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Tomato Spotted Wilt Virus on Pepper *(Capsicum annuum L.)* Plants in Hungary. Molecular Characterization of Wild Type and Resistance Breaking Isolates. Searching for Resistance in *Capsicum* Genus

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Abstract

In Hungary resurgence of Tomato spotted wilt virus (TSWV) has been frequently causing heavy crop losses in pepper production since the mid-nineties. Management of TSWV was at first directed against thrips (using different insecticides or plastic traps), and against weeds as host plants of the virus and the thrips. Later on, *Tsw* resistance gene was introduced into different types of pepper. In 2010 and 2011, sporadically, but in 2012 more frequently a resistance breaking (RB) strain of TSWV on resistant pepper cultivars was observed in the Szentes region (South-East Hungary). The presence of a new resistance breaking strain was demonstrated by virological (test-plant, serological and RT-PCR) methods.

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 was no conserved mutation present in any of the NSs genes of RB isolates from different geographical origins. According to these results, we concluded that the RB isolates evolved separately from a geographic point of view, and also according to the RB mechanism. The gene-silencing suppressor function of NSs protein is also discussed.

In order to find new genetic sources of resistance in *Capsicum* species 89, lines of *Capsicum annuum, C. chinense, C. frutescens, C. chacoense, C. baccatum var. baccatum, C. baccatum var. pendulum* and *C. praetermissum* were tested with TSWV-RB strain isolated in Hungary.

Key words: Tomato spotted wilt virus (WT and RB strains), NSs protein, resistance

Резюме

Периодичната поява на Tomato spotted wilt virus (TSWV) често причинява тежки загуби в производството на пипер от средата на деветдесетте години на миналия век. Контролът срещу TSWV първоначално включва употреба на инсектициди и инсектицидни уловки против трипсовете, пренасящи заразата. Атакувани са и плевелите, които са гостоприемници за вируса и неговите преносители. По-късно в някои типове пипер е внесен ген за устойчивост против TSWV. През 2010 и 2011 спорадично, а в 2012 по-често е наблюдавано преодоляване на устойчивостта (RB) на пиперови сортове в района на Szentes (Югоизточна Унгария). Чрез проучвания, включващи растителен тест, серология и RT-PCR, е доказано присъствието на щам, който преодолява устойчивостта при пипера.

Първоначално неструктурен протеин (NSs), кодиран от малка РНК (S RNA) на TSWV, е проверен като авирулентен фактор за Tsw устойчивост. Анализирани са S RNA на унгарски preodoljá-

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vasti na ustojcsivosta (RB) и диви типове (ili normalnie stamove) (WT) изолати. Направено е сравняване с предишни анализирани изолати на щамове на TSWV, способни да преодоляват устойчивостта (RB), които имат различен географски произход. Филогенетичният анализ показва, че различните RB щамове имат тясно родство с местните WT без консервативна мутация във всички NSs гени на RB изолатите от различните региони. Според резултатите е направено заключение, че изолатите RB частично се различават както по географски произход, така и по механизма на преодоляване на устойчивостта.

Обсъдена е също супресорната функция на NSs протеин.

В опита да бъдат намерени нови генетични източници на устойчивост в пипера 89 линии от *C. annuum, C. chinense, C. frutescens, C. chacoense, C. baccatum var. baccatum, C. baccatum var. pendulum* and *C. praetermissum* са тестирани с изолати TSWV-RB от Унгария.

Introduction

In Hungary, Tomato spotted wilt virus (TSWV) was described in tobacco in 1972 (Ligeti and Nagy, 1972), but the virus was not considered as an important pathogen in vegetable crops. Severe damage to the tomato and pepper production in the Szentes vegetable growing region (South-East Hungary) caused by TSWV infection was first observed in 1995 (Gáborjányi *et al*., 1995). The symptoms were characterized by chlorotic spots, rings, patterns, occasional necrosis on the leaves and fruits, and malformations of the plants (Fig. 1). The introduction and spread of western flower thrips (*Frankliniella occidentalis*), a very efficient TSWV vector, played an important role in TSWV emergence (Jenser and Tusnádi, 1989; Jenser, 1995). Management of TSWV control was at first directed against thrips using different insecticides or plastic traps, and against weeds as host plants of the virus and the thrips. Later on, the *Tsw* resistance gene (Black *et al*., 1996) was introduced into different types of pepper (conical white, long pale green hot and sweet, tomato shape, spice pepper and blocky types) (Csilléry, unpublished). Pepper cultivars carrying the *Tsw* resistance gene upon TSWV inoculation showed necrotic local lesions on the leaves or other parts of the plant without systemic infection (Fig. 2). Thanks to the multicomponent management approaches - the efficient control against thrips and reservoir weed plants, and use of the *Tsw* resistance gene (originated from *Capsicum chinense*) there has been no economical loss caused by TSWV in pepper productions for a long time in Hungary. TSWV (wild type strain, WT) was present in some years and regions without economical effect. For the first time in 2009, again in the Szentes vegetable growing region, virus infection was observed in TSWV resistant pepper varieties, from which resistance breaking isolates of TSWV (TSWV-RB strain) were detected (Salamon *et al*., 2010; Bese *et al.,* 2012; Csilléry *et al*., 2012). The reasons for the appearance of new resistance breaking isolates were that some elements of multicomponent control management were neglected and also some effective chemicals (like Unifos 50 EC) were withdrawn from practice by EU regulation. Since that time TSWV infection could have caused heavy economic losses again. Since it was demonstated that TSWV is able to adapt very rapidly to plant resistance, the *Tsw* resistance gene was broken down only a few years after its deployment in pepper crops (Roggero *et al*., 2002; Thomas-Caroll and Jones, 2003; Margaria *et al*., 2004; Sharman and Persley, 2006).

Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus* (family *Bunyaviridae*) causing an important disease in horticultural and agronomic crops across temperate, subtropical and tropical regions of the world (German *et al*., 1992; Goldbach and Peters, 1994). The virus was first described in Australia at the beginning of the 20th century (Brittlebank, 1919; Samual *et al*., 1930), and is now distributed worldwide, having an extremely broad host range that includes more than 900 plant species (Hanssen *et al*., 2010). High infection rates led to considerable economic losses worldwide, so that TSWV is considered one of the ten most economically destructive plant viruses (Tomlinson, 1987; Goldbach and Peters, 1994). TSWV is transmitted by thrips species in a persistent manner (Whitfield *et al*., 2005). The larval thrips can aquire the virus, which is multiplying in the vector and adult thrips specimens transmit the virus (van der Wetering *et al*., 1996). TSWV has a spherical virion that varies in size from 80 to 120 nm with an enveloped structure. The TSWV genome is composed of three single-stranded linear segments, large (L), medium (M), and small (S), named according to their lengths (Prins and Goldbach, 1998). The L RNA (~9 kb) contains an RNA-dependent RNA polymerase (RdRp) in a negative-sense orientation (Hann *et al*., 1991). In

contrast, the M (~4.8 kb) and S (~3 kb) RNAs each contain two genes, one in positive- and the other in negative-sense orientation (Heinze *et al*., 2001). The M RNA encodes the NSm protein and Gn-Gc glycoproteins, while the S RNA encodes the NSs nonstructural and N nucleocapsid proteins (de Haan *et al*., 1990, Kormelink *et al*., 1992). According to de Ronde *et al*. (2013), 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 (de Ronde *et al*., 2014).

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. Moreover, our aim was to find genetic sources of resistance in *Capsicum* species against resistance breaking strain of TSWV (TSWV-RB).

Materials and Methods

Virus isolates

Fruit samples with typical TSWV symptoms from infected pepper plants (*Capsicum annuum* cv. Brendon and cv. Cibere) were collected in the main pepper growing region of Hungary (Szentes, Szegvár) in 2012 and were tested for presence of TSWV. Two TSWV isolates from cv. Brendon containing the *Tsw* resistance gene (HUP1-2012-RB, HUP2-2012-RB) and one WT (HUP4-2012-WT) isolate derived from 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. 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 (50-GGA CAT AGC AAG ATT ATT TTG ATC CTG-30) and N-Reverse (50-GGG GAT CCA GAGCAA TTG TGT CAA TTT T-30) primers, respectively. The PCR amplification of the 1,404 bp fragment of NSs region was carried out with the primers NSs-Forward (50-GG CTGTAG CAG AGA GCA ATT GTG TCA TAA TTT T-30) and NSs-Reverse (50-GGA CAT AGC AAG ATT ATT TTG ATC CTG-30), while for the amplification of the 1,720 bp 30 fragment containing the N gene and the noncoding regions, N-Forward (50-AAT TTC TCC GCA ATC TAT TTC AGT TG-30) and N-Reverse (50-GGG GATCCA GAG CAA TTG TGT CAA TTT T-30) primers were used. PCR was carried out in 50 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 was examined by the BLAST program of NCBI. The nucleotide and deduced amino acid sequences were aligned with the ClustalW algorithm of the MEGA 6.06 program (Kumar *et al*., 2008). Phylogenetic trees were composed by the Neighbor-Joining method with 1,000 bootstrap replications (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 JN571117.1) were incorporated into the phylogenetic trees as outgroup.

