Nations Food and Agriculture Organization, 180766329 tons of tomatoes were produced on 5030545 hectares worldwide in 2019. Turkey ranks third in the world with a tomato production of 12841990 tons in an area of 181488 ha (FAO 2021), whereas 13204015 tons of tomato production were recorded in 2020 according to the Turkish Statistical Institute (TÜİK 2021).

Among plant pathogenic bacteria, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) causes complete death of tomato plants by showing severe symptoms of canker and wilting in plants. On young plants, when an infection is caused by an inoculum directly placed on the vascular bundle tissue, systemic infection manifests itself with wilting symptoms. In older plants, wilting symptoms appear more slowly and gradually than in young plants. Even though this disease agent has such a high importance, there is no effective cultural, biological or chemical control management. In a fight against the disease, chemical applications protect the plants, but cause negative effects on environment and human health. Considering all these factors, the most important issue would be a permanent and effective controlling of the bacterial pathogen, this is possible with the development of resistant plant varieties (Agrios 2005; Çalış et al. 2013).

The bacterial pathogen *Cmm*, likely other plant pathogens must test for their host's suitability to multiply in the host; if a receptor protein encoded by *resistance* (*R*) gene recognizes elicitor protein encoded from *avirulence* gene of pathogen during interactions between the host and the pathogen are resulting resistance. When the resistance gene recognizes pathogens, it activates defense mechanisms and controls the pathogen (Rathjen and Mofefett 2003; Meyers et al. 2005). In these relationships, called gene-for-gene theory, resistance arises when the *R* genes of the host plant against the *avirulence* genes of the pathogen are in harmony with each other because the plant understands the presence of the pathogen (Lahaye 2004). This resistance mechanism in plants is called systemic immunity or systemic acquired resistance (Wiermer et al. 2005; Grant and Lamb 2006).

There is no reliable resistance gene to bacterial wilt and canker pathogen. Therefore, the study aims to map resistant gene using promising resistant M3-9 and M3-15 plants which were obtained from chemical treated susceptible NCEBR3 tomato plants and to determine polymorphic markers on these plants.

2. Material and methods

2.1. Supply of material

In the study, NCEBR3 tomato line seeds were obtained from Prof Dr. Randy GARDNER at Mountain Horticultural Crops Research and Extension Center, North Caroline State University, USA. The NCEBR3 line is known as North Caroline Early Blight Resistant 3 and is a semi-dwarf table tomato genotype (Nash and Gardner 1988). In previous studies, mutation of NCEBR3 pure line tomato seeds with a single-base mutagen ethyl

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metanosulfonate (EMS, Sigma, Germany) and mutation in the nucleotide sequences on the plant genome resulted in the conversion of cytosine (C) / guanine (G) to thymine (T) / adenine (A). By transforming into (A) nucleotides, 15 M2 mutant plants out of 450 M2 tomato mutant plants were found to be resistant to the disease agent *Cmm2*. Seeds of M3 populations were produced from these plants and plants in the M3-9 and M3-15 mutant families did not show any susceptibility against the *Cmm2* pathogen by pathogenicity tests (Çalış et al. 2013). The seeds of promising resistant mutants M3-9 and M3-15, which were obtained by mutating with EMS chemical mutagen, were obtained from the seed collection in Tokat Gaziosmanpasa University Faculty of Agriculture Plant Protection Department Phytopathology Laboratory.

2.2. Cultivation of plants

The tomato seeds used in the study were sown in vials containing sterile peat in the Tokat Gaziosmanpasa University Faculty of Agriculture Biotechnology greenhouse at 16 hours daytime and 8 hours night length, 23±5ºC temperature and 60% relative humidity conditions. Tomato seeds planted in vials were irrigated at regular intervals to ensure their germination. When the plants reached the stage with 2-3 true leaves, they were planted in 20 cm diameter and 30 cm deep pots containing a mixture prepared by autoclaving 1:1:1:1 ratio of peat: perlite: soil and animal manure at 121ºC for 15 minutes and left to develop in a greenhouse environment.

2.3. Growth of the pathogen in GYCA media and inoculation into test plants

Clavibacter michiganensis subsp. *michiganensis* isolate *2* (*Cmm2*) disease agent with high virulence, was obtained from Professor Hüseyin BASIM (Faculty of Agriculture, Akdeniz University). The pathogen was subcultured on Glucose Yeast Carbonate Agar (GYCA) distilled water was added to the mixture, formed with 2 grams (g) glucose, 5 g peptone, 5 g yeast extract, 40 g calcium carbonate, until it reached 1 liter (L) and the pH of the mixture was brought to 7.2 with either KOH or HCl. Then, 15 g of Agar (Merck, Germany) was added to the mixture and the medium was prepared by autoclaving at 121ºC at 1 atmosphere (atm) pressure for 15 minutes (Oxoid 2013). *Cmm2* bacterial isolates were taken from the stock solutions with the help of a loop, and drawings were made on glass Petri dishes containing GYCA, prepared in sterile conditions. The Petri dishes were wrapped with cling film and kept in an incubator at 28ºC for 3 days, to ensure the growth of bacteria. Inoculation was performed on the plant stems using sterile toothpicks.

