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Emergence of Quarantine *Tobacco ringspot virus* in *Impatiens walleriana* in the Czech Republic

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Abstract

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Tobacco ringspot virus (TRSV) is a quarantine pathogen in Europe. During an official inspection in November 2011, *Impatiens walleriana* plants showing symptoms were found in a nursery in the Czech Republic. The causal agent of the disease was detected as Nepovirus group A by RT-PCR using specific primers of the Nepovirus group. Sequence analysis of PCR fragments confirms that the detected virus is TRSV. TRSV detection in these plants was further confirmed by ELISA and one-step RT-PCR using specific primers. The coat protein (CP) gene of the Czech TRSV isolate was sequenced, and the sequence analysis showed high identity of both nucleotide (99.28%) and amino acid (99.96%) levels compared with other known TRSV isolates from GenBank. Two amino acid motifs characteristic of nepoviruses, FDDY (FDAY) and FWGR (FYGR), were found equally at positions 80 and 497 of the TRSV CP genes, respectively, including the sequences described in this study.

Keywords: TRSV; RT-PCR; quarantine pathogen

Tobacco ringspot virus (TRSV) was first reported in *Nicotiana tabacum* by Fromme *et al*. (1927). It is a member of the genus *Nepovirus* (subgroup A) in the family *Secoviridae* (Le Gall *et al*. 1995; Rott *et al*. 1995). The TRSV genome consists of two polyadenylated positive-sense single-stranded RNA molecules: RNA1, which encodes four non-structural proteins, and RNA2, which encodes the coat protein and an upstream movement protein (Buckley *et al*. 1993; Chandrasekar & Johnson 1998). These two RNA molecules are encapsidated in spherical virions of approximately 28 nm (Diener & Schneider 1966). TRSV has a wide host range, including both woody and herbaceous plants. The virus occurs both in annual and perennial crops, fruit trees, ornamentals, and various weeds (reviewed in Stace-Smith 1985) in nature. It is readily transmissible by sap inoculation and naturally transmitted by the nematode *Xiphinema americanum* and other closely related *Xiphinema* spp. (Lamberti & Bleve-Zacheo 1979). TRSV is transmitted by pollen (Card *et al*. 2007) and the virus is also transmissible by seed at least in twelve species of crop and weed hosts; the frequency of transmission ranges from 3% in *Cucumis melo* to 100% in *Glycine max* (Murant 1983).

TRSV is a quarantine pathogen in Europe (EPPO/CABI 1992), where it is mostly found in herbivorous ornamentals (*Anemone*, *Gladiolus*, *Iris*, *Narcissus*, *Pelargonium*). These recorded incidences in Europe are believed to be associated with the intensive movement of imported plant and planting materials. Due to the wide host adaption of TRSV, its incidence may cause a major phytosanitary concern in Europe and/or elsewhere in the world. The virus was not found in the Czech Republic until September 2011. During a routine phytosanitary inspection, a few mother plants

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of *Impatiens walleriana* with suspicious symptoms were found in ornamental greenhouses. The symptoms were sporadic chlorotic rings and patterns on leaves. A group-specific RT-PCR confirmed that the causal agent of this disease is Nepovirus group A. Further sequence analysis determined the virus species to be TRSV.

MATERIAL AND METHODS

Source of diseased samples. In September 2011 during a routine phytosanitary inspection for the presence of quarantine tospoviruses in a company with ornamental greenhouses, a few mother plants of *Impatiens walleriana* with suspicious symptoms were found. Symptomatic leaves were sampled for laboratory confirmation. The symptoms were sporadic chlorotic rings and patterns on leaves.

Identification and detection of causal agent of the disease

Biological indexing and symptoms evaluation. The diseased leaves of *Impatiens walleriana* were used for mechanical transmission of the virus to *Nicotiana occidentalis* P1, *N. benthamiana,* and *Chenopodium quinoa* plants. The plants were inoculated using a phosphate buffer and celite as an abrasive.