Agrobacterium infiltration

NSs genes of TSWV RB and WT strains were cloned into pBin19 vector and *Agrobacterium tumefaciens* cells were transformed with them. Agrobacteria were cultivated overnight at 28°C in the presence of appropriate antibiotics. The cultures were harvested by centrifugation, and the pellet was resuspended in MES buffer containing 0.01M $MgCl_2$ and acetosyringon. P14 was added to the infiltration solution (OD_{600} 0.4). Final optical density of the *Agrobacterium* cultures containing NSs genes was adjusted at 600 nm (OD_{600}) to 0.5. *Agrobacterium*-mediated transient expression

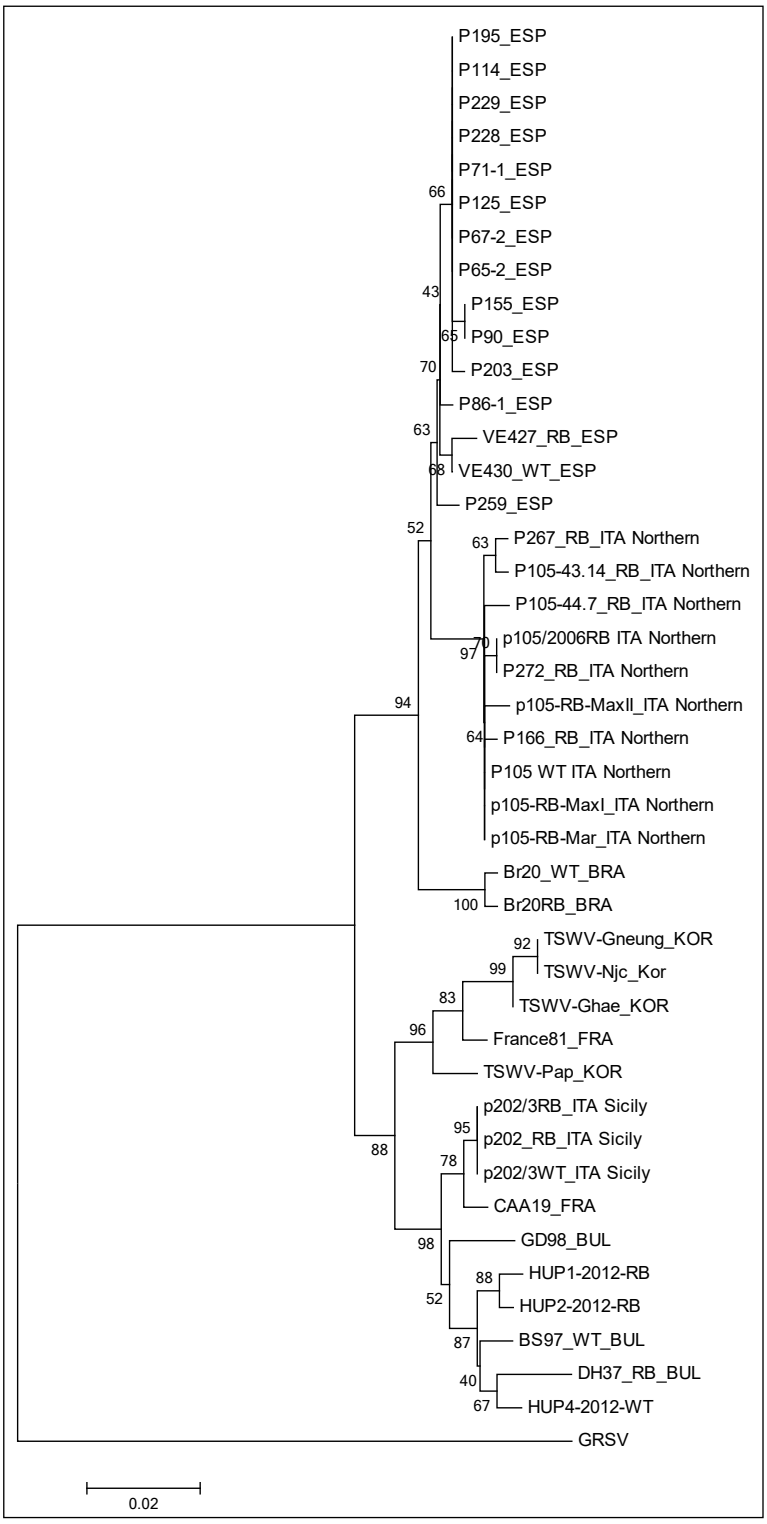

Fig. 1. Phylogenetic tree based on the deduced amino acid sequences of the NSs protein of TSWV. Abbreviations and accession numbers: HUP1-2012-RB : KJ649608; HUP2-2012-RB : KJ649609; HUP4-2012-WT: KJ649611; BS97: AJ418777; DH37: AJ418779; p202/3WT: HQ830187; p202/3RB: HQ830186; p202: DQ398945; GD98: AJ418780; CAA19: FR692822; VE430: DQ376184; VE427: DQ376185; p105: DQ376178; p105-RB-MaxI: HQ839730; p105/2006RB: DQ915946; p267: DQ376180; p105-RB-Mar: HQ839729; p105-44.7: DQ376183; p105-43.14: DQ376182; p105-RB-MaxII: HQ839731; Br20: DQ915948; Br20RB: DQ915947; p166: DQ376179; p272: DQ376181; France81: FR692829; TSWV-Pap: AB643674; TSWV-Ghae: AB643672; TSWV-Gneung: AB643671; TSWV-Njc: AB643673; p86-1: FR693020; p259: FR692932; p65-2: FR693005; p67-2: FR693007; p203: FR692900; p155: FR692871; p125: FR692857; p90: FR693023; p71-1: FR693811; p228: FR692917; p229: FR692918; p195: FR692895; p114: FR692852

on *Capsicum annuum cv Brendon* leaves was conducted by pressure infiltration into the abaxial air space of 4- to 6-week-old plants using a needleless 2-ml syringe. P14 suspension was used for negative control.

Resistance test

89 *Capsicum* items *[Capsicum annuum* (8), *C. chinense* (50), *C. frutescens* (8), *C. chacoense* (2), *C. baccatum var. baccatum* (4), *C. baccatum var. pendulum* (11) and *C. praetermissum* (6)*]* were inoculated at cotyledon stage with TSWV-RB strain. Symptoms were observed in the next 4 weeks.

Results

TSWV isolates were tested on TSWV-susceptible pepper cultivars ('Carma', 'Century', 'Dimentio', 'Skytia'), and pepper cultivars carrying *Tsw* resistance gene ('Celtic', 'Censor', 'Karakter', 'Brendon', 'Bronson', 'Bravia'). TSWV isolates causing necrotic local lesions (HR) on resistant pepper cultivars belonged to a wild type (TSWV-WT) strain, and isolates causing systemic symptoms (chlorotic mosaic and ringspot pattern on the leaves, stunting) on all pepper cultivars belonged to a resistance breaking (TSWV-RB) strain. Three TSWV isolates were selected (HUP1-2012-RB, HUP2-2012-RB and HUP4-2012-WT) for further study. All the three virus isolates induced systemic symptoms (chlorotic or necrotic ringspot) on the inoculated leaves of *N. tabacum* cv. Xanthi-nc plants.

