Phylogenetic analysis of Tomato spotted wilt virus *(TSWV) NSs protein demonstrates the isolated emergence of resistance-breaking strains in pepper*

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Abstract Resurgence of *Tomato spotted wilt virus* (TSWV) worldwide as well as in Hungary causing heavy economic losses directed the attention to the factors contributing to the outbreak of this serious epidemics. The introgression of *Tsw* resistance gene into various pepper cultivars seemed to solve TSWV control, but widely used resistant pepper cultivars bearing the same, unique resistance locus evoked the rapid emergence of resistance-breaking (RB) TSWV strains. In Hungary, the sporadic appearance of RB strains in pepper-producing region was first observed in 2010–2011, but in 2012 it was detected frequently. Previously, the non-structural protein (NSs) encoded by small RNA (S RNA) of TSWV was verified as the avirulence factor for *Tsw* resistance, therefore we analyzed the S RNA of the Hungarian RB and wild type (WT) isolates and compared to previously analyzed TSWV strains with RB properties from different geographical origins. Phylogenetic analysis demonstrated that the different RB strains had the closest relationship with the local WT isolates and there is no conserved mutation present in all the NSs genes of RB isolates from different geographical origins. According to these results, we concluded that the RB isolates evolved separately in geographic point of view, and also according to the RB mechanism.

Keywords *Tomato spotted wilt virus* · Resistance-breaking · Avirulence determinant · Non-structural protein · RNA silencing suppressor

Introduction

After the worldwide spread of western flower thrips (*Frankliniella occidentalis*) *Tomato spotted wilt virus* (TSWV) has been continuously present in pepper-growing regions worldwide. TSWV has extremely broad host range (more than 1,000 plant species), including vegetables, ornamental plants, and weed plants as well [1, 2], and is transmitted by various thrips species in a propagative persistent manner [3, 4]. The most effective control against this pathogen seemed to be the production of resistant cultivars. The application of pepper cultivars bearing *Tsw* resistance gene triggered the rapid emergence of new RB isolates few years after introducing these resistant *Capsicum* cultivars. First, reports on resistance breakdown by newly emerged TSWV RB strains were published from the Mediterranean pepper-producing regions, such as Italy and Spain [5–7]. Then the appearance of RB isolates was detected in other continents and countries, such as Australia and Hawaii [8, 9]. In Asia, TSWV has appeared only a decade ago, and in Korea it was first reported in 2004 [10], but recently the emergence of RB strains was also reported [11, 12].

TSWV is the type species of the genus *Tospovirus*, the only genus in the virus family *Bunyaviridae* to infect and

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A. Almási · Z. Csömör · K. Nemes · K. Salánki (✉) · I. Tóbiás
Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman O. Road 15, 1022 Budapest, Hungary
e-mail: salanki.katalin@agrar.mta.hu

G. Csilléry
Budakert Kft., Bartók B. Road 41, 1114 Budapest, Hungary

Z. Csömör · L. Palkovics
Department of Plant Pathology, Corvinus University of Budapest, Ménesi Road 44, 1118 Budapest, Hungary

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replicate both in plant and insect hosts [13]. The viral genome consists of three single stranded RNA molecules, a large (L RNA), a medium (M RNA), and a small (S RNA) segment, which enables the virus to develop reassortants. The L RNA encodes RNA-dependent RNA polymerase (RdRp) in negative polarity, while the M and S RNAs are ambisense. M RNA encodes the precursor of two glycoproteins (Gn and Gc) that incorporate in the outer envelope/membrane of host-origin and a non-structural protein NSm playing role in the virus movement. From the S RNA, two proteins are translated: the nucleocapsid (N) and a non-structural protein (NSs) [14]. According to de Ronde et al. [15], NSs is the suppressor protein of the host plant gene silencing mechanism and it is responsible for breakdown of the plant's resistance (avirulence factor, avr). The contribution of the different domains of the protein to the gene silencing function and as avr factor was recently analyzed in detail [16].

