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Short communication

Evidence of a tomato spotted wilt virus resistance-breaking strain originated through natural reassortment between two evolutionary-distinct isolates

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ABSTRACT

A *Tsw* resistance-breaking tomato spotted wilt virus field isolate (TSWV-p331) found in northern Italy originated via reassortment from two evolutionary distinct TSWV strains, as revealed by recombination and phylogenetic analysis. Compared to the closest isolate present in the database, p331 NSs protein carries an unusually high number of amino acid substitutions, but no differences in the nucleocapsid protein. Despite these substitutions, p331 NSs is a potent silencing suppressor. As shown by phylogenetic analyses of TSWV nucleocapsid sequences collected over fifteen years, one likely p331 parental lineage has never been detected in northern Italy, allowing speculations on the origin of TSWV-p331.

Tomato spotted wilt virus is the type species of the genus *Tospovirus* in the family *Bunyaviridae* (Scholthof et al., 2011). Members of this species are able to infect more than 1000 plant species worldwide, and cause severe damage to many economically important crops including bean, lettuce, pepper, potato, tobacco, tomato and numerous ornamental species (Pappu et al., 2009). In nature, TSWV is transmitted exclusively by thrips species in the genera *Frankliniella* and *Thrips*; *Frankliniella occidentalis* Pergande, the western flower thrips, is its most efficient vector (Whitfield et al., 2005).

TSWV has enveloped, quasi/spherical particles, 80–110 nm in size, containing three RNA genomic segments, designated large (L), medium (M) and small (S) (Goldbach and Peters, 1996). The L RNA possesses a single open reading frame (ORF) in the viral complementary sense, coding for the viral RNA dependent RNA polymerase (RdRp). The M and S RNAs encodes for two proteins each: specifically, in the viral sense for the non-structural proteins NSm and NSs, respectively, shown to be the movement protein and the silencing suppressor, and in the viral-complementary sense for the glycoprotein precursor (Gn/Gc) and the nucleocapsid (N) protein, respectively (Goldbach and Peters, 1996).

The tripartite genome offers the potential to exchange entire genomic segments among different isolates co-infecting the same plant, giving rise to new TSWV variants. This mechanism is designated as reassortment: first evidence of its occurrence for TSWV was provided by Best in 1961 (Best, 1961). Given the absence of a reverse-genetic system for this virus, reassortment has been used in experimental conditions to map the avirulence determinant necessary to overcome the *Sw5* resistance gene in tomato (Hoffmann et al., 2001) and the *Tsw* resistance gene in pepper (Jahn et al., 2000; Margaria et al., 2007). The same approach was also used in *F. occidentalis* to map the Gn/Gc and NSs proteins as thrips vector transmission determinants (Sin et al., 2005; Margaria et al., 2014a).

Reassortment was shown to occur spontaneously in TSWV (Qiu and Moyer, 1999). The occurrence of extensive reassortment events for isolates from Spain, France and Italy, showed that this mechanism plays an important role in TSWV emergence and epidemics (Tentchev et al., 2011). Reassortment exchanges have been predicted between Asiatic and European TSWV populations (Tentchev et al., 2011), as well as among isolates from Korea (Lian et al., 2013) and New Zealand (Timmerman-Vaughan et al., 2014), and are likely at the origin of one of the two TSWV lineages present in Italy (Margaria et al., 2014b). In this context, it should be mentioned that a broad analysis on reassortment events is still hampered by the

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limited number of full-length TSWV genomic sequences available in GenBank. In fact, no evidence of reassortment or recombination was obtained in a previous analysis with natural TSWV isolates collected from several geographical locations in the United States and Europe (Tsompana et al., 2005), possibly due to the lack of complete genomic sequence data, especially for the L genomic segment.

In Italy, a TSWV population survey carried out at the beginning of the twenty-first century in Apulia region grouped the isolates into two molecularly distinct subpopulation clusters, named TSWV-A or TSWV-D like, according to their similarity with type-isolates from the USA (TSWV-A) or the Netherlands (TSWV-D) (Finetti-Sialer et al., 2002). In this analysis, a collection of isolates sampled during virus epidemics in Apulia from 1999 to 2001 was considered, but no strains from northern Italy were included (Finetti-Sialer et al., 2002). A recent phylogenetic analysis based on the N gene of several isolates from the Mediterranean region, also showed that Italian isolates clustered in two distinct sub-populations, in contrast to other countries, such as France, Spain, and Bulgaria, where TSWV isolates grouped uniformly into one population (Turina et al., 2012). Despite the presence of evolutionary-distinct isolates in the same geographic area, reassortment between isolates from the two clades has not been reported so far in nature in Italy.

Two TSWV isolates from Italy, p105 and p202/3WT, have been recently fully sequenced and characterized (Margaria et al., 2014b). Isolate p105 was collected from a pepper field in Liguria, northern Italy, while p202/3WT was collected in pepper in Sicily, southern Italy. Phylogenetic analyses grouped the two strains into the two separated clades previously reported in Italy (Finetti-Sialer et al., 2002), placing p105 in the TSWV-A clade and p202/3WT in the TSWV-D clade (Margaria et al., 2014b). In summer 2012, an outbreak of TSWV was observed in Carmagnola (Piedmont, Northern Italy), a district where sweet pepper crops are intensively cultivated and where the use of pepper varieties carrying the *Tsw* resistance gene is still limited. Isolate p331 was collected in a field where resistant peppers were grown. This isolate was passaged three times through single-local lesion transfer on *Nicotiana tabacum* cv. White Burley and then on *Capsicum chinensis* PI152225 carrying the *Tsw* gene, where it was still able to overcome resistance as the original field isolate. In this work, we characterized the p331 genome, and we found that molecular markers for the L and S segment grouped consistently with p202/3WT-like isolates (TSWV-D clade), while those for the M segment grouped with p105-like isolates (TSWV-A clade).

