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Short Notes

Increasing diversity of resistance breaking pepper strains of *Tomato spotted wilt virus* in the Mediterranean region

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Summary. *Tomato spotted wilt virus* (TSWV) is an important plant pathogen, causing economic impacts on crop production, especially in vegetable crops, including pepper. Resistance breeding is the most effective technique to manage TSWV epidemics. In pepper, the *Tsw* resistance gene is used. However, rapid emergence of resistance breaking (RB) strains of TSWV has hampered the control of TSWV. RB strains have previously shown clear geographic distribution that parallel each similar wild type (WT) strain. The present study collected pepper-infecting RB TSWV strains in limited districts of Spain and Turkey, and these strains clustered to two main clades based on the NSs protein amino acid sequences. Results verified the coexistence of the different strains in both countries. On the basis of amino acid sequence comparison of the collected isolates, common alteration responsible for resistance breaking was not identified in accordance with the preceding observations. These results emphasize the increasing diversity of the RB TSWV strains.

Keywords. TSWV, *Capsicum annuum*, phylogenetic analysis.

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Tomato spotted wilt virus (TSWV) has a broad host range, including economically important horticultural plants (Adkins *et al.,* 2000; Parrella *et al.,* 2003; Scholthof *et al.*, 2011), and has considerable economic impacts on vegetable production. TSWV has become one of the most important viruses of pepper (Pappu *et al.*, 2009), and pepper breeding for management of this virus has become increasingly important. The main vector of TSWV in pepper cultivation is *Frankliniella occidentalis* (Pergande) in glasshouses or plastic tunnels, while *Thrips tabaci* (Lindeman) has significant impacts in epidemics in open fields.

Management of TSWV based on thrips control is difficult and inefficient. Resistance breeding is a promising virus management strategy, but only the *Tsw* resstance gene is currently available against TSWV in pepper cultivation. Worldwide use of pepper varieties harbouring the *Tsw* gene has resulted in the rapid emergence of resistance breaking (RB) TSWV strains. These strains were first reported in Mediterranean pepper producing regions from Italy (Roggero *et al.*, 1999; 2002) and Spain (Margaria *et al.,* 2004), and

have later been reported from Australia (Thomas-Carroll *et al.,* 2003), Turkey (Deligoz *et al*., 2014), Argentina (Ferrand *et al.,* 2015), and in California, United States of America (Macedo *et al.,* 2019). Although TSWV has been reported in Korea only in the 2000s (Kim *et al.,* 2004), RB strains were identified less than 10 years later (Chung *et al.,* 2012; Hoang *et al.,* 2013).

TSWV is the type member of the genus *Orthotospovirus* (*Tospoviridae*, *Bunyavirales*). The genome of TSWV is composed of three single-stranded RNA segments; the L RNA has negative polarity while the M and the S RNAs are ambisense. The avirulence factor (avr) is the NSs protein in the case of the *Tsw* gene in pepper, translated from the S RNA (Margaria *et al.,* 2007; de Ronde *et al.,* 2013). The NSs protein is multifunctional, also having RNA silencing suppressor (RSS) activity (Takeda *et al.,* 2002; de Ronde *et al.,* 2014). Although an amino acid alteration (NSs T104A) was determined to be responsible for resistance breaking of the HUP2-2012-RB isolate (Almasi *et al.*, 2017), this alteration is not generally present in most of the RB isolates, and other common substitutions responsible for resistance breaking have not been identified. In contrast to RB strains of other viruses, resistance breaking of TSWV in pepper is not linked to a universal specific amino acid alteration of the NSs. Resistance breaking could emerge strain by strain due to substitutions at various amino acid positions.

In each geographic region, the RB and the wild type (WT) strains cluster to the same branch of the phylogenetic tree (Lian *et al.,* 2013; Almasi *et al.,* 2015; French *et al.,* 2016; Macedo *et al.,* 2019), demonstrating the isolated emergence of the RB strains and that virus transport has had a minor role in the general appearance of the resistance breaking strains. However, reassortment and recombination events could play roles in TSWV evolution (Margaria *et al.,* 2015) and in new strain development, as Fontana *et al.* (2020) have recently reported for population structure in the Mediterranean basin.

