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INVESTIGATION ON TOMATO SPOTTED WILT VIRUS INFECTING PEPPER PLANTS IN HUNGARY

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ABSTRACT

In Hungary resurgence of *Tomato spotted wilt virus* (TSWV) frequently causes heavy crop losses in pepper production since the mid nineties. Management of TSWV control was first directed against the 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 from *Capsicum chinense* PI 152225 and PI 159236 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 all the NSs genes of RB isolates from different geographical origins. According to these results, it is concluded that the RB isolates evolved separately in geographic point of view and according to the RB mechanism. In order to find new genetic sources of resistance in *Capsicum* species 89 lines from *Capsicum annuum, C. chinense, C. frutescens, C. chacoense, C. baccatum var. baccatum, C. baccatum var. pendulum* and *C. praetermissum* were tested with the Hungarian TSWV-RB isolate.

Key words: *tomato spotted wilt virus, wild type and resistance breaking strains, NSs protein, resistance.*

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus* (family *Bunyaviridae*), causes an important disease of horticultural and agronomic crops. The virus distributed worldwide is having extremly broad host range and is

now considered as one of the ten most economically destructive plant viruses (Tomlinson, 1987). TSWV is transmitted by thrips in a persistant manner. The virion varies in size from 80 to 120 nm and has spherical enveloped character. The genome of TSWV consists of three ssRNA segments: small (S) and medium (M) RNAs have ambisense coding strategies, whereas the large (L) RNA is of negative polarity (Hann et al., 1991; Prins and Goldbach, 1998). In Hungary TSWV was described in 1972 (Ligeti and Nagy 1972), but the virus was not considered as an important pathogen. In 1995 very severe damage of TSWV infection was observed in tomato and pepper production in the Szentes vegetable growing region (Gáborjányi et al., 1995). The introduction and spread of western flower thrips (*Frankliniella occidentalis*), an efficient TSWV vector, in that time certainly played an important role in TSWV emergence (Jenser, 1995).

Management of TSWV control was first directed against the thrips using different insecticides or plastic traps, and against weeds as host plants/reservoirs of the virus and the thrips. Later on *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 *Tsw* resistance gene upon TSWV inoculation show necrotic local lesions on the leaves or other parts of the plant without systemic infection. In 2010 and 2011 sporadically, but in 2012 more frequently systemic virus symptoms were observed on resistant pepper cultivars in Szentes region (Bese et al., 2012; Csilléry et al., 2012; Salamon et al., 2010). The presence of new resistance breaking strain of TSWV was proved by virological (test-plant, serological and RT-PCR) methods. It was demonstrated that TSWV can adapt very rapidly to plant resistance, and the *Tsw* resistance gene was broken down only a few years after its deployment in pepper crops (Margaria et al., 2004; Roggero et al., 2002; Sharman and Persley, 2006). According to de Ronde et al. (2013, 2014), 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 aim of this research 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 starin of TSWV (TSWV-RB).

MATERIALS AND METHODS

Virus isolates. TSWV isolates originated from pepper cultivars susceptible and resistant against TSWV from Szentes region (South-East Hungary). Fruit samples were collected from plants exhibiting typical symptoms of virus infection such as stunting, mosaic, chlorotic and/or necrotic spots, rings and distortion on the leaves and fruits. The isolates were used for ELISA serological tests, RT-PCR and maintained by mechanical inoculation on *Nicotiana tabacum* cv. Xanthi-nc test plants.

