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Original article

Identification of new sources of resistance to resistance-breaking isolates of tomato spotted wilt virus

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ABSTRACT

The tomato as both a fresh consumption and industrial product is one of the most profitable vegetables and has a large cultivation area in the world. Parallel to intense production activities, Tomato Spotted Wilt Virus (TSWV), like viral diseases, results in significant economic losses every year. Use of resistant cultivars is the most efficient and environmental-friendly method of fighting against these diseases. This study was conducted to develop new tomato genetic resources resistant to TSWV because of the *Sw-5* resistance breaking (RB) isolates that were determined in tomato cultivation areas. In this study, a total of 40 tomato materials including 15 lines, 9 commercial varieties and 16 wild genotypes were by tested with molecular and biological testing methods. Mechanical inoculation method was used for biological testing and SCAR marker was used in molecular analysis. *S. penellii*, *S. chmielewski*, *S. habrochaites*, *S. peruvianum* and *S. sitiens*, LA0716, LA1028, LA1777, LA2744 and LA4110 genotypes were found as resistant against breaking isolates of Tomato Spotted Wilt Virus. These genotypes may be a good resistance source for the future breeding studies in tomato.

1. Introduction

The tomato (*Solanum lycopersicum* L.) belongs to the *Solanaceae* family and has 2n = 24 chromosomes (Peralta et al., 2006). The tomato originated from Peru, Equator, Galapagos Islands and mountainous sections of Chili (Chetelat et al., 2009). There are about 200 diseases which include viruses, bacteria, nematodes and fungi in the tomato (Agrios, 1988; Jones et al., 1991). Among the viruses, Tomato Spotted Wilt Virus (TSWV) was first encountered in 1906 and designated as "spotted wilt of tomato" by Brittlebank in 1919 (Stevens et al., 1992). Tomato Spotted Wilt Virus (TSWV) is placed in the second position among the top 10 virus diseases (Scholthof et al., 2011). TSWV is known to cause an average loss of yield of 1 billion dollars each year and is one of the most intensively studied plant viruses due to the future economic importance of TSWV. TSWV is large geographically widespread over the tomato cultivated lands and a large series of hosts, thus resulting in serious economic losses (German et al., 1992; Krishna Kumar et al., 1993; Mumford et al., 1996). The disease may result in 60–100% yield loss (Roselló et al., 1996). Infected tomatoes exhibit diverse symptoms such as empurpling in veins over the surface of lower leaves and rarely purple spots in between the veins. Normally, in a short period after infection, small yellowish necrotic spots are common over the surfaces of upper leaves. Then, spots get a characteristic bronze color (Roselló et al., 1996). *Frankliniella occidentalis* and *Thrips tabaci* are the most effective vectors in the spread of TSWV, and they are common in field and greenhouse tomatoes (Coutts and Jones, 2005). TSWV is also transmitted by the seeds (Le, 1970; Ming, 1993). The chemical control of TSWV is difficult due to its transmissibility by thrips species, which is a large host. The development and use of resistant cultivars are one of the best alternatives in controlling TSWV dues to its positive impacts on environment and human health (Zitter and Daughtrey, 1989). The resistant varieties against some isolates of TSWV were found in *Solanum esculentum* and *S. pimpinellifolium* (Finlay, 1953; Maluf et al., 1991; Roselló et al., 1997). Resistance was also reported in *S. hirsutum, S. chilense* and *S. peruvianum* (Roselló et al., 1998; Canady et al., 2001; Stevens et al., 1992). However, *S. peruvianum* was found to have broad resistance to all TSWV isolates

