DISTRIBUTION AND GENETIC DIVERSITY OF TOBACCO ETCH VIRUS IN TURKEY AND RESISTANCE OF IMPROVED CAPSICUM LINES

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(Received 9th Jun 2019; accepted 10th Sep 2019)

Abstract. Tobacco etch virus (TEV) belonging to *Potyviridae* is among the most destructive viruses and widespread Worldwide but it has been found only Turkey in Mediterranean Basin. Both dominant and recessive pvr alleles contribute resistance against potyviruses. Prevalence of TEV in main pepper cultivation areas in Turkey and phylogenetic diversity of the isolates based on their coat protein region were determined in this study. Transferring resistance genes both recessive and dominant to susceptible chilli pepper lines was aimed with a breeding program including CM334 and Perennial known as resistance sources against several potyviruses. Two geographically and genetically distinct TEV isolates 1002 and 774 were inoculated to pepper genotypes carrying *Pvr4*, *pvr2*⁺, *pvr2*¹, *pvr2*², *pvr2*³ alleles and 15 lines improved from CM334 carrying *Pvr4*, *pvr2*³ and Perennial carrying *pvr6* allele and QTLs. None of the pvr alleles contribute to resistance to isolate 774 while *pvr2*² provides resistance to isolate 1002. All the pepper cultivation areas are infected by TEV in Turkey mainly Aegean Region and the isolates found in Turkey are close to those from Chinese isolate. Polygenic resistance was transferred by breeding program to line 4 could resist to both Turkish isolates.

Keywords: *polygenic resistance, pvr2 alleles, coat protein, pepper, phylogenetic analysis*

Introduction

Due to widespread cultivation of pepper in the World, harmful pests and diseases caused by fungi, viruses and bacteria of this plant have become prevalent. *Capsicum* spp. widely grown in the world is exposed to 70 viruses and more than 20 important viruses limit its production (Moury and Verdin, 2012; Green and Kim, 1991; Florini and Zitter, 1986). Among these viruses, the most prevalent ones are Tobamoviruses; Potyviruses, Cucumoviruses; and thrips-transmitted tospoviruses and Potyviruses (Moury and Verdin, 2012). Tobacco etch virus (TEV; genus *Potyvirus*; family *Potyviridae*) is an important pathogen in solanaceous crops with 10 described species that are very destructive and distributed with regard to continental pattern (Moury et al., 2005; Janzac et al., 2008). Potyviruses is characterized by flexuous, rod-shaped particles with approximately 10 kb in length single-stranded, positive sense RNA encapsidated coat protein (CP). Tobacco etch virus (TEV) causes up to 70% yield loss in pepper and spread mainly in North and Central America and the Caribbean (Nutter et al., 1989; Ariyaratne et al., 1996; Green and Kim, 1991). The use of resistant genotypes is one of the most efficient methods to protect crops from the yield losses caused by viruses. Using resistant varieties reduce incidence of TEV in pepper cultivated fields and increase fruit yield (Padgett et al., 1990).

Pepper resistance genes and alleles whether dominant or recessive are efficient against several potyviruses. The dominant gene *Pvr4* which originates from *Capsicum annuum* Criollo de Morelos 334 (CM334) confers to a broad range resistance of potyviruses (Kim et al., 2017). *Pvr4* gene provides resistance to PepSMV (Pepper severe mosaic virus) and PTV (Peru tomato mosaic virus), but carrying genotypes this gene are susceptible to ChiVMV (Chilli veinal mottle virus), PVMV (Pepper veinal mottle virus) and TEV

(Janzac et al., 2009). Recessive genes *pvr1* and *pvr2* in pepper have been observed for resistance to potyviruses including tobacco etch virus but many virulent strains of TEV overcome most of these recessive genes (Parrella et al., 2002). Two main alleles from *Capsicum annuum* at the *pvr2* locus have been transferred to modern pepper cultivars (Kyle and Palloix, 1997) and *pvr1* from *C. chinense* (PI 152225) (Venkatesh et al., 2017) so that to provide resistance against potyviruses. Digenic recessive resistance to another potyvirus PVMV has succeeded by combination of recessive allelic series *pvr2* and *pvr6* allele corresponding to the eIF4E and eIF(iso)4E, respectively (Moury et al., 2005; Rubio et al., 2009).