In Hungary, similar to other European countries, TSWV caused severe epidemics in vegetables and ornamental productions due to the introduction of *Frankliniella occidentalis*, the very effective vector of TSWV in greenhouse cultivation [17]. To manage TSWV, disease-resistant pepper cultivars carrying *Tsw* gene were frequently used in the Hungarian pepper-growing regions. However, recently, in 2009–2010 systemic infection was detected on resistant pepper varieties proving the presence of RB isolates. Our aim was to characterize the molecular differences between the WT and the recently emerged RB isolates in the S RNA to determine the potential origin of the RB strains and to identify the mutations in the avr factor responsible for breakdown of the *Tsw* resistance.

Materials and methods

Virus isolates

Fruit samples with typical TSWV symptoms from infected pepper plants (*Capsicum annuum* cv. Brendon and *C. annuum* cv. Cibere) were collected in the main pepper-growing region of Hungary (Szentes, Szegvár) in 2012 (Table 1). The samples were tested for TSWV, *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), and *Potato virus Y* (PVY) by DAS-ELISA to verify the presence of TSWV in fruit samples and to exclude mixed infection using TSWV, TMV, CMV, and PVY antibodies (Art. Ns. 190115, 190125; 190415, 190425; 160612, 160622; 112911, 112921, respectively, Bioreba AG) according to Clark and Adams [18]. Two TSWV strains isolated from *C. annuum* cv. Brendon bearing the TSWV resistance locus (HUP1-2012-RB, HUP2-2012-RB) and one WT (HUP4-2012-WT) isolate derived from *C. annuum* cv. Cibere were mechanically inoculated and maintained on different test plants (*Nicotiana tabacum* cv. Xanthi-nc, *C. annuum* cultivars 'Celtic', 'Censor', 'Carma', 'Century', 'Dimentio', 'Skytia', 'Karakter', 'Brendon', 'Bronson', and 'Bravia') to observe macroscopic symptoms and maintain the isolates (Table 2). Mock-inoculated non-infected plants were used for negative control. For long time storage, samples were kept in a deep freezer (at −70 °C).

Viral RNA extraction, RT-PCR, nucleotide sequence determination

Total RNA was isolated with the Spectrum Plant Total RNA Kit (Sigma) according to the manufacturer's instructions from pepper fruit samples or systemically infected leaves of the test plants. The S RNA was cloned in two segments with overlapping regions. The first strand cDNAs were synthesized with Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Science) using the NSs-Reverse (5′-GGA CAT AGC AAG ATT ATT TTG ATC CTG-3′) and N-Reverse (5′-GGG GAT CCA GAG CAA TTG TGT CAA TTT T-3′) primers, respectively. The PCR amplification of the 1,404 bp fragment of NSs region was carried out with the primers NSs-Forward (5′-GG CTG TAG CAG AGA GCA ATT GTG TCA TAA TTT T-3′) and NSs-Reverse (5′-GGA CAT AGC AAG ATT ATT TTG ATC CTG-3′), while for the amplification of the 1,720 bp 3′ fragment containing the N gene and the non-coding regions, N-Forward (5′-AAT TTC TCC GCA ATC TAT TTC AGT TG-3′) and N-Reverse (5′-GGG GAT CCA GAG CAA TTG TGT CAA TTT T-3′) primers were used. PCR was carried out in 25 µl final reaction volume as follows: amplification consisted of 5 min at 94 °C followed by 35 cycles of 1 min of denaturation at 94 °C, 30 s of annealing at 51 °C, and 3 min of extension at 72 °C, and a final extension cycle for 5 min at 72 °C. PCR products were separated by electrophoresis in 1 % agarose gel stained with ethidium bromide and purified using Silica Bead DNA Gel Extraction Kit (Thermo Science) and cloned into CloneJet (Thermo Science) or pGEM-T Easy Vector (Promega, Madison USA). The sequence determination of the clones was carried out by BAYGEN (Szeged).

