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Short Communication

Evolutionary analysis of tomato *Sw-5* resistance-breaking isolates of *Tomato spotted wilt virus*

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Tomato spotted wilt virus (TSWV) causes severe economic losses in many crops worldwide and often overcomes resistant cultivars used for disease control. Comparison of nucleotide and amino acid sequences suggested that tomato resistance conferred by the gene *Sw-5* can be overcome by the amino acid substitution C to Y at position 118 (C118Y) or T120N in the TSWV movement protein, NSm. Phylogenetic analysis revealed that substitution C118Y has occurred independently three times in the studied isolates by convergent evolution, whereas the substitution T120N was a unique event. Analysis of rates of non-synonymous and synonymous changes at individual codons showed that substitution C118Y was positively selected.

Tomato spotted wilt virus (TSWV) is one of the most economically important plant viruses and causes damage in many agronomic crops worldwide (Adkins, 2000). TSWV is the type member of the genus *Tospovirus*, which contains the only plant-infecting members of the family *Bunyaviridae*. The genome consists of three negative-sense or ambisense RNA segments: segment L encodes a putative RNA-dependent RNA polymerase (de Haan *et al.*, 1991); segment M encodes the cell-to-cell movement protein, NSm (Li *et al.*, 2009), and the precursor of the surface glycoproteins, G_N/G_C, involved in TSWV transmission by thrips (Sin *et al.*, 2005; Naidu *et al.*, 2008); and segment S encodes a silencing suppressor, NSs (Takeda *et al.*, 2002), and the nucleocapsid, N (de Haan *et al.*, 1990).

Controlling TSWV in crop plants has proven difficult because of the wide range of plant hosts (>1000 species) and effective spread of TSWV by thrip vectors (Hanssen *et al.*, 2010). Intense efforts have been made around the world to obtain genetically resistant cultivars against TSWV. Only the dominant genes *Sw-5* and *Tsw* introgressed in tomato (*Lycopersicon esculentum*, =*Solanum lycopersicum*) and pepper (*Capsicum annuum*) cultivars, respectively, have been found to confer resistance to a wide spectrum of TSWV isolates; they have been deployed in commercial cultivars worldwide. *Sw-5* is also effective against two other tospoviruses, *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) (Soler *et al.*, 2003). However, *Sw-5* resistance-breaking (SRB) isolates have been detected in Hawaii, Australia, South Africa, Spain and Italy (Cho *et al.*, 1996; Thompson & van Zijl, 1996; Latham & Jones, 1998; Aramburu & Martí, 2003; Ciuffo *et al.*, 2005) and *Tsw* resistance-breaking (TRB) isolates have been detected in Brazil, USA, Italy, Spain and Australia (Boiteux *et al.*, 1993; Hobbs *et al.*, 1994; Roggero *et al.*, 2002; Margaria *et al.*, 2004; Sharman & Persley, 2006).

Understanding the molecular mechanisms linked to resistance breakdown and the evolutionary processes involved in the emergence of resistance-breaking isolates is a major challenge. This is relevant to the development of more durable and efficient resistance, which would certainly have a considerable economic impact on agriculture. Since a reverse-genetics system based on infectious cDNA clones is not available for TSWV, localization of TSWV avirulence determinants can only be carried out using indirect approaches. Thus, analysis of reassortants generated after co-inoculation of two isolates, one able and the other unable to infect resistant cultivars, revealed that the genetic determinants for pepper *Tsw* resistance breakdown were in the genomic RNA S (Jahn *et al.*, 2000) and in RNA M for tomato *Sw-5* resistance breakdown (Hoffmann *et al.*, 2001). Sequence comparison of the S segment from TRB isolates and isolates unable to infect pepper with *Tsw* showed no differences in the N protein, so the differences observed in the NSs protein (distinct for each isolate) should be the cause of *Tsw* resistance suppression (Margaria *et al.*, 2007). However, expression of both S segment-encoded proteins (N and NS_S) using a potato virus X vector suggested the

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The GenBank/EMBL/DDBJ accession number for the TSWV segment M sequences reported in this paper are FM163370–FM163373 and HM015510–HM015524.