2.4. Molecular markers used

In the study, the chromosome number of the tomato plant was considered in the selection of SSR markers in order to reveal the polymorphism. The tomato plants are diploid species but a haploid set of 12 chromosomes were considered with 24 SSR markers, one for each chromosome see Table 1, which are specialized for the beginning and end of each chromosome. The SSR markers were selected from the markers and maps in the Solanacea Genomics Network and used in our study (Solgenomics 2013).

2.5. DNA isolation from plants

A total of 27 leaf samples were taken from 9 plants each from the promising resistant M3-9 and M3-15 mutants and the original susceptible EBR3 tomato plants at 4-5 true leaf stage. The pellet obtained using the Fermentas DNA isolation kit was dissolved in 100 µL of 1× Tris-EDTA buffer (TE) and stored at -80°C (Fermentas 2014). 1×Tris-EDTA prepared with Tris-HCl (pH:8) 12.1 gL^{-1}, $Na_2EDTA2H_2O$ (pH:8) 3.7 gL^{-1} (Sambrook et al. 1989).

2.6. Classical PCR analyzes

PCR was performed with the modification of the protocol specified by Sambrook et al. (1989) dsH_2O 11.4 µL, $MgCl_2$ 4 µL, 10X Taq Buffer 10 µL, dNTP Mixture 4 µL, Primer Forward 4 µL, Primer Reverse 4 µL, Taq Polimerase 0.6 µL and by adding 2 µL of DNA, total of 40 µL of the PCR mixture was prepared. A Peqlab Pirimus96 (Germany) thermal cycler device was used in the study. Denaturation at 94ºC for 5 minute, 35 cycles at 94ºC, Denaturation at 94ºC for 1 minute, annealing variable with primer for 1 minute, Extension at 72ºC for 1.5 minute, Final Extension at 72ºC for 10 minute and Store 8ºC program was used (Çalış and Topkaya 2011).

2.7. Agarose gel preparation and Gel Imaging

As a result of PCR amplifications, 1% or 2% agarose gels were prepared according to the product size. For preparation 30 mL gel: Agarose 0.37 g, 5XTBE 7.4 mL, dsH_2O 29.6 mL, Buffer (1%): 5XTBE 60 mL, dsH_2O 240 mL and Ethidium Bromide 4 µL were used. 5X Tris/Borate/EDTA (TBE) buffer was prepared by adding 20 mL^{-1} of Tris-Base (pH:8) 54 gL^{-1}, Boric Acid 27.5 gL^{-1} and 0.5 M EDTA (pH:8) used in gel preparation (Sambrook et al. 1989). The PCR products prepared with 5 µL of DNA, 3 µL of loading dye and 7 µL of dsH_2O were loaded into each well on the prepared gel. Promega brand 100 bp ladder (molecular weight marker) was loaded into the first well in the gel. The samples were connected to a direct current source from negative pole through to positive pole and run at 70-160 Volt/cm direct current which varies according to the size of the gel tank. The samples, which were run in horizontal electrophoresis gel tanks, were analyzed in the Vilber Lourmat brand gel imaging system, and printed out on a thermal printer.

3. Results

In pathogenicity tests of M3-9, M3-15 mutant plants are revealing resistance phenotype but their original NCEBR3 plant is susceptible to the *Cmm2* (Figure 1). The resistance status of mutant genotypes was determined by molecular SSR markers and their possible resistance locus were searched on chromosomes. A total of 24 SSR markers were used from each chromosomes and lower parts at haploid 12 chromosomes (Figure 2). The 24 SSR markers were examined for polymorphisms among the NCEBR3 parent, M3-9 and M3-15 mutant plants. There were no band formation or polymorphism found with SSR335 and SSR34 markers (Figure 3) located on chromosome 8 and 10 respectively. The PCR analyses revealed that differences in genetic material of resistant mutants M3-9 and M3-15 and susceptible NCEBR3 original plants identified with SSRB18031 molecular marker produced different amplicons between M3-9 and M3-15 mutants

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Table 1. The 24 SSR markers used in the study for revealing polymorphism between resistant and susceptible plants.

