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Full Length Research Paper

Characterization of resistant tomato mutants to bacterial canker disease

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A small scale ethylmethanesulfonate (EMS) mutation was used to obtain resistant mutant plants to bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* isolate 2 (Cmm2). Susceptible EBR3 tomato line (200) seeds were mutagenised with the chemical EMS. Of the constructed M2 population, 450 seedlings were inoculated with Cmm2. Among the tested M2 plants, 15 M2 plants, not affected by the bacterial pathogen, produced M3 family seeds. 10 seeds from each M3 family were re-inoculated with the Cmm2, M3-9 and M3-15 family plant did not segregate for resistance confirming their resistant phenotypes. The M3-9 and M3-15 family mutant plants were analyzed for secondary metabolites especially phenolics; chlorogenic acid, caffeic acid, p-coumaric acid, rutin hydrate, ferulic acid, quercetin, naringenin and caempherol level in high performance liquid chromatography (HPLC) system. The chlorogenic acid and rutin hydrate levels were increased in M3-9 and M3-15 family mutant plants than susceptible original EBR3 plants. The increased levels of phenolic substances could be associated with resistance phenotypes in the M3-9 and M3-15 mutants' plants.

Key Words: Tomato, ethylmethanesulfonate mutagenesis, resistant mutants, bacterial canker.

INTRODUCTION

Bacterial canker and wilt disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is a destructive tomato disease causing severe economic losses in worldwide tomato growing areas. The gram-positive actinomycete bacteria enter the plant through natural openings and wounds colonies on xylem vessels and lead to typical disease symptoms: light brown discoloration of the vascular tissues, wilting of leaves, sometimes one-sided asymmetric and necrotic lesions on leaves, canker lesions on stems and petioles (Davis et al., 1984; Agrios, 1997). If plant infection occurs from seed or seedlings a systemic disease infection will develop and the plant will die, otherwise, if infection occurs at late stage of plant development plants will be able to survive and generate fruits that can have bird's eye spots (Fatmi et al., 1991; Agrios, 1997).

Several hypotheses were proposed to explain how Cmm bacteria induce bacterial canker and wilt during infection. It is unknown which strategies are utilized to obtain necessary proteins, sugars and other substantial materials from host cells (Gartemann et al., 2003). One of the hypotheses is that the bacteria are mainly located in the xylem where the bacteria caused physical impairment of water transport so that it may increase severe water stress resulting in wilting. The Cmm bacterial avirulence (*Avr*) genes have being not identified yet but proteobacteria encode exopolysaccharides (EPS) that is necessary for plugging of xylem vessels generating a condensed matrix around the bacteria for protecting against dehydration (Leigh and Coplin, 1992) and acting as ion exchangers: importing minerals and nutrients around the bacteria (Niehaus et al., 1993). The bacterial EPS is considered a main component protecting the bacteria from plant defense reactions by either detoxification of phytoalexins or reactive oxygen species

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(Kiraly et al., 1997). Furthermore, the EPS are able to block not only plant surveillance systems using agglutinins and lectins (Young and Sequeira, 1986) but also mediate adhesion to abiotic and biological surfaces to promote colonization and infection on host cells (Tharaud et al., 1997). Additionally, Cmm produces several extracellular enzymes such as polygalacturonase, xylanase and serine proteases that are able to degrade plant cell walls and attack xylem vessels and neighbouring parenchymatic cells (Beimen et al., 1992; Gartemann et al., 2003; Chalupowicz et al., 2010).

The Cmm bacteria contain two plasmids where they encode proteins called effectors allowing high-titer bacterial colonization and production of significant amount of proteins (Jahr et al., 2001; Gartemann et al., 2003). Several bacterial *Avr* genes are able to interact counterpart plant *R* genes using either gene-for-gene (direct) or guard (indirect) theories (Van Oijen et al., 2007; Jones and Dangl, 2006; Buttner and Bonas, 2006; Da Cunha et al., 2007). This is inadequate for the pathogen to disclose its presence to the tomato plant by secreting *Avr* gene products that are recognized by resistant (*R*) gene encoded proteins (Gartemann et al., 2003). To date, there is no fully resistant tomato cultivar identified to bacterial canker and wilt disease because host targets of Avr products of Cmm bacteria are still unknown, and their probable locations in host cells are not been determined yet (Gartemann et al., 2003).