In order to characterize p331 genomic segments, we used five molecular markers located along the whole genome: the Gn/Gc, NSs and N markers consisted of a fragment in the open reading frame (ORF) of the corresponding gene, being 692, 587 and 774 nt in length, respectively; the RdRp and NSm markers, beside a fragment of the corresponding gene, included also a portion of the 3′-untranslated region and intergenic region, respectively, and were 310 and 878 nt in length. Total RNA was extracted from systemically infected *N. benthamiana* leaves using the "Spectrum™ Plant Total RNA Kit" (Sigma–Aldrich, St. Loius, MO, USA). Reverse transcription was performed using the specific reverse primer (Supplementary Table 1) with the "Thermoscript™ RT-PCR" kit (Invitrogen, Grand Island, NY, USA), and followed by PCR using PolyTaq polymerase (Polymed, Florence, Italy) and primers reported in Supplementary Table 1. Amplification products were cloned using the "pGEM-T Easy vector" system (Promega, Madison, WI, USA) and sequenced (see Supplementary Table 1 for GenBank accession numbers). A first analysis of possible recombination events was performed using Recombination Detection Program 4.16 (RDP4) software (Martin and Rybicki, 2010). For this purpose, the sequences of the five markers of each isolate were concatenated together in a single sequence file, in the following order: RdRp, NSm, Gn/Gc, NSs and N marker, giving rise to an artificial full-length contig of about 3300 nt. Markers for p105 and p202/3WT isolates were retrieved by selecting the same target regions from the sequences available in GenBank (Margaria et al., 2007, 2014a,b). Isolates p105 and p202/3WT were chosen for comparison because they are the closest isolates present in the database for which full length genomic sequence is available (Margaria et al., 2014b). Sequences were aligned using MEGA6 software according to the Clustal W algorithm (Tamura et al., 2013), and then run in RPD software, at default parameter values. Three recombination events were consistently found by different detection algorithms: recombination event number 1 concerned the whole S segment markers (N and NSs), and event number 2 concerned the whole L segment marker (Table 1). Both events were detected at stringent *P*-value scores (lowest confidence was at E-05), and identified p202/3WT as the contributing parental for p331 for the L and S genomic segments. A third recombination event identified the p105 as the major parent contributing to p331 for the whole M segment, at stringent *P* values (Table 1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.11.012.

Similarity percentages and potential reassortment analysis among the isolates in study was further performed by scanning of the concatenated marker sequences with SimPlot v. 3.5.1 (Lole et al., 1999), at default values. As shown in Fig. 1, p331 high sequence similarity was found with p202/3WT in the L (RdRp marker) and S (NSs and N marker) segments, while for the M segment, the highest similarity was between p331 and p105. We further obtained similarity matrix scores for the markers in study using MatGat v. 2.03 (Campanella et al., 2003): sequence analysis confirmed higher percentage scores between p331 and p202/3WT for the L and S segment markers (98.4, 99.5 and 99.6% for RdRp, NSs and N markers, respectively), and between p331 and p105 for the M markers (97.8 and 90.5%, for Gn/Gc and NSm, respectively) (Supplementary Table 2). The nucleotide alignments of the five markers are provided in Supplementary Fig. 1.

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Phylogenetic analyses according to the Neighbor-Joining method (Saitou and Nei, 1987) using the alignment of the concatenated markers for each genomic segment, grouped p331 in the p202/3WT clade for the L and S segment, and in the p105 clade for the M segment (Fig. 1). Together, these results support different origin for the genomic segments of p331 and consistently provide direct evidence that p331 was derived through reassortment between two evolutionary distinct TSWV isolates; the two possible parental isolates used for comparison are both unable to overcome *Tsw* resistance.

Presence of a TSWV-D-like strain was never witnessed before in northern Italy, and therefore we decided to investigate a number of isolates from northern Italy present in the PLAVIT (Plant Viruses Italy, http://www.wfcc.info/ccinfo/index.php/collection/by_id/1057/) collection. For this study, we newly sequenced the N gene of seven isolates collected during TSWV outbreaks in northern Italy (Piedmont, Liguria and Lombardy regions) from 1997 to 2014. Sequences were used for phylogenetic analysis, together with the sequence of TSWV isolates from northern and southern Italy previously characterized (Margaria et al., 2007; Turina et al., 2012; Zindovic et al., 2014). Nucleocapsid protein sequences showed high similarity among all isolates (not shown), as expected; in fact, amino acid changes in this protein are thought to be deleterious for the virus (Tentchev et al., 2011). However, phylogenetic analysis on the nucleotide sequences showed that all the isolates from northern Italy grouped with p105, with the exception of isolate p331 which grouped in the p202/3WT clade (Fig. 2), suggesting that this