Two supplementary figures are available with the online version of this paper.

opposite, i.e. that the N protein is the avirulence factor for *Tsw* resistance in pepper (Lovato *et al.*, 2008). Presently, the genetic determinants involved in tomato *Sw-5* resistance breakdown are unknown. Moreover, although data on variability and evolution of TSWV have been reported (Tsompana *et al.*, 2005), evolution with respect to resistance breakdown has never been investigated for TSWV, which could be useful in understanding the factors involved in resistance durability (García-Arenal & McDonald, 2003; Janzac *et al.*, 2009; Fraile & García-Arenal, 2010).

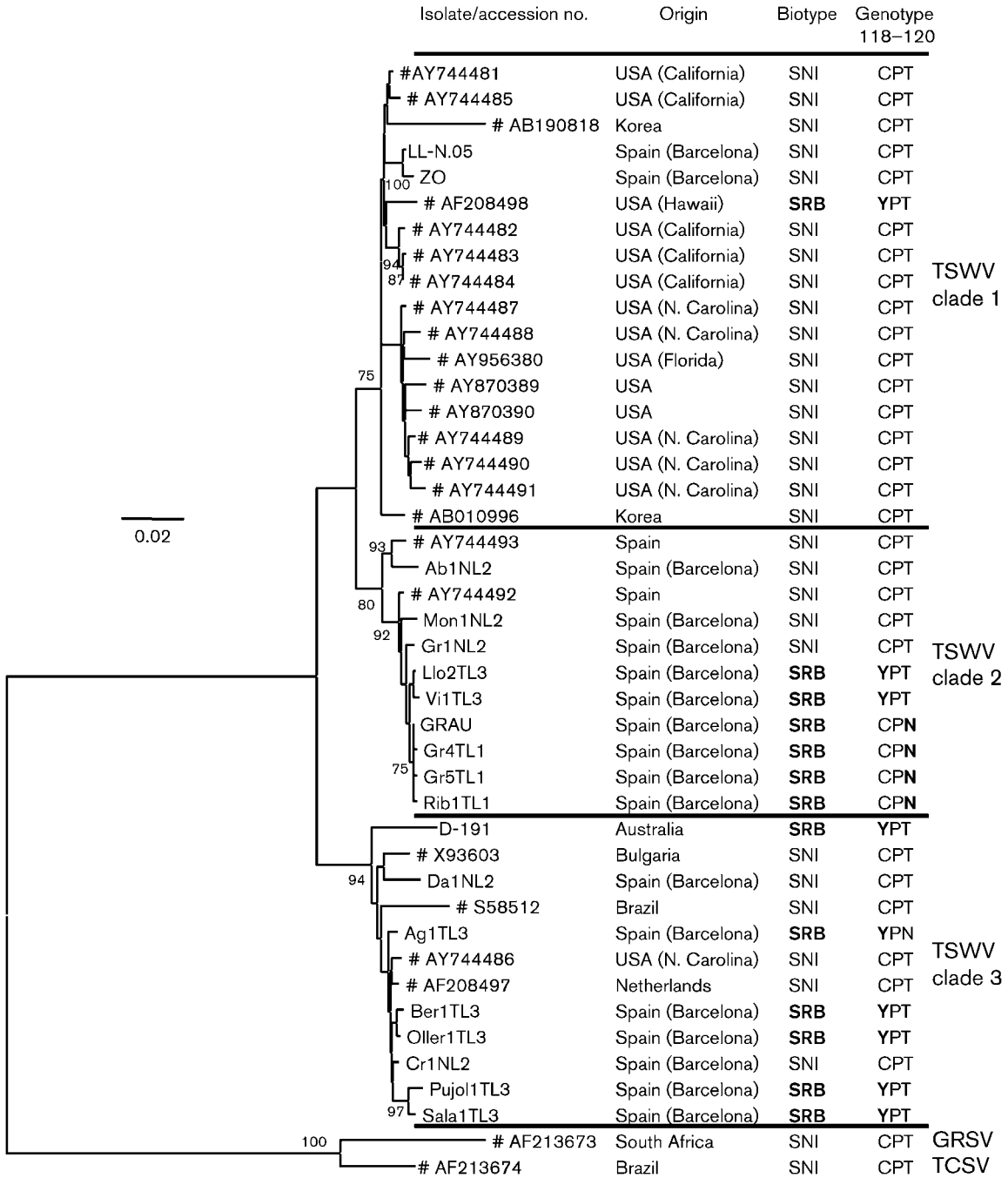

Fig. 1. Maximum-likelihood phylogenetic tree based on sequences of the NSm gene from 41 TSWV isolates, using GRSV and TCSV as an outgroup. Numbers at nodes indicate percentage bootstrap values obtained from 1000 replications (only values $\geqslant$75 % are shown). Branch lengths are proportional to genetic distance. Sequences retrieved from GenBank have '#' preceding the accession number. Biotypes are SNI (*Sw-5* non-infecting) and SRB (*Sw-5* resistance-breaking). The genotype indicates the amino acids at positions 118–120 of NSm; substitutions that correlated with resistance breakdown are in bold.

In this paper, we present evidence that resistance conferred by the gene *Sw-5* can be overcome by two different single amino acid substitutions in the TSWV NSm protein. Our analysis indicated convergent evolution and a role for positive selection in *Sw-5* resistance breakdown, whereas recombination was not relevant.

Eighteen TSWV isolates were collected from tomato crops in Barcelona province, Spain, between 2002 and 2004. The infectivity of these isolates and isolate D-191 from Australia (Department of Agriculture of Western Australia) was evaluated in tomato cultivars 'Verdi', with *Sw-5*, and 'Marmande', without *Sw-5* (provided by Semillas Fitó, Barcelona, Spain). The isolates were classified into two biotypes: SRB, represented by 11 Spanish isolates and the Australian isolate, and *Sw-5* non-infecting (SNI), represented by seven Spanish isolates unable to overcome *Sw-5* resistance. The complete sequences of the M segment of these TSWV isolates were determined as described previously (Tsompana *et al.