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(Paterson et al., 1989; Maluf et al., 1991; Kumar and Irulappan, 1992; Stevens et al., 1994). It was reported that the resistance in *S. peruvianum* is controlled by a single dominant gene *Sw-5* which is more stable and less isolate specific (Stevens et al., 1992, Stevens et al., 1995). Therefore, this resistance source has been widely used in tomato breeding programs (de Oliveira et al., 2018). The *Sw-5* has been genetically mapped between the markers CT71 and CT220 on chromosome 9 (Stevens et al.1995). In addition, Spassova et al. (2001) found that the *Sw-5* gene has five alleles along the chromosome 9, named *Sw5-a* to *Sw5-e*, and among them, *Sw5–b* is the functional allele for conferring resistance to TSWV. Molecular markers such as RFLP, RAPD, CAPs and SCAR are linked to *Sw-5* locus (Stevens et al, 1995; Chagué et al., 1996, Smiech et al., 2000; Langella et al., 2004; Dianese et al., 2010). The presence of *Sw-5* gene in tomato plants confers resistance to TSWV by a hypersensitive defense response that causes local lesions on the leaf, preventing the spread of the virus from the infection site through the plant (Aramburu et al., 2000). In addition, some isolates like TSWV6 from Spain and Italy have been reported to overcome the resistance provided by Tsw gene (Saidi and Warade, 2008). Aramburu and Martí (2003) reported that five isolates broke the Sw-5 resistance in north-east Spain. It was reported that the Sw-5 resistance breaking (RB) isolates were determined in tomato cultivation areas in Antalya province (Fidan and Sari, 2019a,b). As a result of the efforts to obtain a new resistance sources, *Sw-7* gene was determined which was conferred by a single dominant gene not linked to *Sw-5* and *S. chilense* was used as a source of this resistance gene (Stevens et al., 2007). It is important to identify different resistance sources against the TSWV virus (Saidi and Warade, 2008).

The aim of the present study was to determine the reactions of different wild tomato genotypes and lines against TSWV with molecular and biological tests to find new resistance sources.

2. Material and methods

2.1. Material

Forty tomato genotypes consisting of 15 pure lines, 9 commercial hybrids and 16 wild genotypes were used as plant material. The experiment was conducted at the Akdeniz University Faculty of Agriculture (36° 53́ 58.7544″ and 30° 39́ 4.7556″) (Table 1).

2.2. Method

2.2.1. Mechanical inoculation

Resistance breaking (RB) isolate (TSWVAntRB) was obtained from Akdeniz University Faculty of Agriculture Plant Protection.

2.2.2. Biological testing (TSWV inoculation)

As an inoculation material, diseased plant samples were supplied from virology laboratories of Plant Protection Department of Akdeniz University. Plant samples were initially subjected to ELISA test, and the isolate from tomato plant with a high disease intensity was used. ELISA testing used in identification were conducted in accordance with "Agdia TSWV Reagent Set". Seeds of tomato genotypes were sown for TSWV inoculation, and initial inoculations were performed when the 2–3 true leaves of the plants were seen. The phosphate buffer (1 lt 0.01 M) used in inoculation was prepared by supplementing 1 lt water with 5.253 g KH_2PO_4 (MA = 136.09) and 10.93 g $Na_2HPO_4{\cdot}2H_2O$ (MA = 177.99) (pH 7.0). Resultant solution was then supplemented with 1% Na_2-NO_3 and 0.1% Merchapto ethanol and preserved at + 4 °C. The source of inoculum was prepared by adding 200 g phosphate buffer to 100 g diseased plant sample.

Plant samples were placed into plastic mortars and smashed roughly as to pass the plant juice into the buffer. Plant residues were filtered through to remove them from the solution and carborundum powder was spattered to bruise leaf tissue for intrusion of disease agent into the solution. During these preliminary and inoculation processes, inoculum source was kept in ice. Inoculation was performed by rubbing the 2–3 true leaves with a sponge. Following this process, water was sprayed over the plants to prevent dry outs. A week later, a second mechanical inoculation was performed. Inoculations were performed in a controlled greenhouse at 26–30 °C temperatures and 60–70% relative humidity. The experiment was conducted in a completely randomized block with two replicates, and ten plants were tested each replicate and non-inoculated plants from each tomato materials were used as control plants. Scoring was carried out 21 days after the first inoculation.

Table 1
Plant material and origin of the tomato genotypes used in the experiment.