Enzyme-linked immunosorbent assay (ELISA). The sample was tested by ELISA for the presence of *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), several other viruses known to infect Impatiens plants (*Alfalfa mosaic virus* – AMV, *Broad bean wilt virus 1* – BBWV 1, *Cucumber mosaic virus (*CMV), *Tobacco mosaic virus* – TMV, and *Tomato mosaic virus* – ToMV) and TRSV.

All ELISA tests were performed using an ELISA kit (Adgia, Elkhart, USA) according to the manufacturer's instructions.

RNA preparation. RNA was isolated from the leaves of all the above mentioned plants using a commercially available extraction kit, RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with a modification according to Mekuria *et al*. (2003). 200 mg of either fresh or frozen leaf tissue was ground into a fine powder in liquid nitrogen, mixed with 2.0 ml of extraction buffer containing 4.4% (w/v) PVP-40 (Sigma, St. Louis, USA) and 1% (w/v) sodium metabisulphite, and briefly vortexed. 500 µl of the homogenate was mixed with 60 ml of 20% (w/v) sarkosyl (*N*-lauroylsarcosine; Sigma) and incubated at 70°C with agitation for 10 minutes. The contents were then transferred to a QIA shredder mini column and centrifuged at 14 000 rpm for 5 minutes. The column flow-through (350 ml) was mixed with 315 ml of 95% ethanol, and the remainder of the protocol was performed according to the manufacturer's instructions. RNA was stored at –20°C (or at –80°C for long-term storage). The quality and quantity of isolated RNA were determined by spectrophotometry at 260, 230, and 280 nm.

Virus identification by RT-PCR and sequencing. Two-step RT-PCR was performed for detection of the virus using primers specific to Nepovirus group A and group B as described in Wei and Clover (2008). The first strand cDNA was synthesised by reverse transcription of total RNAs using the Nepovirus subgroup A and subgroup B reverse primers (NepoA-R and NepoB-R) and *Avian myelobastosis virus* (AMV) reverse transcriptase (Promega Corp., Madison, USA). Amplification of cDNA was carried out using the proofreading Ex Taq™ polymerase (TakaRa, Kyoto, Japan) with the primer pair NepoA-F/NepoA-R (for subgroup A) and NepoB-F/NepoB-F (for subgroup B) (Table 1). The reaction was conducted under the following conditions: an initial denaturation at 94°C for 3 min, then 35 cycles at 94°C for 45 s (denaturation), 50°C for 30 s (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 3 minutes.

The PCR products were analysed by 1% agarose gel electrophoresis; staining was done by SYBR Green (Invitrogen, Carlsbad, USA). PCR fragments were also analysed by sequencing of amplicons using reverse and forward primers of each subgroup. The sequences were analysed using the software Clustal W, v. 1.7 (Thompson *et al*. 1997), Sequencher 4.8 (Gene Codes Corp., Ann Arbor, USA), and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Virus detection by one-step RT-PCR. One-step RT-PCR for multiplex virus detection was performed with the One-Step-RT-PCR kit (Qiagen) as described by Kundu (2003) using specific primers to TRSV designed by Fuchs *et al*. (2010). The One-Step-RT-PCR mixture containing 5 µl of the 5× Qiagen One-Step-RT-PCR buffer, 10 nM of each dNTP, 1 µl of the Qiagen One-Step-RT-PCR enzyme mixture, 5 µl of Q-solution, and 6 pM of reverse (MF0505-21-R) and forward (MF05-21-F) primers (Table 1) was prepared in 1 µl of RNA and adjusted to 25 µl with RNase-free water. The reaction was conducted in a thermocycler (Bio-Rad, Hercules, USA) as follows: a RT step at 50°C for 30 min and an initial PCR activation step at 95°C for 15 min followed by

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Table 1. Primers used for target genes of TRSV