*, 2005) and analysed together with equivalent sequences from 22 TSWV isolates (one being SRB) and sequences from GRSV and TCSV isolates retrieved from GenBank (accession numbers, origins and biotypes are shown in Fig. 1).

First, the nucleotide and translated amino acid sequences were aligned using the CLUSTAL algorithm implemented in the MEGA 4.0 program (Kumar *et al.*, 2008). The only changes common to all SRB isolates were located in the NSm amino acid sequence (Supplementary Figs S1 and S2, available in JGV Online). All SNI isolates of TSWV, GRSV and TCSV had 118C (amino acid C at position 118) and 120T, whereas SRB TSWV isolates had 118Y (substitution C118Y) or substitution T120N. Both amino acid substitutions require only one nucleotide change (Table 1), which suggests that these mutants can be easily generated. The two amino acid substitutions found in TSWV SRB isolates in this work contrast with those observed for pepper *Tsw*, which showed amino acid substitutions and deletions in different positions (78, 85, 174, 255, 267, 408, 427 and 437) for different TRB isolates (Margaria *et al.*, 2007), although we cannot rule out the possibility that amino acid substitutions other than C118Y and T120N are involved in *Sw-5* resistance breakdown. Resistance breakdown has also been associated with a small number of amino acid substitutions for different proteins from several other plant viruses (Meshi *et al.*, 1989; Tsuda *et al.*, 1998, Karasawa *et al.*, 1999; Matsumoto *et al.*, 2009). Thus, it is plausible that plant resistance genes have evolved to recognize conserved motifs essential for virus infection (in which a small number of substitutions are allowed) to minimize the generation of resistance-breaking mutants (Janzac *et al.*, 2009). Indeed, resistance against plant viruses has been efficient and durable (García-Arenal & McDonald, 2003).