To understand the host responses during the tomato-Cmm interaction and their resistance mechanism, the tomato is a good model plant with extensive genomic data available at Sol Genomics Network (http://solgenomics.net/). Reverse genetics gives the opportunity to target a specific sequence for genetic analyses requiring the means to selectively mutate or identify a mutation in the chosen gene. Mutagens have caused a high number of random mutations and screening systems allow discovering induced mutations in sequence DNA targets (McCallum et al., 2000; Triques et al., 2007). The imperfectly matched DNA known as heteroduplex DNA is subject to detect a mutation controlling resistance to the bacterial canker and wilting pathogen.

Plant secondary metabolites are produced when plants are under stress conditions like drought, insect and disease infections. Some of these compounds provide resistance to some specific agents like essential oils for insect attack. Tomato plants are reported to synthesize phenolic compounds for protection against plant pathogenic bacteria.

In this study, characterization of the resistant tomato mutants, obtained from a screening from mutagenised susceptible tomato cultivar (EBR3) seeds with a chemical mutagen ethylmethanesulfonate (EMS) were carried out. Additionally, phenolic compounds levels were determined in both original EBR3 tomato and promising mutant tomato (M3-9 and M3-15) plants.

MATERIAL AND METHODS

Plant materials

Tomato seeds of EBR3 tomato cultivars were kindly provided by Prof. Dr. Randolph G. Gardner (Horticultural Science, North Carolina State University, Raleigh, USA). The tomato seeds were sown in 6 × 30 × 50 cm plastic seed trays containing turf (Potground, Klasmann, Germany). The seed trays were placed in a glasshouse at 24±5°C with 50% relative humidity, with 16 h day, and 8 h night conditions. Germinated seedlings were placed on turf containing pots at 4 to 5 real leaf stages of tomato plants.

Ethylmethanesulfonate (EMS) mutagenesis

The EBR3 tomato line was enhanced susceptible to *Clavibacter michiganensis* subsp. *michiganensis* isolate 2 (Çalış unpublished results). The EBR3 tomato line seeds were soaked on Whatman paper for 10 h. The imbibed 200 tomato seeds were transferred into an Erlenmeyer bottle containing 100 ml dsH_2O, and immediately 0.5% (500 µl) EMS (Sigma, Germany) solution was added, and then the bottle was incubated for 12 h with gentle shaking in fume hood at room temperature. The EMS solution was removed from the seeds; they were extensively rinsed under running water for 5 to 10 min. The seeds were dried in an incubator at 37°C for 3 h and then sown in plastic seed trays at the same day. The EMS mutation provides broad range of alleles in a relatively small population because there are many viable mutants in each line (Emmanuel and Levy, 2002; Menda et al., 2004).

Screening of mutant plants

After EMS mutagenesis, all the 200 seeds were sown in plastic trays and germinated seedlings were transferred into plastic pots containing sterile turf. The M1 plants were allowed to set seeds with self-pollination in a glasshouse. Whole tomatoes were harvested and their seeds were pooled for establishing 4000 M2 plant seeds. From the constructed M2 seeds, 600 M2 seeds were sown into plastic trays: 100 seeds did not germinate and the rests viable 500 seedlings were transferred into plastic pots at four to five true leaf stages. Meanwhile, 20 original EBR3 tomato seedlings were transferred into plastic pots at the same age. Inoculated 15 M2 plants were not affected from the Cmm2 and set tomatoes for producing M3 mutant family seeds. From each individual 15 M3 families, 10 seedlings were re-inoculated with the Cmm2 using original EBR3 plants as controls with dsH_2O as negative controls; the rest of the 450 M1 plants were inoculated with the bacteria as described below.

Bacterial strains

The most virulent isolate of *C. michiganensis* subsp. *michiganensis* (Cmm2) obtained from Prof. Dr. Hüseyin Basim (Akdeniz University, Plant Protection Department, Antalya, Turkey) was used in these studies. The bacteria were grown either in nutrient agar (NA: Merck, VM984550823) or glucose yeast carbonate agar (GYCA) medium at 28°C. The GYCA medium contained 5 g glucose, 5 g yeast extract, 40 g calcium carbonate and 15 g agar per liter of water at pH 7.2 (Lelliott and Stead, 1987).