In this study, spreading of TEV in the main open-field and protected pepper cultivation areas in Turkey has been determined and genetic diversity of CP region of some isolates compared with World isolates. Improved pepper lines from Perennial and CM334 resistance sources carrying *pvr* alleles were selected as resistant to two isolates determined distinct both genetically and geographically in this study. The genetic diversity of TEV infecting pepper was analysed in Turkey, to understand the diversity of TEV populations and to estimate the response of improved pepper genotype transferred both recessive resistance allele *pvr2*³, *pvr6* and dominant gene *Pvr4* from resistant donors and the impact of geographic location on that structure. Transferring resistance genes against potyviruses both recessive and dominant to susceptible chili pepper lines was aimed with a breeding program including CM334 and Perennial as resources of resistance and Sena chili pepper variety which were commercial and susceptible to pathogen.

Materials and methods

Breeding population

First four years of breeding program resistant sub-populations were improved so that collecting resistance alleles. These populations were derived from crosses between the resistant donor Criollo de Morelos 334 (CM334) and Perennial inbred lines resistant to several potyviruses. CM334 (male) carrying *Pvr4* and *pvr2*³ genes and Perennial (female) carrying *pvr2*³ and *pvr6* genes were crossed and 106 F₂ individuals were self-pollinated for three years. Selected as new resistant sources were crossed by Sena which was suitable for paprika production but susceptible variety against infections of TEV. F₁ progeny were not backcrossed to susceptible recipient (Sena) but self-pollinated and resistant individuals backcrossed to susceptible recipient and self-pollinated again. After two backcrossing and self-pollination alternately, selected 15 lines were mechanically inoculated by two TEV isolates 1002 and 774.

Field surveys and collecting of TEV isolates

TEV isolates were collected from June to August in 2013 and 2014 (open field) and March to May 2013 and 2014 (protected cultivation) with hierarchical (nested) sampling method (Ben Khalifa et al., 2009; d'Urso et al., 2003) from important pepper growing areas in Turkey. The factors was assessed by hierarchically partitioning the sample population among region, (West, South and East), cultivation system (open and protected) and sites (11 provinces) and samples were collected from 51 fields and 39 greenhouses. The pepper production values and sampling number of provinces were given in Table 1. Totally 1525 leaf samples and/or small pepper fruits were randomly collected from plants having symptoms related with viral infection.

Table 1. Prevalence of tobacco etch virus infections determined by DAS-ELISA and RT-PCR in the main pepper-growing areas, Turkey

Province	Pepper production of sampled provinces (tons)	Number of samples ^b	ELISA		RT-PCR	
			Number	%	Number	%
Antalya ^a	270,127	193 (21)	9	5	0	0.0
Balikesir	61,046	39 (4)	0	0	2	5.1
Bursa	133,438	183 (7)	42	22.9	70	38.2
Çanakkale	130,063	256 (8)	52	20.3	49	19.1
Hatay	102,665	294 (5)	36	18.6	9	3.0
Izmir	132,519	131 (7)	0	0.0	2	1.5
Kilis	55,000	93 (4)	2	2.1	4	5.3
Manisa	169,787	143 (8)	0	0.0	8	5.5
Mersin ^a	227,086	75 (18)	8	10.6	5	6.7
Samsun	257,306	60 (4)	3	5.0	0	0.0
Şanlıurfa	67,583	58 (4)	0	0.0	2	3.4
Total	1,606,620	1525 (90)	152	9.9	151	9.9