Phylogenetic and sequence analysis

The nucleotide homology of the Hungarian and other TSWV strains retrieved from the GenBank (Online Resource 1) was examined by the BLAST program of NCBI. The nucleotide and deduced amino acid sequences were aligned by the ClustalW algorithm of the MEGA 6.06 program [19]. Phylogenetic trees were composed by the Neighbor-Joining method with 1,000 bootstrap replications

Table 1 Hungarian *Tomato spotted wilt virus* (TSWV) isolates used in this study

Isolates	Type[a]	Origin	Plant	Date of collection	Accession number
HUP1-2012-RB	RB	Szegvár, Hungary	*C. annuum* 'Brendon'	28.06.2012	KJ649608
HUP2-2012-RB	RB	Szegvár, Hungary	*C. annuum* 'Brendon'	28.06.2012	KJ649609
HUP4-2012-WT	WT	Szentes, Hungary	*C. annuum* 'Cibere'	28.06.2012	KJ649611

[a] *RB* resistance-breaking, *WT* wild type

Table 2 *Capsicum annuum* cultivars used in test plant experiments

	Test plant species	Cultivars	TMV resistance	*Tsw* resistance gene present
1	*Capsicum annuum*	Celtic	L3	+
2		Censor	L3	+
3		Carma	L3	−
4		Century	L4	−
5		Dimentio	L3	−
6		Skytia	L3	−
7		Karakter	L3	+
8		Brendon	L3	+
9		Bronson	L3	+
10		Bravia	L3	+

(MEGA 6.06 program) with the entire viral proteins. The amino acid sequences of the N and NSs proteins of the *Groundnut ringspot virus* (GRSV) gained from the NCBI GenBank (accession numbers AF251271 and JN571117.1, respectively) were incorporated into the phylogenetic trees as outgroup.

Results

Virus isolation and pathological characterisation

Fruit samples with typical TSWV symptoms showing positive reaction only to TSWV and negative result to all other examined viruses (TMV, CMV, and PVY) in the DAS-ELISA (data not shown) were selected for further experiments.

Ten commercially available pepper cultivars (Table 2) and *N. tabacum* cv. Xanthi-nc plants were inoculated with two TSWV strains isolated from *C. annuum* cv. Brendon bearing the TSWV resistance locus (HUP1-2012-RB, HUP2-2012-RB) and one WT (HUP4-2012-WT) isolate derived from *C. annuum* cv. Cibere (Table 1). The negative control (mock-inoculated non-infected) plants showed no symptoms (Fig. 1a, b). All the pepper cultivars carrying *Tsw* resistance gene (*C. annuum* cvs. 'Celtic', 'Censor', 'Karakter', 'Brendon', 'Bronson', 'Bravia') reacted with HR to the HUP4-2012-WT isolate showing local necrotic lesions on the inoculated leaves 4–7 days post inoculation (dpi) and this TSWV strain did not spread systemically (Fig. 1h). On the TSWV-susceptible pepper cultivars (*C. annuum* cvs. 'Carma', 'Century', 'Dimentio', 'Skytia'), HUP4-2012-WT isolate did not induce HR and systemic symptoms (chlorotic mosaic and ringspot pattern on the leaves, stunting) appeared 14–16 dpi (Fig. 1g). On the leaves of all the different genotypes of pepper cultivars infected with HUP1-2012-RB and HUP2-2012-RB isolates, local necrotic lesions were not detected but systemic symptoms were observed on the upper non-inoculated leaves (Fig. 1c–f). All the three virus isolates induced systemic symptoms (chlorotic or necrotic ringspot) on the inoculated *N. tabacum* cv. Xanthi-nc plants.