Chromosome	Marker	Nucleotide Sequence	Annealing Temperature	Position
1	SSR92	F5'-AAGAAGAAGGATCGATCGAAGA-3' R5'-TCATGACCACGATACTACATGTTTC-3'	50ºC	0.00 cM
1	SSR65	F5'-GGCAGGAGATTGGTTGCTTA-3' R5'-TTCCTCCTGTTTCATGCATTC-3'	50ºC	159.00 cM
2	SSR96	F5'-GGGTTATCAATGATGCAATGG-3' R5'-CCTTTATGTCAGCCGGTGTT-3'	50°C	36.50 cM
2	SSR22	F5'-GATCGGCAGTAGGTGCTCTC-3' R5'-CAAGAAACACCCATATCCGC-3'	60ºC	99.00 cM
3	SSR86	F5'-AGGGCAACAAATCCCTCTTT-3' R5'-GGAGACGAG GCTGCTTACAC-3'	50°C	54.00 cM
3	SSR27	F5'-CCCAAATCA AGGTTTGTGGT-3' R5'-TCAGATGCCACCACTCTCAG-3'	50°C	169.00 cM
4	SSR638	F5'–TGTTGGTTGGAGAAACTCCC-3' R5'-AGGCATTTAAACCAATAGGTAGC-3'	50°C -60°C	58.00 cM
4	SSR214	F5'-AAATTCCCAACACTTGCCAC-3' R5'-CCCACCACTATCCAAACCC-3'	50°C	95.00 cM
5	SSR13	F5'-GGGTCACATACACTCATACTAAGGA-3' R5'-CAAATCGCGACATGTGTAAGA-3'	50°C	27.00 cM
5	SSRB18031	F5'AGACTCAGTCCCGAACAAGTTGAAG-3' R5'ACATTACACTAAACCCCCAATTGCC-3'	55°C	119.00 cM
6	SSR47	F5'-TCCTCAAGAAATGAAGCTCTGA-3' R5'-CCTTGGAGATAACAACCACAA-3'	50°C	6.50 cM
6	SSR350	F5'-GGAATAACCTCTAACTGCGGG-3' R5'-CGATGCCTTCATTTGGACTT-3'	55°C	101.00 cM
7	SSR241	F5'-TCAACAGCATAGTGGAGGAGG-3' R5'-TCCTCGGTAATTGATCCACC-3'	55°C	0.00 cM
7	SSR45	F 5'-TGTATCCTGGTGGACCAATG-3' R5'-TCCAAGTATCAGGCACACCA-3'	50°C	60.00 cM
8	SSR335	F5'-CCTCTCCATTCTGTGGTGGT-3' R5'-AACCGTCCTCGATTTCACAC-3'	55°C	49.70 cM
8	SSRB105694	F5'-AAGCCAAAGTGGAAGAACTCAAGG-3' R5'-CTCGTAAAACGTTCATCAATCTCGC-3'	53°C	87.00 cM
9	SSR69	F5'-TTGGCTGGATTATTCCTGTTG-3' R5'-GCATTTGATAGAAGGCCAGC-3'	50°C	24.90 cM
9	SSR599	F5'-GATTTCTCATGGAGAATCAGTC–3' R5'-TCCCTTGATCTTGATGATGTTG-3	55°C	109.0 cM
10	SSR34	F5'-TTCGGATAAAGCAATCCACC–3' R5'-TCGATTGTGTACCAACGTCC–3'	50°C – 45°C	25.30 cM
10	SSR479	F5'-TGTAAGAGTGTCTGCCTGCAC–3' R5'-ATGGGTTCGGGTTAGCTCTT –3'	52°C	86.00 cM
11	SSR136	F5'-GAAACCGCCTCTTTCACTTG–3' R5'-CAGCAATGATTCCAGCGATA –3'	50°C	11.00 cM
11	SSR67	F5'-GCACGAGACCAAGCAGAT TA–3' R5'-GGGCCTTTCCTCCAGTAGAC–3'	50°C	24.00 cM
12	SSR44	F5'-TCATCTGCAATTCATGGCTC–3' R5'-AGGTCAAGGATGTGCTTCCC–3'	45°C	60.00 cM
12	SSR345	F5'-AAGCCAAGCTCGAACCTGTA-3' R5'-ATCCATGCTGTCGCTTTCAT-3'	60°C	72.50 cM

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Figure 1. Stem sections ofM3-15 and M3-9 resistant mutants and susceptible NCEBR3 tomato plants. The plants are inoculated with *Clavibacter michihganensis* subsp. *michiganensis* isolate 2 at 15 days post inoculations.