^aProtected cultivation areas

^bNumber of visited sampling areas in the bracket

Serological analysis and molecular characterization of TEV pathotypes

Samples were analyzed by DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay) with antibodies against TEV (Agdia, France) and sequenced CP cistrons. Total RNAs were extracted with the Tri-Reagent kit (Molecular Research Centre Inc.) from fruit flesh or leaf (0.5 g) of collected pepper plants. Viral populations were characterized by two steps reverse transcription-polymerase chain reaction (RT-PCR) amplified with Avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA) and Taq DNA polymerase (Promega Corp.) and primers reported by Buzkan et al. (2015). Degenerated primers were designed as reverse primer positions from 9273 to 9297 (CCCTAATAGTGTGTGTCATGTTACGG) and the forward primer positions from 8538 to 8560 (TGCTGAYGCYGGYAAGAAGAAAG) produced a 760 nucleotide amplicon for TEV CP cistron. RT-PCR products were sequenced with primers corresponding to viral polarity by Medsantek (İstanbul, Turkey). CLUSTAL W program (Thompson et al., 1994) were used to align nucleotide sequences of CP coding regions of TEV and sequences retrieved from databanks were compared with Turkish isolates. MEGA version 6.06 software (Tamura et al., 2013) was used to select appropriate nucleotide substitution model with MODELTEST (Posada and Crandall, 1998). Neighbor joining, maximum likelihood and maximum parsimony methods were used to estimate nucleotide diversity and phylogenetic construction and evaluation with 1000 bootstrap resampling.

Biological characterization of TEV pathotypes, inoculation and screening improved pepper lines

Selected two different TEV isolates in relation to their geographical origin (774 from Hatay and 1002 from Gaziantep) and corresponding to phylogenetic tree (Fig. 2) were mechanically inoculated on the pepper genotypes possessing *pvr2* alleles Yolo Wonder (YW), Yolo Y (YY), Florida VR2 (VR2) and W4. YW genotypes. YY, VR2 and W4

are homozygous for the *pvr2*⁺, *pvr2*¹, *pvr2*² and *Pvr4* resistance genes, respectively. Isolate 774 was selected as viral material because of its resistance breaking ability over *pvr2*² allele from Florida VR2 confers resistance to TEV isolates (Caranta et al., 1997). Isolate 1002 is not be able to overcome *pvr2*² allele. Two TEV isolates were multiplied on *Nicotiana* to obtain high titer inoculum for tests on *C. annuum*. *Nicotiana* leaves showing severe symptoms of the TEV were weighed as 1 g and homogenized in 4 ml of 0.03 M phosphate buffer (pH 7.0) supplemented with 2% (wt/vol) diethyldithiocarbamate, active charcoal at 20 mg/ml, and Carborundum at 20 mg/ml. Inoculum multiplied on tobacco plants were inoculated on the two cotyledons of 15 seedlings from each pepper genotype. *C. annuum* test plants were inoculated manually at cotyledon leaf stage with same as well as in tobacco plants. Symptoms were recorded between 14 and 30 days postinoculation (dpi) and genotypes without symptoms were re-inoculated by two isolates 1002 and 774. Virus multiplication in the pepper genotypes were assessed by DAS-ELISA after one month post-inoculation (Moury et al., 2004). Genotypes were considered as positive and susceptible when their absorbance values at 405 nm was higher than three times that of negative control (Murphy et al., 1998).

Results and discussion

Distribution of tobacco etch virus in main pepper cultivation areas in Turkey

DAS-ELISA and two-steps reverse transcription polymerase chain reactions (RT-PCRs) were used to assess Tobacco etch virus both open field and protected pepper cultivation areas in Turkey (Fig. 1). Total 1525 fruit and/or leaf samples from different provinces of Turkey were tested. Tobacco etch virus was most prevalent in Bursa and Çanakkale provinces in open field pepper cultivation areas. According to ELISA signals TEV did not spread through Balıkesir, İzmir and Şanlıurfa provinces. But RT-PCR results indicated that TEV was also prevalent in these provinces. Assessing both ELISA and RT-PCR results TEV was widespread all the pepper cultivation areas both open field and protected (Table 1). In Mediterranean Basin TEV was only presence in Turkey (Moury and Verdin, 2012). Americas, Africa and Asia especially China also were infected with TEV (Green and Kim, 1991). Some of the pepper cultivation areas with local varieties nearly whole of the field infected with TEV in Çanakkale province located western side of the Turkey. Benner et al. (1985) and Abdalla et al. (1991) indicated that the incidence of the TEV can be reach nearing %100.