Sequence analysis of the Hungarian TSWV isolates

The nucleotide and the deduced amino acid sequences of the complete S gene of three Hungarian TSWV strains isolated from pepper were deposited to the GenBank database (for the accession numbers see Table 1). Sequence similarities of the NSs genes were compared to the sequences of WT and RB isolates, predominantly originated from pepper (except for the three Bulgarian isolates, see Online Resource 1) from distinct geographical locations. Nucleotide sequence identity among the Hungarian isolates was 99 %, while compared to other isolates this value varied between 95 and 99 %.

Phylogenetic tree was constructed based on the deduced amino acid sequences of the NSs and N genes of the Hungarian and the selected isolates (Online Resource 1) from the GenBank (Figs. 2, 3). The phylogenetic tree based on the NSs protein consisted of two main clusters that were divided again into 2–2 more branches (Fig. 2). One of the main clusters contains all the Spanish and most of the Italian (from North Italy) isolates (in two branches) and two Brazilian isolates. The other main cluster builds up from the Korean and French isolate as a subcluster. The other subcluster contains the Italian isolates from Sicily (South Italy) and other French isolate together with a Bulgarian isolate. All the Hungarian isolates forms a branch within this subgroup together with three Bulgarian isolates (two of them isolated from tobacco and one from tomato). The structure of the phylogenetic tree based on the

◀**Fig. 1** Macroscopic symptoms of *C. annuum* test plants (16 days post inoculation) infected with the different TSWV isolates. **a** Mock-inoculated susceptible control pepper cultivar. **b** Mock-inoculated resistant control pepper cultivar (carrying *Tsw* gene). **c** Susceptible pepper cultivar inoculated with HUP1-2012-RB TSWV isolate. **d** Resistant pepper cultivar (carrying *Tsw* gene) inoculated with HUP1-2012-RB TSWV isolate. **e** Susceptible pepper cultivar inoculated with HUP2-2012-RB TSWV isolate. **f** Resistant pepper cultivar (carrying *Tsw* gene) inoculated with HUP-2012-RB TSWV isolate. **g** Susceptible pepper cultivar inoculated with HUP4-2012-WT TSWV isolate. **h** Resistant pepper cultivar (carrying *Tsw* gene) inoculated with HUP4-2012-WT TSWV isolate

N protein was similar to that of the NSs protein (Fig. 3). In all the different groups the RB and WT isolates are present and there is no separation according to this feature/property. In addition, all the RB isolates locate nearby the corresponding WT isolate.

Amino acid (aa) sequences of the NSs protein (467 aa) were compared among the WT and RB isolates (Fig. 4). Several changes were present only in the three Hungarian isolates at positions 122 (A to D), 137 (T to K), 174 (M to T), 450 (G to R), and 459 (P to S). The Hungarian RB isolates (HUP1-2012-RB, HUP2-2012-RB) had two aa substitutions compared to the WT Hungarian isolate (HUP4-2012-WT) at positions 104 and 461 (A instead of T). Substitution at position 104 has occurred only in case of the Hungarian RB isolates, while at the 461 position it was present both in other WT and RB isolates retrieved from the GenBank. Two aa substitutions were found only in case of HUP4-2012-WT at positions 282 (A to V) and 284 (I to V). HUP1-2012-RB had two changes at positions 287 (H to Q) and 330 (H to Y), while HUP2-2012-RB had one substitution at position 356 (H to Y). No specific aa change was identified in all the RB strains from different geographical origins.

Discussion

The rapid adaptation of TSWV to pepper resistance and breakdown of the *Tsw* resistance gene facilitated the determination of the avr factor (avr) of TSWV, and the study of the evolutionary aspects of emergence of new strains [15, 20–27]. However, contradictory results, lacking information and methodological difficulties hinder or delay advances in these questions. In addition, the mechanism(s) and the viral determinant(s) of 'gene-for-gene' resistance based on *R* genes vary depending on the virus species, strains, and host plant [28]. In many viruses, different genes or gene products (CPs, movement proteins, viral polymerases, genome-linked proteins, etc.,) were identified as the avr factor that elicits HR. Generally, RB of (usually dominant monogenic) *R* gene-mediated resistance can be coupled with specific point mutations of the avr factor [29].