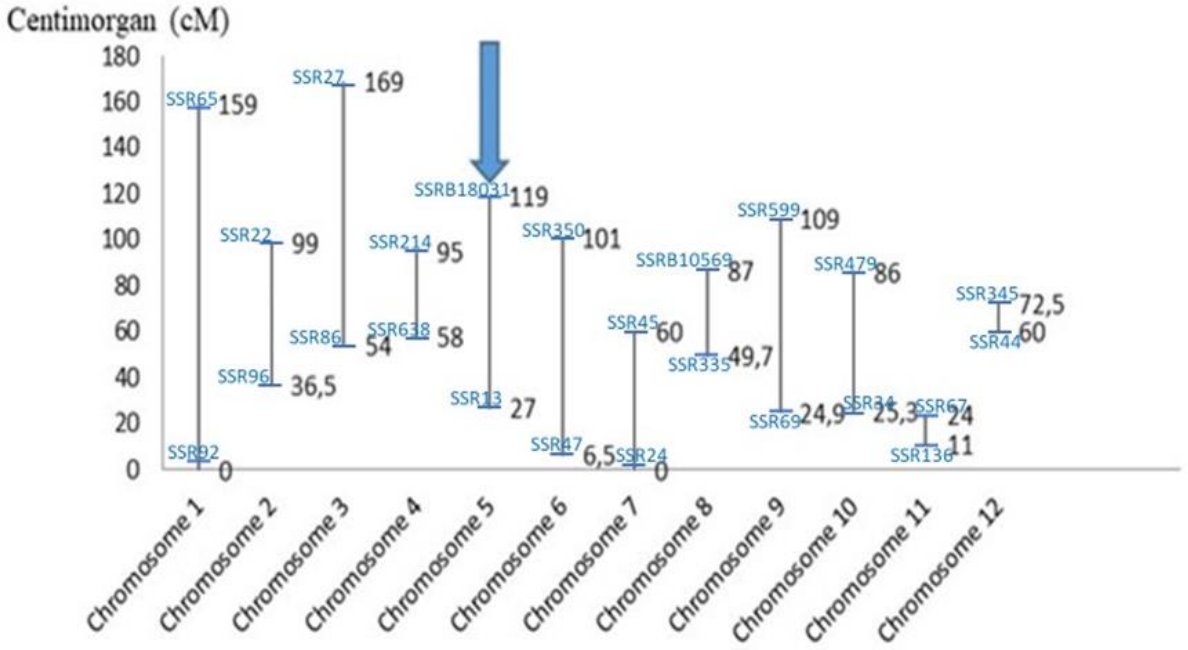


Figure 2. Chromosome distribution of *Solanum lycopersici* specific simple sequence repeat (SSR) markers.

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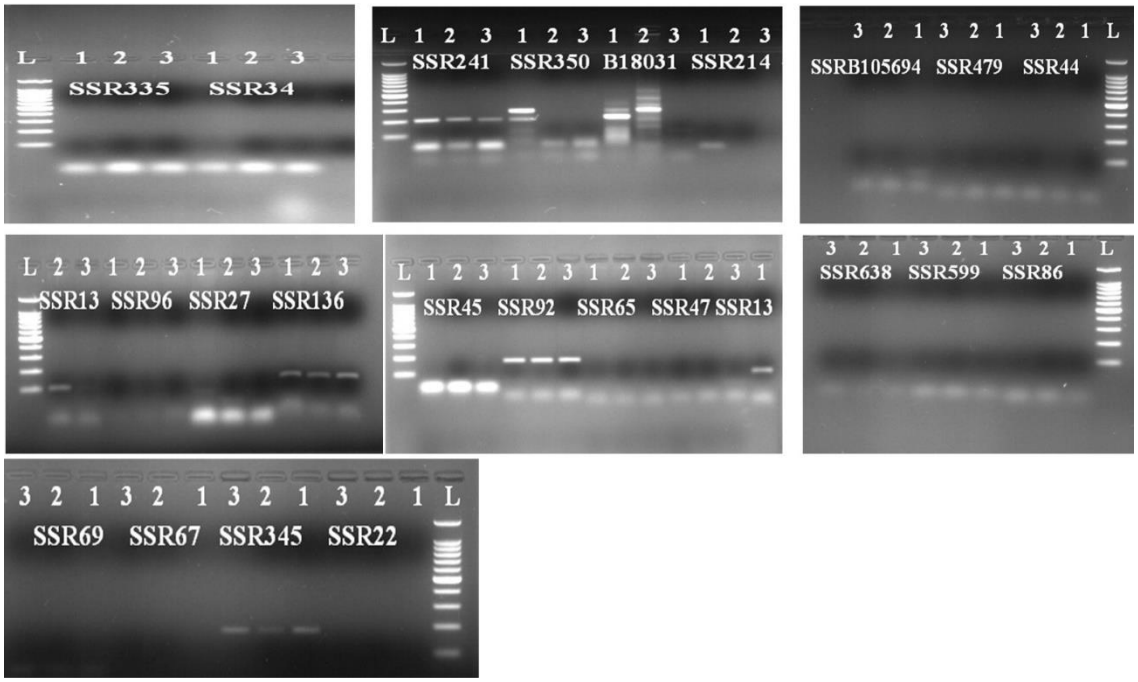