Plant inoculation

The Cmm2 bacterial strain was stored in 30% glycerol at -80°C until used. The inoculums of Cmm2 were prepared from two days-old

bacterial cultures on GYCA plates. The bacteria were picked using a sterile toothpick, then the toothpick was immediately initiated into plant stem (above first true seed) at 4 to 5 real leaf stages. For the control plant's inoculation, the toothpick was dipped in distilled sterile water and then was initiated into plant stem. In pathogenicity tests, 450 M2 and 10 original EBR3 tomato plants were inoculated with the Cmm2 and 50 M2 and 10 original EBR3 original tomato plants were inoculated with dsH_2O as negative controls. The inoculated plants were kept in a glasshouse at 23±5°C temperature with 60% relative humidity, and at 16/8 h day/night conditions. Symptoms were checked every day in the first week, 14, 21 and 28 days post inoculation.

Sample preparation for chromatography

Samples were prepared from 20 plants leaves at indicated days post inoculation (dpi) with aqueous methanol (Merck, Germany) for high performance liquid chromatography (HPLC) analysis. Mutant (M3-9 and M3-15) leaves were pooled and analyzed; the control tomato leaves were separately analyzed. Acidified aqueous methanol at a final concentration of 75% methanol (v/v) and 0.1% formic acid (v/v) was considered to be the most suitable solvent for efficient extraction of a wide range of secondary metabolites from tomato leaves. All leaf samples were homogenated with liquid nitrogen using mortar and pestle and 100 mg of leaf samples were accurately weighed and extracted with methanol (10 mL) in test tube for 6 h. After the filtration over syringe type filter (Chromtech, 13 mm, 0.22 µm), the filtrate was injected into HPLC system for analysis. The results were expressed as mg phenolics/kg fresh leaf. The samples were prepared according to De Vos et al. (2007).

High performance liquid chromatography (HPLC) analysis

Phenolic compounds, chlorogenic acid, caffeic acid, p-coumaric acid, rutin hydrate, ferulic acid, quercetin, naringenin and caempherol levels for original and mutant tomato plant were measured with HPLC; A Perkin Elmer Series 200 liquid chromatography system (Perkin Elmer, USA) equipped with UV detector. The analytes were separated on a Phenomenex Kromasil 100A C18 (250x4.60 mm, 5 µm) column. The column temperature was maintained at 40°C using a water bath (Wisebath, WB-22, Korea). The mobile phase consisted of acetonitrile (A) and water containing 2.5 % formic acid (B). The gradient conditions were: initial, 0 to 3 min, held at A to B (5:95, v/v); 3 to 8 min, linear change from A to B (5:95, v/v) to A to B (10:90, v/v); 8 to 13 min, linear change from A to B (10:90, v/v) to A to B (15:85, v/v); and 13 to 15 min, isocratic elution A to B (15 to 85, v/v); 15 to 22 min, linear change from A to B (15:85, v/v) to A to B (25:75, v/v); 22 to 37 min, linear change from A to B (25:75, v/v) to A to B (50:50, v/v); 37 to 40 min, isocratic elution A to B (100-0, v/v). The mobile phase flow rate was set at 1 ml/min and the injection volume was 20 µl.

Statistical analysis

Using the SPSS statistical package (SPSS for Windows 17.0), an analysis of variance (ANOVA) was performed to estimate genetic resistance. Chi-square analyses were conducted to estimate recessive and dominant ratios for phenotypic resistance.

RESULTS

EMS mutation was used to induce point mutation for obtaining resistant tomato plants against bacterial canker disease caused by *C. michiganensis* subsp. *michiganensis* in the EBR3 tomato line. The EMS mutagenesis is reliable, efficient, and well understood but is very difficult to characterize resistance responses because of the point mutation. Establishment of the mutant population took three years. Seeds of EBR3 were treated with 0.5% EMS that caused lethal dose (LD_{15}) reduction in seed germination to obtain high mutation rates with minimizing M1 and M2 sterility. The resistant mutant phenotypic screens were performed on the M2 population because M1 seeds had mutation on only one allele; therefore, whole M1 seeds were sown to allow crossing with each other for establishment of homozygous M2 seeds (Figure 1). We focused the screening on 600 M2 families with pathogenicity tests, all M2 seeds were sown on turf and 500 M2 seeds were germinated. The mutation treatments resulted in 16.6% of lethal mutations because 100 seeds did not germinate or produce albino seedlings. Among the 500 remaining seedlings, 450 seedlings and 10 original EBR3 seedlings were inoculated with Cmm2, and the rest of the 50 M2 and 10 EBR3 original seedlings were mock-inoculated with distilled sterile water. Among the 450 inoculated M2 plants, only 15 plants survived and produced fruits at 30 dpi with light wilting symptoms while the other M2 mutant plants inoculated with Cmm2 were already dead at 15 dpi (Figure 1).