In the case of TSWV, two main resistance genes are used in the breeding programs. On *Lycopersicon peruvianum Sw5* resistance gene was identified and introgressed into tomato [30, 31], while on pepper cultivars (*C. annuum*) the *Tsw* gene is used. In both cases, RB strains of TSWV have been reported from distinct parts of the world [32–36]. The genetic determinant for breakdown of *Sw-5*

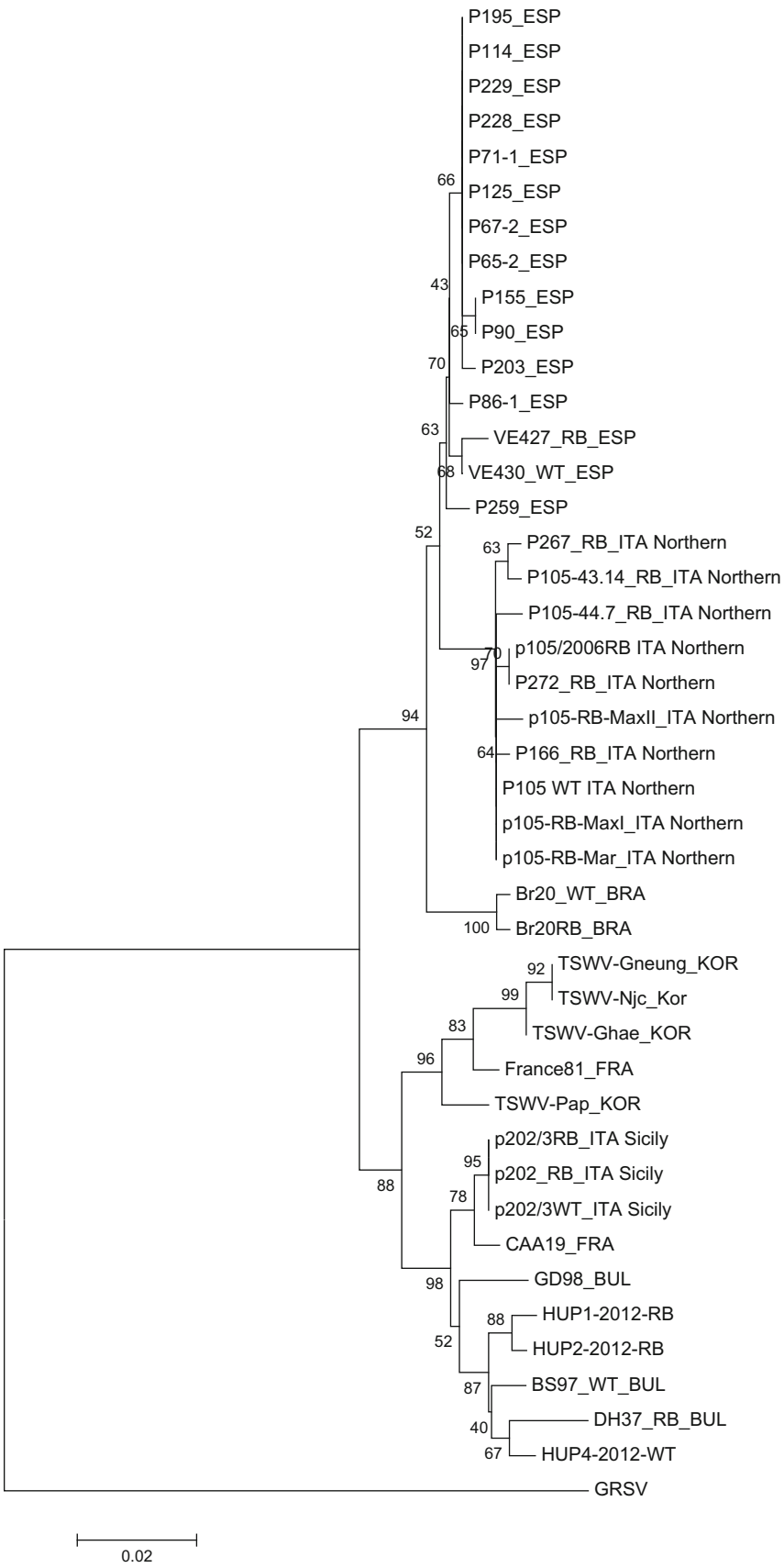

Fig. 2 Phylogenetic tree based on the deduced amino acid sequences of the NSs protein of TSWV. Hungarian RB and WT strains and other strains selected from the GenBank (for accession numbers see Online Resource 1)

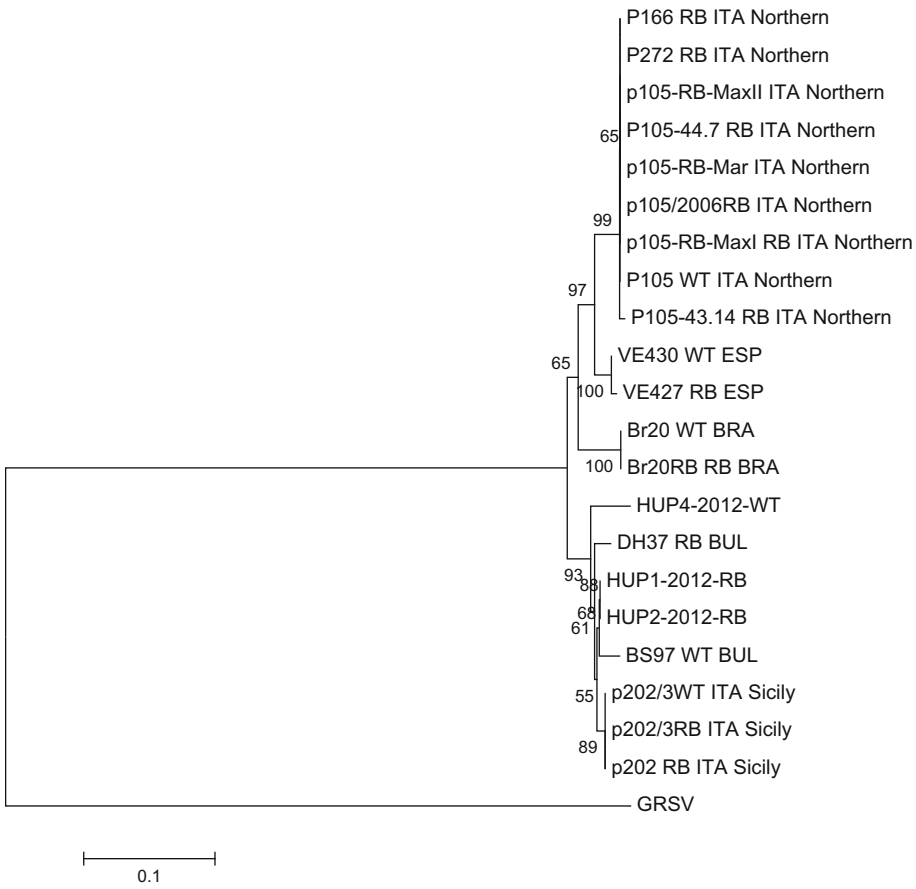

Fig. 3 Phylogenetic tree based on the deduced amino acid sequences of the N protein of TSWV. Hungarian RB and WT strains, and other strains selected from the GenBank (for accession numbers see Online Resource 1)

resistance in tomato was localized to NSm gene/protein of TSWV that has a role in virus movement [37], while in the case of *Tsw* gene, the NSs protein was identified as the avr factor. The role of the NSs protein as a suppressor of the post transcriptional gene silencing (PTGS) was proved by various research groups [21, 22]. Although there are TSWV strains that are able to break resistance in tomato as well as in pepper, but the two functions are independent. Different viral small RNAs (vsiRNAs) accumulation was identified among TSWV RB—breaking *Sw5* gene-mediated resistance—and normal strains in *Solanum lycopersicum* inbred lines and differences in RNA interference processes could be detected by deep sequencing [38, 39].