RNA extraction and RT-PCR. Total RNA was extracted from leaves of *N. tabacum* cv. Xanthi-nc plants systemically infected by TSWV or from infected pepper fruits using the Spectrum Plant Total RNA Kit (Sigma) following the manufacturer's instructions. RT-PCR reactions for synthesis of first-strand cDNA were performed with Revert Aid H Minus First Strand cDNA Synthesis Kit

(Thermo Science) using NSs-Reverse primer. The PCR amplification of the 1,404 bp fragment of NSs region was carried out with the primers NSs-Forward (5'-GG CTGTAG CAG AGA GCA ATT GTG TCA TAA TTT T-3') and NSs-Reverse (5'-GGA CAT AGC AAG ATT ATT TTG ATC CTG-3'), PCR reaction was performed in 25µl – 50 µl final volume. PCR products were electrophoresed in 1% agarose gel and stained with ethidium bromide.

Phylogenetic and sequence analysis. The nucleotide homology of the Hungarian and other TSWV strains retrieved from the GenBank was analyzed/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. Phylogenetic trees were composed by the Neighbor-Joining method with 1,000 bootstrap replications (MEGA 6.06 program) with the entire viral proteins. *Groundnut ringspot virus* (GRSV) was 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. Final optical density of the *Agrobacterium* cultures containing NSs genes was adjusted at 600 nm (OD600) to 0.5. *Agrobacterium*-mediated transient expression 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) és C. *praetermissum* (6)] were inoculated at cotyledon stage with TSWV-RB strain. Symptoms were observed in the next 4 weeks.

RESULTS AND DISCUSSION

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 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 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 has occurred only in the

case of the Hungarian RB isolates. 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 (Figure 1).

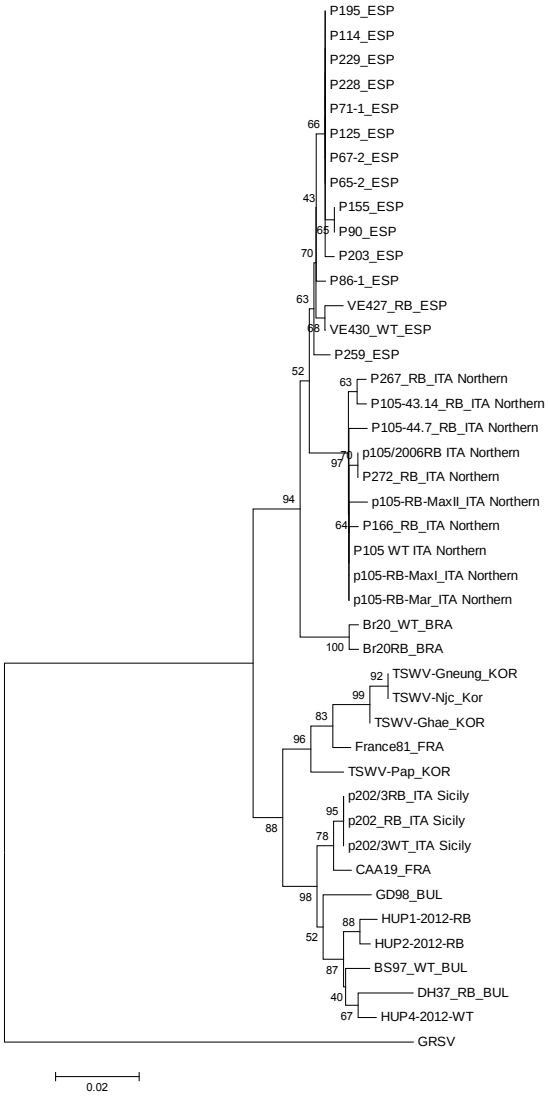

Figure 1. Phylogenetic tree based on the deduced amino acid sequences of the NSs protein of TSWV.

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;

One of the two main clusters consists of Spanish, the Northern Italian, and the two Brasilian 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. Amino acid differences in NSs protein of TSWV-WT and TSWV-RB strains from different geographical locations are different. The Brasilian WT and RB strains are different in aa position of 174 and 255, the Spanish at 84 and 407, North and South Italian strains at 424 and 427 respectively, while Hungarian WT and RB strains differ in 104 and 461 aa. 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.