Confirmation of M2 mutant phenotypes

The promising 15 M2 plants set tomatoes generated M3 seeds. 10 M3 plants from each individual 15 families were re-inoculated with Cmm2 to confirm resistant phenotypes obtained from M2 population. Among the tested 15 families, only the M3-9 and M3-15 family plants revealed resistant phenotypes; they did not segregate for resistance to bacterial canker pathogen, generating tomatoes at 30 dpi. These results show that verification step is essential for M3 phenotypes.

Chlorogenic acid and rutin hydrate levels enormously increased in mutants

The Cmm3 inoculated M3-9, M3-15 mutants, and original EBR3 tomato plants were analyzed in HPLC system to measure levels of chlorogenic acid, caffeic acid, p-coumaric acid, rutin hydrate, ferulic acid, quercetin, naringenin and caempherol in leaves of the tomato plants. The mock inoculated mutants and original EBR3 tomato samples were also measured for the same phenolic substances in HPLC system. The HPLC results reveal that chlorogenic acid and rutin hydrate levels were increased 6.2 and 13.6 times higher in M3 mutants, respectively (Table 1). These compared results were obtained between inoculated mutant leaf and inoculated orginal EBR3 leaf samples (Table 1).

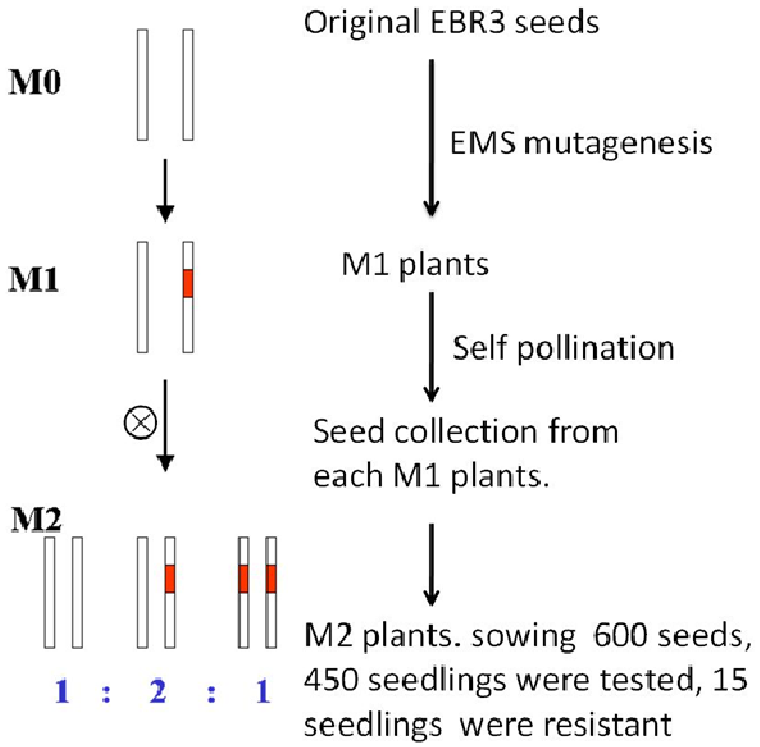

Figure 1. Establishment of M2 mutant population using chemical ethylmethanesulfonate on susceptible original EBR3 tomato line seeds. The M2 mutant plants were inoculated with bacterial canker disease pathogen Cmm2 to identify resistant phenotypes.

Table 1. Inductions of phenolic compound levels were analyzed in HPLC system at a time course experiment.

Standard	Days post inoculated					
	0	4	6	14	21	28
Cmm2 ınoculated promising M3 plants						
Chlorogenic acid	20.93	45.94	44.17	81.64	562.22	851.47
Cafeic acid	-	-	1.96	-	-	-
P-coumaric acid	1.95	2.29	4.09	6.49	13.97	-
Rutin hydrate	141.71	235.71	264.62	775.59	1371.01	20688.70
Ferulic acid	1.71	3.10	62.00	1.94	1.75	37.13
Quercetin	1.72	-	2.12	2.19	2.01	2.31
Naringenin	-	-	-	-	-	1.48
Caempherol	-	-	-	-	-	-
Cmm2 inoculated EBR3 plants						
Chlorogenic acid	31.60	53.65	62.45	76.46	120.34	136.82
Cafeic acid	-	1.76	-	-	-	-
P-coumaric acid	-	2.31	-	2.15	1.57	1.77
Rutin hydrate	109.84	262.50	311.42	320.34	316.23	1518.97
Ferulic acid	1.75	1.71	1.77	1.91	3.53	23.05
Quercetin	-	-	-	-	1.45	2.31
Naringenin	-	1.09	-	-	-	1.23
Caempherol	-	-	-	-	-	-