Genotype	Species	Origin	Genotype	Species	Origin
4	*S. lycopersicum*	BATEM	Torry F1	*S. lycopersicum*	SYGENTA SEED
6	*S. lycopersicum*	BATEM	Verty F1	*S. lycopersicum*	MULTI SEED
38	*S. lycopersicum*	BATEM	LA0116	*S. lycopersicum*	TGRC
70	*S. lycopersicum*	BATEM	LA0121	*S. pimpinellifolium*	TGRC
15	*S. lycopersicum*	BATEM	LA0247	*S. neorickii*	TGRC
228 2/1	*S. lycopersicum*	BATEM	LA0369	*S. pimpinellifolium*	TGRC
A218	*S. lycopersicum*	BATEM	LA0716	*S. penellii*	TGRC
9	*S. lycopersicum*	BATEM	LA1028	*S. chmielewski*	TGRC
31	*S. lycopersicum*	BATEM	LA1777	*S. habrochaites*	TGRC
34	*S. lycopersicum*	BATEM	LA1930	*S. chilense*	TGRC
50	*S. lycopersicum*	BATEM	LA1959	*S. chilense*	TGRC
141	*S. lycopersicum*	BATEM	LA1969	*S. chilense*	TGRC
191	*S. lycopersicum*	BATEM	LA2157	*S. arcanum*	TGRC
207/1	*S. lycopersicum*	BATEM	LA2623	*S. lycopersicum*	TGRC
229 1/2	*S. lycopersicum*	BATEM	LA2744	*S. peruvianum*	TGRC
Yeliz F1	*S. lycopersicum*	MONSANTO SEED	LA2931	*S. chilense*	TGRC
7870 F1	*S. lycopersicum*	PROTO SEED	LA3667	*S. lycopersicum*	TGRC
Tayfun F1	*S. lycopersicum*	ANTALYA SEED	LA4110	*S. sitiens*	TGRC
Vitellio F1	*S. lycopersicum*	SYGENTA SEED			
Bigmek F1	*S. lycopersicum*	MARS SEED			
İpekce F1	*S. lycopersicum*	BATEM			
Landolina F1	*S. lycopersicum*	SYGENTA SEED			

Batem = Bati Akdeniz Agricultural Research Institute, Tgrc = Tomato Genetics Resources Center, Monsanto, Proto, Antalya Seed, Sygenta, Mars Seed = Private Sector.

2.2.3. Molecular marker and PCR Amplifications

DNA was extracted from fresh leaves using a modified CTAB extraction protocol (Doyle and Doyle, 1990). Extraction buffer, which consisted of 1.4 M of NaCl, 20 mM of EDTA, 100 mM of Tris–HCL (pH 8), 2% CTAB, and 0.2% of beta-mercapto ethanol, was added in 0.6 mL of 0.2 g of fresh tomato tissue. The suspension was mixed with vortex and incubated at 60 °C for an hour. Next, chloroform–isoamyl alcohol (24:1) extraction was added to the solution, which was mixed with vortex for 10 s and centrifuged at 10000 rpm for 3 min. The supernatant was transferred to a fresh tube, and cold isopropanol (−20 °C) was added inside the micro tubes. The pellet formed after centrifugation at 13,100gn for 10 min was washed twice with 0.75 mL of 76% ethanol and 10 mM of ammonium acetate, and then re-suspended in sterile distilled water. The solution was incubated at 37 °C for 1 h and, afterwards stored at − 20 °C until use.

Sw5-2 primer in Table 2 was used for identification of *Sw-5* gene expressing resistance to TSWV (Dianese et al., 2010). Amplifications were performed in thermal cycler in a 10 μL final volume, containing 1 μL genomic DNA, 1X reaction buffer, 0,6 mM of $MgCl_2$, 0.7 mM of each dNTP, 0.5 μM of each primer and 0,1 μL of Taq DNA polymerase. For the marker of *Sw-5*, after initial denaturation for 2 min at 94 C, the PCR profile was as follows: 28 cycles of 30 s at 94 ^{0}C, 1 min 50 ^{0}Cs, 30 s 72 °C and a final extension of 5 min at 72 °C. PCR products were separated on a 1.5% agarose gel (Sigma, St. Louis, MO) and, visualized with ethidium bromide under UV light. In this study, the genotypes were identified as homozygote and heterozygote-resistant or susceptible according to their locus (Table 2).