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. The NSs protein of TSWV-WT strain caused HR on infiltrated leaves while NSs protein of TSWV-RB caused no necrosis. To determine which nucleotide or aa changes in NSs led to RB and how other functions altered, needs further mutational analysis.

CONCLUSION

Searching for resistance to TSWV-RB strain testing of 89 *Capsicum* items was carried out [*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)]. 85 items were susceptible and 4 *C. baccatum* var. pendulum items showed HR-like symptoms. Further study is necessary to clear the genetic background and the possibility to use these items in resistance breeding.

REFERENCES

Bese, G., Krizbai, L., Horváth, J., Takács, A., (2012). Resistance breaking strain of *Tomato spotted wilt virus*, (TSWV) on resistant pepper cultivars in Hungary. Internat. Symp. on Current Trends in Plant Protection, Belgrade, Serbia 2012. pp. 239-241.

Black L.L., Hobbs H.A., Kammerlohr D.S. (1996). Resistance of *Capsicum chinense* lines to *Tomato spotted wilt virus* from Louisiana, USA, and inheritance of resistance. *Acta Horticulturae* 431,393-401.

Csilléry G., Almási A., Tóbiás I. (2012). Occurence of resistance breaking strain of *Tomato spotted wilt virus* on resistance papper cultivars in Hungary. The 21st Internatioanl Pepper Conference, Florida, November 4-6 2012, pp 27.

de Ronde, P. Butterbach, D. Lohuis, M. Hedil, J.W.M. van Lent, R. Kormelink (2013). *Tsw* gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*. Mol. Plant Pathol. 14, 405–415.

De Ronde D., Pasquier A., Ying S., Butterbach P., Lohuis D., Kormelink R. (2014). Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppressor. Molecular Plant Pathology, 15 (2), 185-195.

Gáborjányi R., Csilléry G., Tóbiás I., Jenser G. (1995). *Tomato spotted wilt virus:* A new threat for pepper production in Hungary. IXth Eucapia Meeting, Budapest, 159-160.

de Haan P., Kormelink R., de Oliveira R.R., van Poelwijk F., Peters D., Goldbach R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen Virol 72: 2207-2216.

Jenser G. (1995). The role of the *Thysanoptera* species in the spread of *Tomato spotted wilt tospovirus*. Növényvédelem 31, 541–545.

Ligeti L., Nagy Gy. (1972). A Lycopersicum vírus 3 dohányültetvényeink új, veszedelmes kórokozója. Dohányipar: 41–43.

Margaria P., Ciuffo M., Turina M. (2004).Resistance breaking strain of *Tomato spotted wilt virus* (*Tospovirus*, *Bunyaviridae*) on resistant pepper cultivars in Almeria, Spain. Plant Pathol. 53. 795.

Prins M., Goldbach R. (1998). The emerging problem of tospovirus infection and nonconventional methods of control. Trends in Microbiology 6: 31-35.

Roggero P., Masenga V., Tavella L. (2002). Field isolates of Tomato spotted wilt virus overcoming resistance in pepper and their spread to other hosts in Italy Plant Disease. 86, 950-954.

Salamon P., Nemes K., Salánki K. (2010). A paradicsom foltos hervadás vírus (*Tomato spotted wilt virus*, TSWV) rezisztenciatörı törzsének első izolálása paprikáról (*Capsicum annuum* L) Magyarországon. Növényvédelmi Tudományos Napok pp 23.

Samuel, G., Bald, J.G., Pittman, H.A. (1930). Investigation on 'spotted wilt' of tomatoes in Australia. Commonw. Coun. Sci. Ind. Res. Bull 44, 8-11.

Sharman M., Persley D.M. (2006). Field isolates of *Tomato spotted wilt virus* overcoming resistance in Capsicum in Australia Australasian Plant Pathology. 35, pp. 123-128.

Tomlinson J.A. (1987). Epidemiology and control of virus diseases of vegetables. Annals of Applied Biology 110: 661-681.