Diseased fruit samples of various pepper cultivars were collected from important pepper growing areas in Turkey and Spain (Table 1). The samples were tested for the most relevant pepper infecting viruses (TMV, PVY, CMV TSWV) by DAS-ELISA, using Bioreba antisera according to the supplier's instructions. All samples were negative for TMV, PVY, and CMV, but positive for TSWV. *Nicotiana tabacum* L. cv. Xanthi test plants were inoculated and the symptom phenotypes confirmed the results of the ELISA tests. Test plant assays with susceptible *Capsicum annuum* cv. Galga plants and TSWV resistant *C. annuum* cv. Brody plants further confirmed that all of the collected TSWV isolates were of the resistance breaking phenotype (Figure 1).

Total nucleic acid was extracted from diseased pepper fruits (White and Kaper, 1989). First-strand cDNAs were synthesized (RevertAid Reverse Transcriptase, Thermo Scientific), followed by the amplification of the NSs genes by RT-PCR using specific primer pairs TSWV-NSs SacI For 5'-GGGAGCTCAGAGCAATTG TGTCATAATTTTATTCTTAATCAAACCT–3' and TSWV-NSs BamHI Rev 5'-GGGGATCCGGACAT-AGCAAGAATTATTTTGATCCTGAAGCATATG–3' (Almasi *et al.,* 2015). The PCR products were cloned into pGem®-T Easy vector (Promega) according to standard protocols. Nucleotide sequences of five clones of each isolate were determined (Biomi Ltd.), and were deposited to the GenBank (Table 1).

Relationships between the different pepper infecting isolates were determined by phylogenetic analysis based on the deduced amino acid sequences of the NSs proteins. The maximum likelihood tree was composed

Table 1. *Tomato spotted wilt virus* isolates collected from pepper and characterised in this study. Isolate name, and origin location, host pepper cultivar, and GenBank accession numbers are indicated.

Isolate	Location	*Capsicum annuum* cultivar	GenBank Accession No.
P1 Alm	El Ejido, Almería, Spain	Icaro	MK922146
P2 Alm	El Ejido, Almería, Spain	Souleria	MK922147
P3 Alm	El Ejido, Almería, Spain	Olimpiakos	MK922148
P4 Alm	El Ejido, Almería, Spain	Icaro	MK922149
P5 Ant	Demre, Antalya, Turkey	Doddo (ex. Greeno)	MK922150
P6 Ant	Demre, Antalya, Turkey	Benino	MK922151
P7 Ant	Demre, Antalya, Turkey	Belissa	MK922152
P8 Ant	Kumluca, Antalya, Turkey	Souleria	MK922153
P9 Ant	Demre, Antalya, Turkey	Briot	MK922154
P10 Ant	Kumluca, Antalya, Turkey	Benino	MK922155
P11 Ant	Kumluca, Antalya, Turkey	ESC 15218	MK922156

Figure 1. Host symptoms of the isolated TSWV strains mechanically inoculated on resistant (*Capsicum annuum* cv. Brody) and susceptible (*C. annuum* cv. Galga) pepper cultivars.

using the Mega 7.0 software (Kumar *et al.,* 2016; Nei and Kumar, 2000) (Figure 2). The different pepper strains of TSWV retrieved from the GenBank (Table 2) and the strains isolated in the present study clustered in two main clades. Clade I built up of Spanish, Northern Italian and two Brazilian (RB and WT) strains, while Clade II consisted of strains from Southern Italy, Hungary, France and Korea (Figure 2). Consistent with previous studies, sequences of the pepper strains of TSWV clustered according to their geographic localization (Kim *et al.,* 2004), although this could evolve due to host and environmental factors (Jiang *et al.,* 2017). Except for one strain (P1Alm ESP), the isolates collected in the present study from Spain did not cluster together with previously collected Spanish isolates (Clade I), but they were located in Clade II, close to the South-Italian and Hungarian strains (P2Alm ESP, P3Alm ESP, P4Alm ESP). The strains collected from Turkey also clustered into different main clades. Except for one strain (P5Ant), the Turkish isolates were located on Clade I (P6Ant TUR, P7Ant TUR, P8Ant TUR, P9Ant TUR, P10Ant TUR, and P11Ant TUR). The P5Ant TUR isolate was located on Clade II, close to Spanish isolates characterized in this study (Figure 2). To date, the NSs gene sequence originating from pepper has not been recorded from Turkey. Recently, the coding region of the nucleocapsid protein (N gene) of an RB strain was determined from the cropping period of 2015 (Güneş and Gümüş, 2019). The N gene sequence of the Turkish RB strain clustered together with the Clade II isolates.