3. Results

3.1. Biological testing results

Mechanical inoculation technique is a simple and quick method to screen a number of tomato genotypes simultaneously (Roselló et al., 1997). Symptoms were initially identified as small black spots over the upper leaves, then general dwarfing and dry out in plants. Plants were scored based on the presence or absence of the symptoms (Oguz, 2010). The results of mechanically tested tomato genotypes by TSWV mechanically was given in Table 3. Symptoms include numerous small brownish ringspots (see photo 1 Fig. 1b and c), that may be so prevalent that the leaves exhibit a bronzed appearance, purpling and upward rolling of leaves and stunting of leaves and plants. According to biological testing, 15 genotypes resistant and 25 genotypes susceptible genotypes were determined. The first detectable TSWV symptoms were observed in Ipekce F1 plants which were used as susceptible controls in experiment.

3.2. Molecular testing results

The *Sw5-2* marker was used in screening for *Sw-5* gene. Molecular marker results are given in Table 3. According to molecular selection, tomato genotypes were evaluated susceptible, homozygote resistance and heterozygote resistance 10, 23 and 7 respectively. Heterozygote resistant genotypes were yielded bands at 464–575 bp and 510–575 bp, susceptible genotypes and homozygote resistant genotypes were yielded bands at 464 bp and 575 bp respectively (Fig. 2).

4. Discussion

The *Sw-5* gene was conferred as dominant resistance to Tomato Spotted Wilt Virus and originated from *S. peruvianum* (Stevens et al., 1995). Environmental conditions such as high soil temperature is one of the most important factors in disturbing the resistance of the gene because foe example *Mi* gene loses its effectiveness at soil temperatures above 28 °C (Hu et al., 2015). De Ronde et al. (2019) describes a new class of temperature-sensitive resistance-breaking TSWV isolates that can be break up to 28 °C. Disease symptom development on leaves were determined at five days after inoculation (Fig. 1). As a result of molecular analyses on leaf samples that didn't show disease symptoms, the presence of infection was confirmed (Fidan and Sari, 2019a,b).

Present findings of molecular markers were similar with the results of Dianese et al. (2010). Three bands with different sizes were obtained in the PCR reaction. The first group (Stevens', 'Viradoro' and 'Santa Clara R' cultivars) was *S. peruvianum* 'PI 128660′, and the genotypes bearing *Sw-5* resistance gene homozygous yielded bands only at 575 bp. The second group ('Nemonetta' and 'Ohio 8245′ sensitive genotypes) yielded bands at 510 bp. The third group ('IPA-5′ isogenic line and 'Santa Clara S' cultivar and 6 selfed lines obtained from commercial cultivars) yielded bands at 464 bp. Researchers indicated the marker they used as co-dominant.

Although 14 tomato genotypes had *Sw-5* gene, seven tomato lines (15, 9, 31, 50, 141, 191, 229 1/2) and seven wild genotypes (LA0369, LA1930, LA1959, LA1969, LA2157, LA2931 and LA3667) showed disease symptoms in the experiment (Table 3). Although *Sw-5* reported as stable resistance against TSWV (Gullino et al., 2020) and this gene is deployed in commercial cultivars worldwide (Pappu et al., 2009), hypersensitive reactions were observed in studies even if this gene was present (Aramburu et al., 2000). When the plants are infected with disease, necrotic local lesions may appear on inoculated leaves even on plants carrying *Sw-5* gene (Jahn et al., 2000).

In addition, some studies have reported that high aggressivity and virulence isolates overcame the resistance conferred by *Sw-5* gene. Therefore, it is necessary to continue searching for new sources of resistance (Roselló et al., 1997). However, plants carrying *Sw-5* gene indicate that a small percentage of plants can be infected (Roselló et al., 2001). *Sw-5* resistance-breaking (SRB) isolates have been detected in Australia, Italy, Spain, California and Turkey (Latham and Jones, 1998; Aramburu and Martí, 2003; Ciuffo et al., 2005; Batuman et al., 2017; Deligoz et al., 2014). Fidan and Sari (2019a,b) identified the cause of the resistance-breaking genetic mutations on the virus genome, and a new resistance source is needed to protect the tomato from new RB strains. Our results confirm the *Sw-5* resistance-breaking isolates of TSWV.