De Ronde et al. [15] succeeded to prove directly the independent role of NSs protein both in *Tsw* gene-mediated resistance and in RNA silencing suppression by transient expression [16]. For this reason, we chose S RNA segment of the Hungarian TSWV pepper strains to determine differences between the RB and WT isolates in nucleotide and (deduced) aa sequences. In the literature and in the GenBank, there are limited numbers of RB and WT TSWV strains isolated from pepper from the same geographic locations or data are not complete. Isolates from the GenBank containing most of these data were chosen to compare variations or mutations occurring in RB pepper strains. Similarly, to the results of Margaria et al. [24], we detected several mutations both in NSs and N proteins, but there were not any of them common with all the RB strains. Controversially, with several plant viruses that interfere plant resistance by a 'gene for gene' interaction and the emergence of RB strains can be connected to specific point mutations (at given aa positions), TSWV RB strains evolve by different point mutations/substitutions at various aa positions. In case of the avr factor, i.e., NSs protein, the Hungarian RB and WT strains differed only in two positions. The phylogenetic analysis supported the hypothesis that TSWV RB strains has been developed locally, and the worldwide trade and transport of plant propagating material seem not to contribute to the expansion of RB strains. Based on the phylogenetic analysis of the NSs protein of several TSWV pepper strains, it can be concluded that cluster differentiation relies mostly on the geographic origin. One of the two main clusters consists of all the Spanish, the northern Italian, and the two Brazil strains (further divided into different subgroups) regardless of the strain type, i.e., RB or WT. The other main branch contains the Korean, French, and Italian strains from Sicily. In this case, reassortment may have contributed to the genetic