Figure 3. The PCR amplifications performed using 24 Simple Sequence Repeats (SSR) molecular markers and their amplicons were run on 2% agarose gel.1: M3-9, 2: M3-15, 3: NCEBR3, L: 100 bp Ladder.

but no amplification product was formed in susceptible NCEBR3 plant samples (Figure 3). The SSRB18031 molecular marker is located on chromosome 5 and the marker has been distinguished in mutants and original tomato plants. In the use of SSR214 and SSR27 molecular markers, they consisted of different size bands with M3-9 and M3-15 resistant mutant plants, no band formation was observed in the susceptible NCEBR3 plant (Figure 3). Another SSR13 marker located on chromosome 5 produced a band M3-9 resistant mutant but it did not form a distinct band, neither M3-15 resistant mutant nor in susceptible NCEBR3 plant (Figure 3). The SSR136, SSR92 and SSR345 markers amplified same size band on resistant M3-9, M3-15 and susceptible NCEBR3 plants. Apart from these, SSRB105694, SSR479, SSR44, SSR96, SSR27, SSR65, SSR47, SSR638, SSR599, SSR86, SSR69, SSR67 and SSR22 molecular markers did not amplify any bands on resistant M3-9, M3-15 and susceptible NCEBR3 original plants (Figure 3).

4. Discussion

For the genetic control of tomato bacterial canker and wilt disease, M3-9 and M3-15 resistant mutants found as a result of chemical mutation, the two resistant mutants were amplified and their genotypes were searched for polymorphisms with their original susceptible parent. In previous studies, *Cmm2* inoculated M3-9 and M3-15 resistant plants' extracts were analyzed in High Pressure Liquid Chromatography (HPLC) system. In HPLC analysis, chlorgenic acid and rutin hydrate were increased 6.7 and 13 times higher in resistant plants than in susceptible NCEBR3 plants respectively (Bayan 2011). Additional inoculation tests of M3-9 and M3-15 plants were carried out with 3 different *Cmm* isolates obtained from Tokat tomato production areas in the Black sea region, Turkey (Çalış et al. 2015). This phenotypic knowledge led us to search polymorphism to locate possible resistance gene(s) on genotypes of mutants compared with susceptible NCEBR3 plants. Molecular analyses with 24 SSR markers were randomly chosen from each terminal part of chromosomes and their molecular analyses were associated with SSR13 and SSRB18031 markers at chromosome 5 (Figure 2 arrow indicates). The results reveal that a mutation occurred on M3-15 and M3-9 resistant plants but the susceptible NCEBR3 plant did not have an amplified band. The chemical mutation might create a mutation on M3-9 and M3-15 plants and control resistance to the *Cmm2* pathogen. These results should be verified with constructing individual mapping populations with the M3-9 and M3-15 plants.

SSR markers provide high efficiency and accuracy in detecting DNA difference in any plant. SSR markers are preferred in population genetics and gene mapping studies because they require a low level of DNA, have high levels of polymorphism and codominant features (Powell et al. 1996). All these attributed to use the codominant SSR markers where SSRB18031 and SSR13 markers have polymorphism between resistant mutant and susceptible NCEBR3 plants indicating phenotypes of the plants are matched in genotypes with these markers.

5. Conclusions

In this study, genetic polymorphisms were investigated in resistant M3-9 with M3-15 mutants and susceptible NCEBR3 plants. Altogether 24 SSR markers selected from each end of haploid 12 tomato chromosomes, were used to determine a possible resistance locus using PCR based molecular analyses. The PCR amplification method revealed polymorphisms with SSRB18031 and SSR13 molecular markers in M3-9, M3-15 mutants and susceptible NCEBR3 plants. As a result of the polymorphisms the resistance locus is on chromosome 5 because the two SSR markers are located on the chromosome. Further fine mapping studies should uncover possible resistance locus, which controls resistance to the bacterial canker and wilting pathogen.

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