Genetic diversity of tobacco etch virus

The phylogenetic tree of the CP coding region displayed that the Turkey was clustered three subgroups. Isolate 1002 from Gaziantep province and 774 from Hatay were distinct (0.025 p distance) from each other based on their CP (coat protein) sequences (Fig. 2). This distinction was sourced from threonine to alanine substitutions on 57th position on CP region (T57A). Two haplotypes were defined according to the amino acid sequence of the CP corresponding to isolate 1002 (KR024266.1) and 774. Other distinction based on amino acid substitutions were originated by A49S (isolate 1496), N6S (isolate 851), and S1T (isolate 861) among the Turkish isolates. Clustering of other Turkish isolates was based on synonymous substitutions of nucleotides. All of the survey area only isolates of Hatay (774 to 1506) could be sequenced. Single amino

acid substitution of CMV-CP (Cucumber mosaic virus-Coat Protein) induced chlorosis on tobacco (Shintaku, 1991; Shintaku et al., 1992). Mochizuki et al. (2008) reported that substitution of the amino acid in the coat protein of Melon necrotic spot virus preventing from transmitting by *Olpidium bornovanus* zoospores. Dolja et al. (1994) indicated that mutation of CP of TEV affects movement of the virus in the plant.

Figure 1. Survey area of tobacco etch virus isolates on open and protected cultivation field in Turkey

The TEV virus infections in surveyed regions are widespread through Turkey in main pepper grown fields. They are built a distinct phyletic group from the other isolates in the World related with CP regions. This group is clustered to three subgroups because of amino acid substitution. TEV was presence in only Turkey among the Mediterranean countries (Moury and Verdin, 2012). Americas (USA, Canada, El Salvador, Puerto Rico, Jamaica, Mexico) Africa (Nigeria) and Asia especially China also were infected with TEV (Olawale et al., 2015; Ariyaratne et al., 1996; Green and Kim, 1991). The nucleotide sequences of isolates from these countries except Nigeria have been registered in GenBank. Potato virus Y (PVY) is widespread in the World, while TEV isolates occur mainly in North and Central America and in the Caribbean (Caranta and Palloix, 1996). Bootstrap values associated to the branches of the tree supported the existence of three major clades (Fig. 1). The first clade includes TEV isolates from Turkey and SD1 isolate AY787757 from China. The second one includes Chinese, Jamaican and Mexican TEV isolates. Several Chinese and Jamaican isolates grouped in same cluster but also they built distant clades. Complete sequences of the TEV isolates (MK688996, MK688997 and MK680813) from Trinidad and Tobago have been recorded recently. These isolates clustered more distantly from Turkish isolates in the third clade. However the closest isolate to Turkish ones from the world was SD1 registered as AY787757 from China (Fig. 2).

Virulence properties TEV isolates against resistance sources and improved pepper lines

Two isolates (774 and 1002) clustered different group both molecular related their CP region and geographic origin were evaluated against improved pepper lines and

homozygous reference pepper genotypes for the *Pvr4*, *pvr2¹*, *pvr2²* and *pvr2⁺* genes (or alleles) (Fig. 3). On the basis of ELISA results assessed one month post-inoculation on apical leaves, both TEV isolates multiplied in and infected to susceptible Yolo Wonder carrying *pvr2⁺* Yolo Y; *pvr2¹* and W4; *Pvr4* and many of the improved lines selected as resistant. Florida VR2 carrying *pvr2²* alleles resisted to isolate 1002 but infected by isolate 774. Improved lines 62 and 63 also resisted to isolate 1002 but resistance failed by inoculation of isolate 774 except two plants of line 63 (Table 2). Only one improved line 4, showed resistance to both isolates. *pvr2²* contributes resistance to TEV but virulent strains are overcome resistance *pvr2* alleles providing restriction of cell-to-cell movement or inhibition of viral coat protein accumulation (Pallard et al., 2002). *Pvr4* did not provide any resistance against TEV (Janzac et al., 2009).

Table 2. Numbers of resistant and susceptible individuals of improved lines and genotypes carrying *pvr* alleles to tobacco etch virus infections determined by DAS-ELISA

Improved Lines and genotypes carrying <i>pvr</i> alleles	TEV isolate 1002		TEV isolate 774	
	R	S	R	S
3	0	15	0	15
4	15	0	15	0
6	0	15	0	15
8	0	15	0	15
11	0	15	0	15
23	0	15	0	15
25	0	15	0	15
27	0	15	0	15
54	0	15	0	15
61	0	15	0	15
62	15	0	0	15
63	15	0	2	14
64	0	15	0	15
69	0	15	0	15
W4 (<i>Pvr 4</i>)	0	15	0	15
Florida VR2 (<i>pvr2²</i>)	15	0	0	15
Yolo Y (<i>pvr2¹</i>)	0	15	0	15
Yolo Wonder (<i>pvr2⁺</i>)	0	15	0	15