The resistance breaking phenotype of a virus strain can be confirmed by test plant assay, since resistant pepper cultivars bearing the *Tsw* gene respond with hypersensitive response (HR) to the inoculation with WT strains while TSWV infection on susceptible pepper cultivars induce systemic symptoms. In the case of an RB strain, systemic symptoms are induced on both of the cultivars. The NSs gene was identified as avr factor (Margaria *et al.,* 2007; de Ronde *et al.,* 2013), so characterization of the differences in the amino acid sequences of the NSs proteins is crucial for determining the background of resistance breaking nature of the isolates.

The pairwise comparisons of the eleven unique NSs sequences showed similarity between 93.3 and 99.8% among the isolates. Previous studies demonstrated isolated emergence of the RB strains, so the amino acid

Table 2. GenBank accession numbers of the TSWV strains selected in this study for phylogenetic analyses.

Strain	Origin	Strain type	GenBank accession number
P71-1	Spain	*	FR693011
P67-2	Spain	*	FR693007
P65-2	Spain	*	FR693005
P229	Spain	*	FR692918
P228	Spain	*	FR692917
P195	Spain	*	FR692895
P114	Spain	*	FR692852
P125	Spain	*	FR692857
P155	Spain	*	FR692871
P90	Spain	*	FR693023
P203	Spain	*	FR692900
P86-1	Spain	*	FR693020
VE427	Spain	RB	DQ376185
VE430	Spain	WT	DQ376184
P259	Spain	*	FR692932
P105-43.14	North-Italy	RB	DQ376182
P267	North-Italy	RB	DQ376180
P105-RB-MaxII	North-Italy	RB	HQ839731
P105-1	North-Italy	RB	DQ376177
P105/2006RB	North-Italy	RB	DQ915946
P272	North-Italy	RB	DQ376181
P105-44.7	North-Italy	RB	DQ376183
P105	North-Italy	WT	DQ376178
P105-RB-Mar	North-Italy	RB	HQ839729
P105-RB-MaxI	North-Italy	RB	HQ839730
P166	North-Italy	RB	DQ376179
BR20WT	Brazil	WT	DQ915948
BR20RB	Brazil	RB	DQ915947
TSWV-Gneung	Korea	*	AB643671
TSWV-Njc	Korea	*	AB643673
TSWV-Ghae	Korea	*	AB643672
France-81	France	*	FR692829
TSWV-Pap	Korea	*	AB643674
HUP4-2012-WT	Hungary	WT	KJ649611
HUP2-2012-RB	Hungary	RB	KJ649609
P170	South-Italy	WT	DQ431237
CAA19	France	*	FR692822
p202/3WT	South-Italy	WT	HQ830187
p202/3RB	South-Italy	RB	HQ830186
p202	South-Italy	RB	DQ398945

Strain type: RB resistance breaking, WT wild type, * not known.

variations of the NSs proteins of RB strains (isolated in this study) were compared to the NSs proteins of the WT strains located in closest position on the phylogenetic tree. This showed the greatest similarity to the different strains (for accession numbers see Table 2).