Average results were presented in inoculated M3-9 and M3-15 mutant plants and average results were obtained from ten replicates of inoculated EBR3 plants.

Table 2. Inductions of phenolic compound levels were analyzed in HPLC system. Distilled sterile water used for inoculations and the mock inoculated samples were prepared at the indicated time.

Standard	Days post inoculated					
	0	4	6	14	21	28
Mock ınoculated promising M3 plants						
Chlorogenic acid	37.472	33.844	90.514	109.27	190.24	265.707
Cafeic acid	-	1.418	1.979	-	-	
P-coumaric acid	-	-	2.119	2.782	1.727	
Rutin hydrate	218.8	228.58	221.06	451.83	745.13	1206.202
Ferulic acid	1.668	4.126	2.751	2.019	4.119	7.527
Quercetin	-	1.277	1.633	1.53	1.502	1.88
Naringenin	-	-	-	-	-	
Caempherol	-	-	-	-	-	1.863
Mock inoculated EBR3 plants						
Chlorogenic acid	1	58.545	15.547	1.131	-	
Cafeic acid	23.625	-	-		-	
P-coumaric acid	-	-	-	1.783	-	
Rutin hydrate	30.255	46.912	107.61	106.33	362.78	363.852
Ferulic acid	9.135	8.594	1.686	1.875	1.689	1.705
Quercetin	1.847	-	-	-	-	
Naringenin	-	-	1.123	-	-	1.094
Caempherol	-	-	-	-	-	

On the other hand, similar association was not measured for chlorogenic acid in mock inoculated M3 mutant and the original EBR3 leaf samples (Table 2). The chlorogenic acid level was increased in mock inoculated mutants but its level was decreased in EBR3 plants (Table 2). Rutin hydrate level was increased 3.3 times higher in the mock inoculated mutant plants than mock inoculated original EBR3 plants in time course experiments (Table 2).

DISCUSSION

In the study, susceptible tomato line (EBR3) seeds were mutagenised with EMS that causes point mutations in the tomato cultivar. The M2 mutant plants have been screened to identify resistant phenotypes against the bacterial canker pathogen Cmm2. The small scale screen yielded 15 M2 mutants which were used to establish M3 family plants. The M3-9 and M3-15 family plants did not segregate for resistance and survived with Cmm2 inoculation. The two family plants were selected because these plants synthesize very high level phenolics compounds against the bacterial canker pathogen. A new reverse genetics method has been developed to identify and isolate EMS mutants for plant resistance genes. The method is based on the amplification of the target gene in pools of plants from the EMS population. The PCR products reveal the mismatched heteroduplexes formed after annealing from original and mutant plants. Various methods were used to detect mismatched heteroduplexes. These high-throughput methods are targeting induced local lesions in genomes (TILLING) with denaturing high power liquid chromotography (McCallum et al., 2000a), and gel-based method where an endonuclease (CelI) cleaves mismatched heteroduplexes and the cleavege product are separated with electrophoresis (Colber et al., 2001). Recently, the Osbourn group (Field et al., 2011; Chu et al., 2011) have revealed operon-like gene clusters that are required for the synthesis and modification of secondary metabolites. Starting from this point, if secondary metabolites are synthesized by gene clusters, specifically designed primers could be revealed gene clusters which increase secondary metabolites such as phenolic compounds in PCR detection system. The resource described here will be able to combine with whole genome sequence of the tomato genome. Here, we describe that increased levels of phenolic substances were already associated with resistance phenotypes to bacterial canker disease. This

association between phenotypes and secondary metabolite levels will enable us to detect mutated tomato genes controlling resistance to the bacterial disease.

Full characterization of tomato mutants are required to understand the role of secondary metabolites in resistance. If resistant phenotype associated with secondary metabolic gene clusters, the gene clusters will be able to locate the resistant gene on chromosomes of mutant plants.

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