Related with resistance provided by the *Pvr4* dominant gene to potyviruses is not effective to prevent TEV (Janzac et al., 2009; Moury and Verdin, 2012). CM334 originated *Pvr4* and *pvr2³* resistance also is not effective for TEV (Janzac et al., 2009). Line 4 was improved from CM334 and Perennial which was carrying recessive alleles *pvr2³* and *pvr6* (Caranta et al., 1997) expressing resistance to TEV. Caranta and Palloix (1996) reported that resistance to several potyviruses can be controlled by recessive alleles and/or by a dominant allele from Criollo de Morelos 334 and by polygenic resistance in Perennial. In this study Line 4 which has been improved as resistant to both 1002 and 774 TEV isolates probably has *pvr2³* alleles from CM334 and *pvr6* Perennial along with some QTL (Quantitative trait loci).

Figure 2. Phylogenetic tree of Turkish Tobacco etch virus (TEV) isolates and registered nucleotides in NCBI corresponding to 687 nucleotides of the CP coding region with 1000 bootstrap replicates. Isolates 1002 and 774 in the frame were used to determine resistance of the improved lines

Figure 3. ELISA optical density absorbance at 405 nm of improved lines to 1002 and 774 isolates one month post inoculation

pvr2² and *pvr6* allele combinations were identified as providing resistance source to another potyvirus PVMV (Rubio et al., 2009). Systemic infection of TEV isolate 774 breaking down *pvr2²*-mediated resistance could be prevented by the combination between *pvr2³* and *pvr6* alleles. Lines possessing the *pvr2²* and *pvr6* genes resist to systemic infection by PVMV or ChiVMV potyviruses (Moury et al., 2005). Because of the lack of resistance ability of the *pvr2²* allele against isolate 774 it is suggested that TEV resistance resulted from complementary effects between recessive alleles transferred both resistant and susceptible parents. With respect to the pepper-TEV interaction, not much more alleles were characterized that confer complete resistance to TEV while eleven chromosomal regions were found to be associated with quantitative resistance to PVY (Caranta et al., 1997). Also heterozygosity of *pvr2* alleles has been found to increase resistance efficiency against potyviruses (Moury et al., 2005; Rubio et al., 2009). Kang et al. (2005) demonstrated that TEV (both highly transmitted by aphids and non wilting isolates) infectivity could be blocked by wild-type eIF4E in *pvr1* (234) and *pvr1²*. Murphy et al. (1998) indicated that *pvr1* does not confer resistance to Mex21 TEV isolate. Finally, pathogenic processes of TEV and potyviral resistance interactions determine resistance of improved lines.

Conclusion

Viral pathogens threaten to crop production world wide as a consequence of the evolution of viral genomes to overcome resistance genes in plants and to adapt vector organisms causing epidemics. In the last decade resistance breaking Tomato spotted wilt virus isolates have overcome *Tsw* (Tentchev et al., 2011), a local strain of Paprika mild mottle virus have broken *L3* mediated resistance (Luria et al., 2018) and poleroviruses exclusively transmitted by aphid have adapt to whiteflies (*Bemisia tabaci*)

as a vector (Ghosh et al., 2019) and Zucchini yellow mosaic virus has appeared in peppers (Verma et al., 2019).

Potyvirus is in the top ten list of plant viruses (Rybicki, 2015) and pepper (*Capsicum annuum* L.) affected by five major potyviruses including tobacco etch virus which has resistant breaking abilities on both many recessive *pvr* alleles and dominant *Pvr* genes. Conclusively, our results further emphasize the threat to pepper breeders and growers around the world especially in Turkey that evolution of virulent TEV isolates and the possibility of combining recessive alleles will be able to provide resistance to infection of tobacco etch virus. The mechanisms of multigenic resistance can be explored further and combination of resistance alleles against TEV need to be searched by associating host pathogen interactions.

Acknowledgements. This project was supported by General Directorate of Agricultural Research and Policies with TAGEM/13/ARGE/21 project number. Thanks to anonymous reviewers for their constructive comments.

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