Sequence similarities of the NSs genes were compared among the sequences of WT and RB isolates, originated from pepper 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%. Amino acid (aa) sequences of the NSs protein (467 aa) were compared among the WT and RB isolates

Several mutations/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 occurred only in the case of the Hungarian RB isolates. A phylogenetic tree was constructed based on the deduced amino acid sequences of the NSs genes of the Hungarian and the selected isolates from the GenBank (Fig. 1).

One of the two main clusters consists of Spanish, the Northern Italian, and the two Brazilian l strains (further divided into different subgroups) regardless of the strain type, i.e., RB or WT. The other main branch contains the Korean, Hungarian, Bulgarian and Italian strains from Sicily. The phylogenetic analysis supported the hypothesis that TSWV RB strains have been developed locally, and the worldwide trade and transport of plant propagating material do not seem to contribute to the expansion of RB strains.

The NSs proteins were tested for their avirulence (Avr) activity by triggering of HR (necrosis) on *Capsicum annuum* cv Brendon (Tsw+) plants in *Agrobacterium* transient expression assay (Fig. 2).

NSs-RB NSs- Control

Fig. 2. Symptoms on leaves of Brendon pepper variety (containing *Tsw* resistance gene) after *Agrobacterium* transient expression assay with NSs proteins of TSWV-WT (HR) and TSWV-RB (no HR).

Fig. 3. HR-like symptoms on *C. baccatum var. pendulum* items after inoculation with TSWV-RB strain.

To determine which nucleotide or aa changes in NSs led to RB and how other functions altered, further (mutational analysis) investigation/analysis is needed. In the search for resistance to TSWV-RB strain, 89 *Capsicum* items were tested *[Capsicum annuum* (8), *C. chinense* (50), *C. frutescens* (8), *C. chacoense* (2), *C. baccatum var. baccatum* (4), *C. baccatum var. pendulum* (11) and *C. praetermissum* (6)*]*. Eighty-five items were susceptible and 4 *C. baccatum var. pendulum* items showed HR-like symptoms (Fig. 3). Further study is necessary to clear the genetic background and the possibility to use these items in resistance breeding.

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