It was determined that the symptoms on some varieties were evaluated very late and the plants didn't show any symptoms until the fruit stage (Mandal et al., 2017).

Table 2
Primer sequence for identification of *Sw-5* gene.

Gene	Primer Sequence	Homozygote resistant (bp)	Heterozygote-resistance (bp)	Susceptible (bp)	Literature
Sw-5	F:AAT TAG GTT CTT GAA GCC CAT CT R:TTC CGC ATC AGC CAA TAG TGT	575	464–575, 510–575	464, 510, 464–510	Dianese et al., 2010

Table 3
Result of to mechanical inoculation and molecular test of TSWV of the genotypes.

Genotype	Species	Biological Test	Molecular Test	Elisa Test
4	*S. lycopersicum*	S	S	+
6	*S. lycopersicum*	S	S	+
38	*S. lycopersicum*	S	S	+
70	*S. lycopersicum*	S	S	+
15	*S. lycopersicum*	S	R	–
228 2/1	*S. lycopersicum*	R	R	–
A218	*S. lycopersicum*	S	S	+
9	*S. lycopersicum*	S	R	–
31	*S. lycopersicum*	S	R	–
34	*S. lycopersicum*	R	R	–
50	*S. lycopersicum*	S	R	–
141	*S. lycopersicum*	S	R	–
191	*S. lycopersicum*	S	R	–
207/1	*S. lycopersicum*	R	R	–
229 1/2	*S. lycopersicum*	S	R	–
Yeliz F1	*S. lycopersicum*	R	HR	–
7870 F1	*S. lycopersicum*	R	HR	–
Tayfun F1	*S. lycopersicum*	R	HR	–
Vitellio F1	*S. lycopersicum*	R	HR	–
Bigmek F1	*S. lycopersicum*	R	HR	–
Ipekce F1	*S. lycopersicum*	Susceptible Control	S	+
Landolina F1	*S. lycopersicum*	S	S	+
Torry F1	*S. lycopersicum*	S	HR	–
Verty F1	*S. lycopersicum*	S	S	+
LA0116	*S. lycopersicum*	S	S	+
LA0121	*S. pimpinellifolium*	R	R	–
LA0247	*S. neorickii*	R	R	–
LA0369	*S. pimpinellifolium*	S	R	–
LA0716	*S. penellii*	R	R	
LA1028	*S. chmielewski*	R	R	
LA1777	*S. habrochaites*	R	HR	
LA1930	*S. chilense*	S	R	
LA1959	*S. chilense*	S	R	
LA1969	*S. chilense*	S	R	
LA2157	*S. arcanum*	S	R	
LA2623	*S. lycopersicum*	S	S	
LA2744	*S. peruvianum*	R	R	
LA2931	*S. chilense*	S	R	
LA3667	*S. lycopersicum*	S	R	
LA4110	*S. sitiens*	R	R	

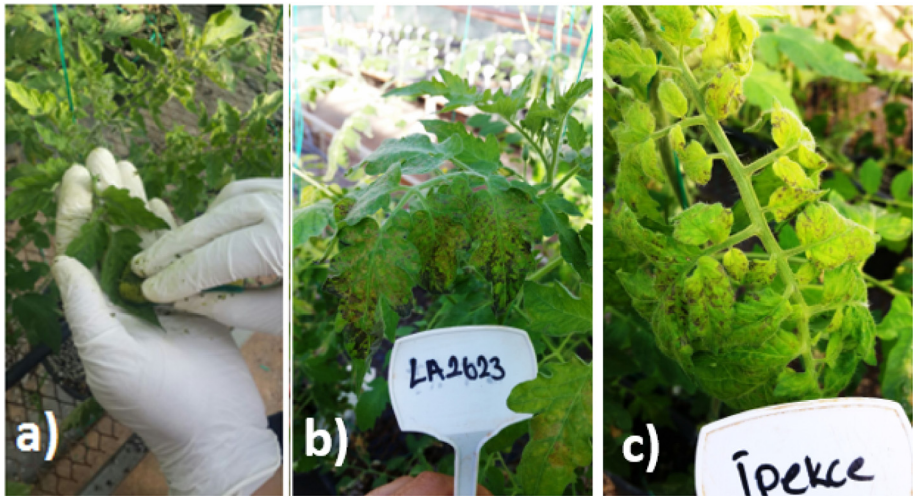

Fig 1. Mechanical inoculation on plants and disease symptoms, (a) Mechanical inoculation, (b) (c) Disease symptoms development on leaves.