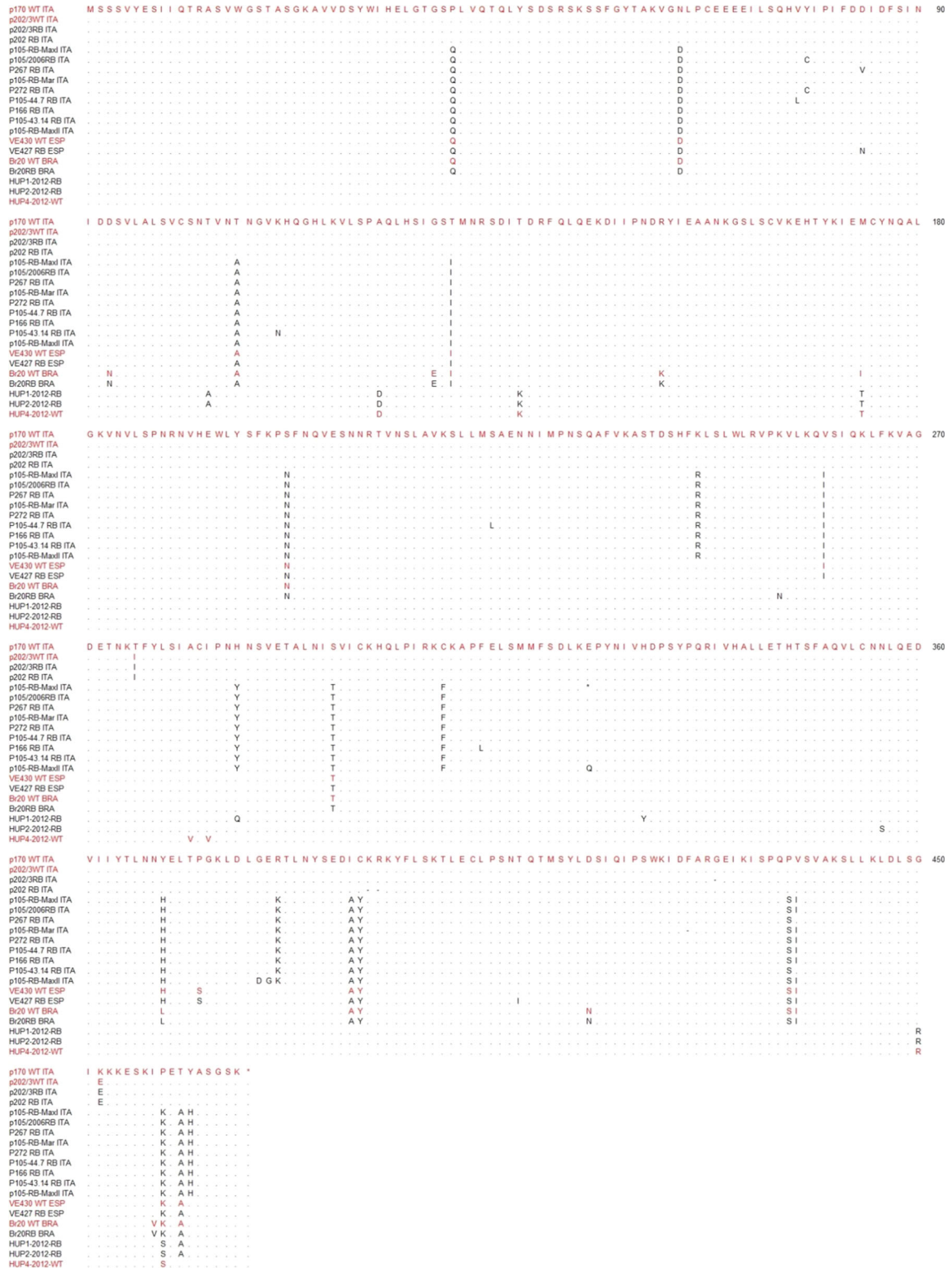

Fig. 4 Deduced amino acid sequences of the NSs protein of TSWV. Hungarian RB and WT strains and other strains selected from the GenBank (for accession numbers see Online Resource 1)

differentiation and first diversification [27], but the very tight relation in the same tree branch of the WT and RB isolates indicates that RB strains have developed in tight location. The division of the Italian strains can be explained by the different origins as they were collected from the northern and from the southern region of Italy,

respectively. None of the RB strains of different geographic origins, just as in case of the WT strains, is found in the same branch. All the Hungarian strains shared most similarity with Bulgarian strains collected from tobacco and tomato plants (S RNA segment sequence of any Bulgarian isolate from pepper is not deposited at the GenBank), and unfortunately there is no data available regarding to their reaction on different pepper cultivars. Regarding the origin of the TSWV isolates present in Hungary, the most likely that TSWV originally was introduced from the Balkan Peninsula due to the tobacco cultivation, as TSWV was first detected from tobacco in Hungary in 1972 [40]. Tentchev et al. [27] presented two possible theories for TSWV resurgence worldwide, from which the so-called 'local re-emergence' scenario suites more likely to our results. Several publications are focusing on population genetics and evolution of TSWV. The analyses underline the impact of genome reassortment in TSWV diversification [41–44]. Reassortment plays a role in strain emerging of TSWV rather than recombination that is far more uncommon in negative-sense RNA viruses [25, 45]. We analyzed only the S RNA of the retrieved strains; therefore, we cannot state anything about the implication of reassortment in the evolving processes of the Hungarian RB strains. It can be excluded, that there was any recombination event during the Hungarian RB strain evolution (recombination analysis was done, data not shown) because the phylogenetic trees of the NSs and N proteins are very similar. The only viral factor that may affect resistance could be the mutations in the NSs gene and protein as the avr determinant. To determine which nucleotide or aa changes in NSs led to RB and how other functions altered, needs further (mutational analysis) investigation.

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