Primer name	Sequence 5' → 3'	References
NepoA-F (forward)	ACDTCWGARGGITAYCC	Wei and Clover (2008)
NepoA-R (reverse)	RATDCCYACYTGRCWIGGCA	
NepoB-F (forward)	TCTGGITTTGCYTTRACRGT	
NepoB-R (reverse)	CTTRTCACTVCCATCRGTAA	
MF05-21-R	CAATACGGTAAGTGCACACCCCG	Fuchs *et al.* (2010)
MF05-22-F	CAGGGGCGTGAGTGGGGGCTC	
Oligo (dT)	AATTCGCGGCCGC$(T)_{15}$	Zadeh and Foster (2001)
P1	GGCTCGAGCCATGGGTGCTGTGACAGTTGTTC	

35 cycles at 94°C for 30 s (denaturation), at 50°C for 1 min (annealing), and at 72°C for 1 min (extension). After the last cycle, a final extension step at 72°C for 10 min was added.

The PCR products were analysed in 2% agarose gel electrophoresis; staining was done by SYBR Green (Invitrogen, Carlsbad, USA).

Viral sequence determination and analysis. Two-step RT-PCR was performed for the amplification of RNA 2 segment of TRSV as described in Zadeh and Foster (2001). The first strand cDNA was synthesized by reverse transcription of total RNAs using the oligo (dT) primer and *Avian myelobastosis virus* (AMV) reverse transcriptase (Promega, Madison, USA). Amplification of cDNA was performed using the proofreading Ex Taq™ polymerase (TakaRa, Kyoto, Japan) with the primer pair oligo dT/P1 (Table 1). The reaction was conducted under the following conditions: 30 cycles at 94°C for 1 min (denaturation), at 55°C for 1 min (annealing), at 72°C for 2 min (extension), and a final extension at 72°C for 10 minutes.

An aliquot (4 µl) of each amplification reaction was mixed with 1 µl of 6× loading dye (Fermentas, MBI, Amherst, USA), electrophoresed in high resolution 1.5% agarose gel, and run in TAE buffer. The bands were visualised and photographed under UV (312 nm) after SYBER safe (1 µg/ml) binding to the DNA fragments. A 1 kb marker (Fermentas, MBI) was included on the gel. The rest of the DNA of the positive samples was purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Purified DNA was cloned into pGEM-T easy vector (Promega, Madison, USA) according to the manufacturer's instructions. Plasmids were purified using QIAprep (Qiagen), and six clones were sequenced with the universal vector primers T7+SP6 (Macrogen, Seoul, Korea). Sequencher™ 4.8 (Genes Codes Corp., Ann Arbor, USA) software was used to assemble and view sequences and check for base-calling errors. Phylogenetic and sequence analyses were performed with Clustal-X (Thomson *et al.* 1997) using the Neighbour-joining method (Saitou & Nei 1987) and MEGA 4 (Tamura *et al.* 2007).

RESULTS

Detection of *Tobacco ringspot virus* in *Impatiens walleriana*

During an official inspection in November 2011 symptomous *Impatiens walleriana* plants were found in a nursery in the Czech Republic. Several viruses known to infect *Impatiens* such as *Tomato spotted wilt virus* and *Impatiens necrotic spot virus*, *Alfalfa mosaic virus, Broad bean wilt virus 1, Cucumber mosaic virus, Tobacco mosaic virus,* and *Tomato mosaic virus* were tested by ELISA. All tests for the viruses were negative. The mechanical inoculation of the diseased leaf in *Nicotina occidentalis* P1 or *N. benthamiana* showed systemic symptoms, and *Chenopodium quinoa* had a local lesion (Figure 1). The symptoms in the herbaceous plants and electron microscopy by negative staining of the leaf samples (results are not shown) indicate the possible causal agent to be *Nepovirus*. The *Impatiens* and the herbaceous host plants were tested by RT-PCR using Nepovirus subgroup-specific primers for subgroups A, B, and C (Wei & Clover 2008). The specific PCR product of Nepovirus subgroup A was detected in all tested plants (Figure 2). The mother Impatiens plant and its several progeny and New Guinea hybrid Impatiens plants from the same orchards were also tested by RT-PCR using Nepovirus subgroup A primers. Subgroup A was detected in all Impatiens plants but not in the Impatiens New