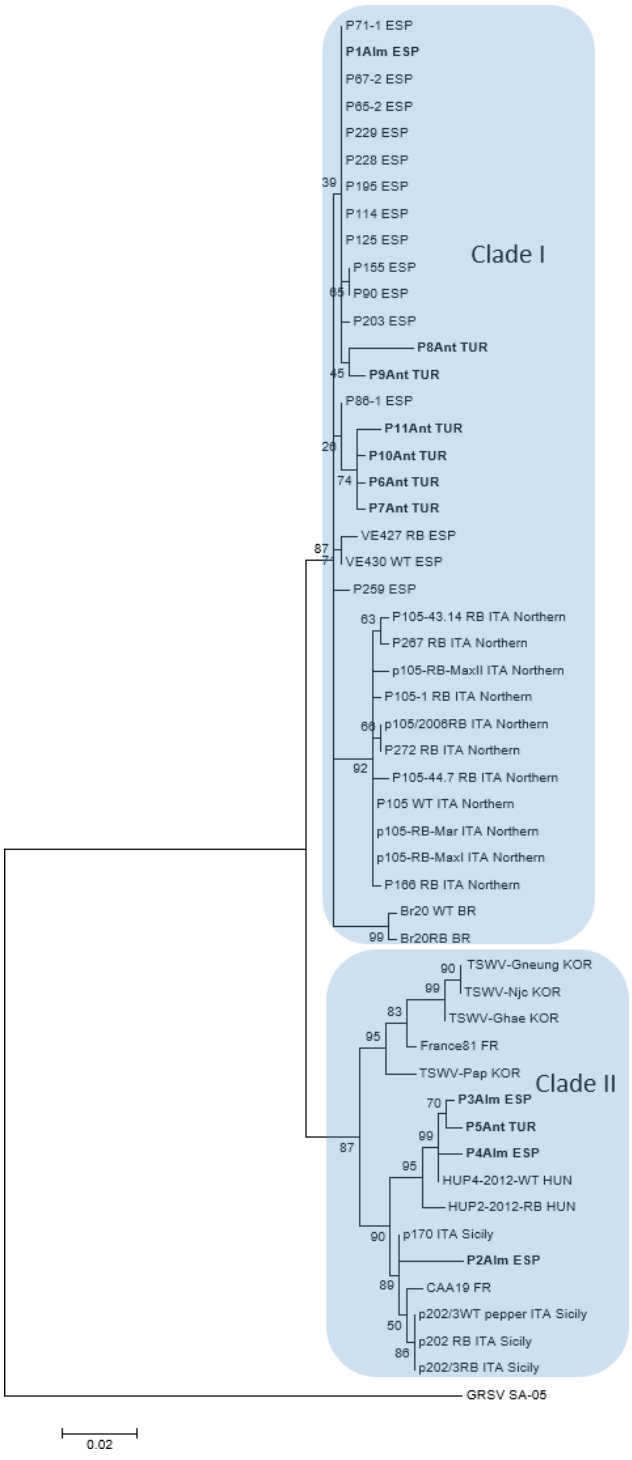

Figure 2. Phylogenetic tree based on the deduced amino acid sequences of the NSs protein of TSWV strains originated from pepper Maximum likelihood tree (1000 bootstrap replicates) was composed of TSWV strains retrieved from GenBank (see accession numbers in Table 2) and newly isolated strains in bold font (Table 1). *Groundnut ringspot virus* strain SA-05 (GenBank acc. number JN571117) was used as the outgroup. Two main clades (Clade I and Clade II) are indicated.