Recombination was examined with the GARD program of the HYPHY package (Kosakovsky Pond & Frost, 2005a) available at the Datamonkey server (http://www.datamonkey.org) because (i) recombination is an evolutionary mechanism that can create greater genome diversity and adaptability, as well as remove deleterious mutations (Nagy, 2008), and (ii) it affects the analysis of molecular selection and phylogenetic inference (see below). No recombination event was found in the M segment of the analysed TSWV isolates. This was somewhat expected, as recombination in negative-sense RNA viruses is much rarer (Chare *et al.*, 2003) than in positive-sense RNA viruses (Chare & Holmes, 2006). By contrast, reassortment of RNA segments seems to play a role in TSWV evolution (Qiu & Moyer, 1999).

Phylogenetic relationships of TSWV isolates, including GRSV and TCSV isolates as an outgroup, were inferred using the maximum-likelihood method implemented in the PHYML program (Guindon & Gascuel, 2003) available at the Phylemon server (http://phylemon.bioinfo.cipf.es). This was performed using the GTR substitution model with a gamma distribution with 16 classes for rate heterogeneity estimated with the program ModelTest (Posada & Crandall, 1998). Significance of nodes was estimated using 1000 bootstrap replicates. The two coding regions of the M segment, G_N/G_C and NSm, gave essentially the same phylogenetic topology, confirming the absence of recombination; thus, only the NSm tree is shown (Fig. 1). Three main clades were observed, in agreement with a previous phylogeny of 17 SNI TSWV isolates (Tsompana *et al.*, 2005). Clade 1 was composed of 17 SNI TSWV isolates [two from Korea, 13 from USA (five from California, five from North Carolina and one from Florida) and two from Barcelona, Spain] and one SRB isolate from Hawaii, USA. Clade 2 was composed only of Spanish isolates: five SNI and six SRB isolates. Clade 3 was composed of six SNI isolates (one from Brazil, one from the Netherlands, one from Bulgaria, one from North Carolina, USA, and two from Barcelona, Spain) and six SRB TSWV isolates (one from Australia and five from Barcelona, Spain). The three clades each contained TSWV SRB isolates with the C118Y substitution, from Spain,

Table 1. Amino acid and nucleotide differences correlated with ability of TSWV isolates to infect tomato cultivars with the resistance gene *Sw-5*

The amino acid (single-letter code) and nucleotides at codons 118 and 120 of TSWV protein NSm are shown. Substitutions responsible for amino acid changes correlated with the ability to overcome *Sw-5* resistance are highlighted in bold.

Virus	Isolates (*n*)	Biotype	Codon 118	Codon 120
GRSV	1	SNI	C (TGT)	T (ACA)
TCSV	1	SNI	C (TGT)	T (ACA)
TSWV	26	SNI	C (TGT)	T (ACT)
TSWV	2	SNI	C (TGT)	T (ACC)*
TSWV	9	SRB	Y (T**A**T)	T (ACT)
TSWV	4	SRB	C (TGT)	N (A**A**T)

*Synonymous nucleotide substitution.

Hawaii and Australia, and TSWV SNI isolates, suggesting that *Sw-5* resistance breakdown has occurred independently at least three times by convergent evolution. This is not unique, as breakdown of host resistance by independent evolutionary lineages have also been reported for *Beet necrotic yellow vein virus* (Acosta-Leal *et al.*, 2010). Clade 2 included TSWV SRB isolates from Spain that had the T120N substitution and showed very low genetic divergence, suggesting a unique resistance-breakdown event. The close genetic relationship between some TSWV isolates from different continents suggests long-distance migration, probably by unintentional traffic of TSWV-infected plants or thrips.

Finally, selection across the coding region of the NSm protein was studied by estimation of rates of non-synonymous and synonymous changes at each codon using three complementary maximum-likelihood methods (Kosakovsky Pond & Frost, 2005b) available at the Datamonkey server: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and random effects likelihood (REL). Only concordant results for the three methods were considered.