In studies to find different genetic resources, *Sw-7*, which is a single dominant gene, has been identified as a resistance gene source identified from *S. chilense* (Stevens et al., 1994). This wild genotype is suitable for use as a resistance source against TSWV in field conditions (Canady et al., 2001). It has also been determined that *Sw-7* is not associated with *Sw-5*. (Stevens et al., 2007).

Padmanabhan et al. (2019) determined that the *PR5* gene controls the strength and extensibility of the plant primary cell wall, and this gene restricts virus movement from cell to cell through induction of callose deposition in the cell wall, resulting in resistance to TSWV. As a result, virus particles do not cause the systemic infections.

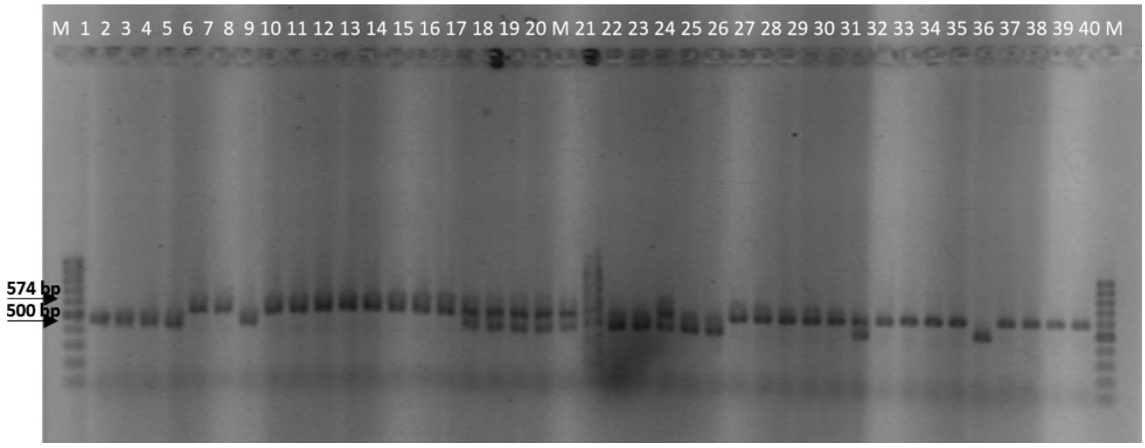

Fig 2. Agarose gel electrophoresis representing PCR products obtained by *Sw-5–2* primer pair. M: 100 bp DNA ladder; homozygous resistant alleles (574 bp); heterozygous resistant alleles known as resistant tomato varieties (574–510 bp); without *Sw-5* allele (500 bp).

According to the results, new resistance sources were determined against TSWV from the tomato germplasm which include *S. penellii*, *S. chmielewski*, *S. habrochaites*, *S. peruvianum* and *S. sitiens.* The genotypes LA0716, LA1028, LA1777, LA2744 and LA4110 respectively can be used as a resistance source in breeding studies.

5. Conclusion

Tomato Spotted Wilt Virus (TSWV) is one of the most destructive viruses in the world, and it is known to cause damage on cultivated plants such as pepper, tomato, eggplant and lettuce. *Sw-5* gene refers to resistance of this disease, but *Sw-5* resistance-breaking (SRB) isolates have been detected in some countries. The *Sw-5* resistance-breaking isolates of TSWV cause a big problem in production areas because this disease can remain hidden until the harvest period. Due to this situation, it can be a source of new infections by keeping its existence in the greenhouse throughout the period. Producers can be affected negatively in terms of loss of yield and time. It is extremely necessary to search for new sources of resistance. This new resistance source will be useful for the development of TSWV resistant cultivars in tomato.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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