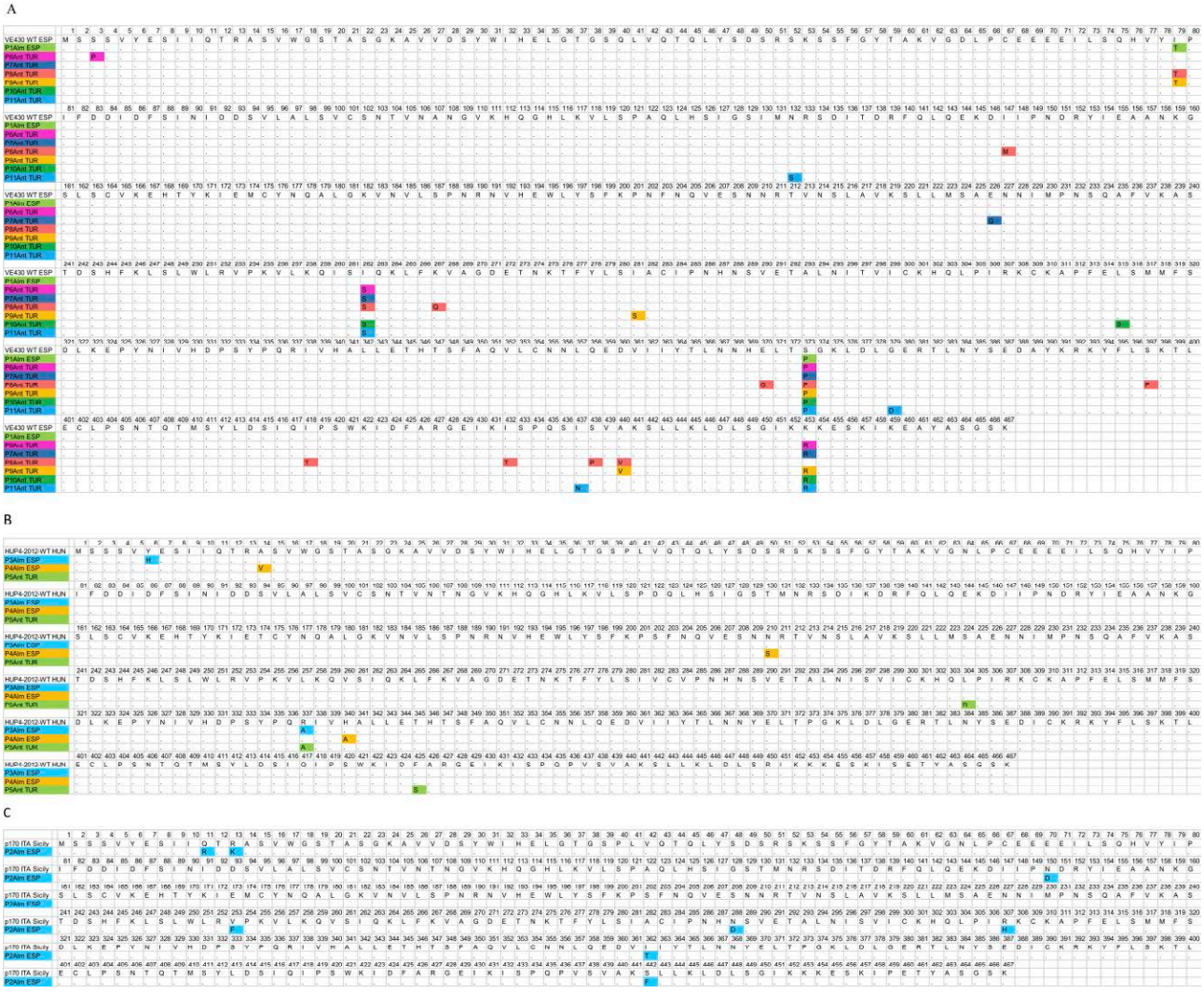

Figure 3. Amino acid sequence comparison of the NSs proteins of the isolated TSWV strains. The NSs amino acid sequences of RB strains (isolated in this study) were compared to the NSs amino acid sequences of the WT strains located in closest position on the phylogenetic tree. **A**: Strains P1Alm ESP, P7Ant, TUR, P8Ant TUR, P9Ant TUR, P10Ant TUR and P11Ant TUR are compared to the Spanish VE 430 WT ES strain. **B:** Strains P3Alm ESP, P4Alm ESP and P5Ant TUR are compared to the Hungarian HUP4-2012-WT HUN strain. **C:** Strain P2Alm is compared to the Italian P170 WT strain.

Strains P1Alm ESP, P7Ant TUR, P8Ant TUR, P9Ant TUR, P10Ant TUR and P11Ant TUR were compared to the Spanish WT strain VE 430 WT ESP. For strains P3Alm ESP, P4Alm ESP and P5Ant TUR, the Hungarian WT strain HUP4-2012-WT was chosen for comparison while P2Alm ESP was compared to the p170 ITA Sicily strain (Figure 3). The numbers of differences were between two and 11 amino acids. For strains P1Alm ESP and P3Alm ESP, only two (respectively, I79T, S373P and Y6H, R337A) substitutions were recognized. The NSs of P4Alm ESP and P5Ant TUR contained three amino acid alterations. P6Ant TUR, P7Ant TUR, and P10Ant TUR had four amino acid residues differences. P9Ant TUR differed in five amino acid residues, P11Ant TUR in six, and P2Alm ESP in eight. The most substitutions were recognized for P8Ant TUR, with 11 amino acid differences. In accordance with previous results, no conserved amino acid changes were identified in the different RB isolates. The recently published single point mutation responsible for resistance breaking (T104/A) (Almási *et al.*, 2017), was not present in any of the isolates.

To date, the phylogenetic relationships of the RB TSWV pepper strains showed the closest similarity to

the WT TSWV strains with the same geographical origin (Tsompana *et al.,* 2004; Lee *et al.,* 2011; Tentchev *et al.,* 2011; Almási *et al.,* 2015). The RB strains analyzed in the present study clustered into diffuse positions on the phylogenetic tree, indicating the currently occurring increasing diversity of RB TSWV strains. This emphasizes the expansion and simultaneous occurrence of the different TSWV strains infecting pepper in the Mediterranean basin.

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