Most codons of NSm were found to be under neutral evolution, whereas 34 negatively selected codons were identified (Fig. 2), which could be involved in functional or structural domains. NSm is involved in cell-to-cell movement, tubule formation, symptomatology, host-range determination and interactions with the TSWV N protein, plant proteins and RNA (Soellick *et al.*, 2000; Li *et al.*, 2009).

Positive selection was only detected at codon 118 (SLAC $P=0.115$, FEL $P=0.076$ and REL Bayes factor=36900.6), supporting our finding that the substitution C118Y could be an adaptation to overcome resistance conferred by the dominant gene *Sw-5*. Positively selected mutations have also been identified in *Potato virus Y*, involved in overcoming recessive resistance (Moury *et al.*, 2004), and in animal and human viruses that escape the immune system (Haydon *et al.*, 2001; Ross & Rodrigo, 2002). It is also possible that substitution T120N had been positively selected to overcome *Sw-5* resistance, but the small number of additional mutations between these isolates might have precluded its detection by the statistical methods used. Interestingly, except for the four TSWV SRB isolates with 120N described here, all NSm sequences available for the genus *Tospovirus* (99 isolates from 16 virus species) have 120T, suggesting that this amino acid could have an important function and would be under strong negative selection in the absence of the *Sw-5* gene.

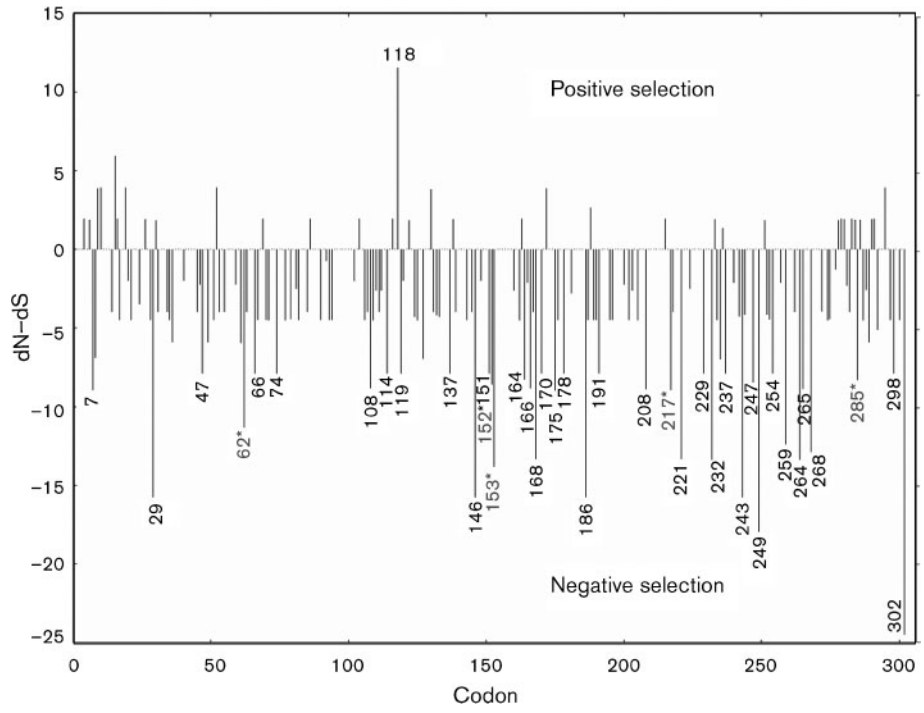

Fig. 2. Codon or amino acid sites of the protein NSm under positive or negative selection. The vertical axis represents normalized dN−dS and the horizontal axis represents codon position. Numbers above plotted spikes indicate codon positions under positive selection (dN>dS) and numbers below spikes indicate sites under negative selection (dN<dS) that were statistically significant by the three statistical tests SLAC, FEL and REL; asterisks indicate negative selection that was statistically significant only by the SLAC and FEL tests.

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