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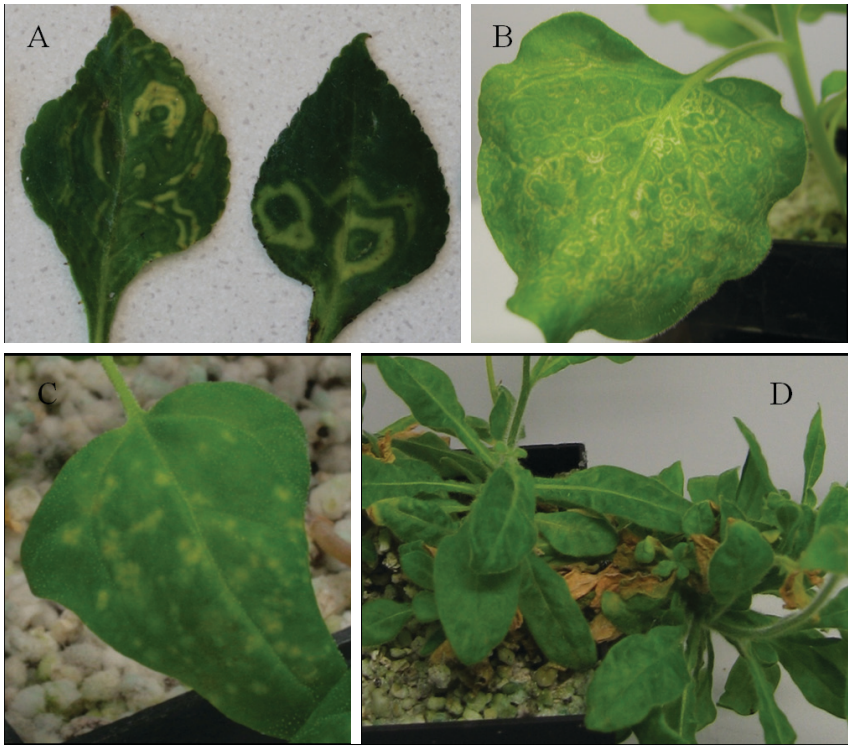

Figure 1. TRSV symptoms: (A) Chlorotic rings and patterns on leaves of *Impatiens walleriana*; (B) local concentric chlorotic lesions, later necrotic rings and systemic leaf deformation and dwarfing on *Nicotiana benthamiana*; (C) local chlorotic spots, necrosis of the top of the plant, and subsequent stunting of lateral shoots of *Chenopodium quinoa*; and (D) chlorotic rings and spots and systemic necrosis, leaf deformation and dwarfing on *N. occidentalis* P1.

Guinea hybrids plants (Table 1 and Figure 2). The PCR products were subjected to sequence analysis, and the mutilated sequence analysis showed a similarity with TRSV sequences from Gene Bank. The TRSV detection in these samples was tested again by ELISA with TRSV antiserum, which confirmed the PCR and sequenced-based detection of the virus (data not shown). The detection of TRSV was again confirmed by one-step RT-PCR (Figure 3) using specific primer pairs described in Fuchs *et al.* (2010).

The presence of TRSV in all lots of mother plants of *I. walleriana* and the absence of any other potential hosts (including mother plants of Impatiens New Guinea hybrids) suggest that TRSV was introduced into the premises with infected plants and that it was then spread among *I. walleriana* plants by vegetative propagation. This assumption is further supported by the investigation of soil samples for the presence of a nematode vector, *Xiphinema americanum* (absent from the Czech Republic), which yielded negative results. Hence, in the absence of nematode vectors, TRSV did not spread to other host species in the greenhouse.

TRSV sequence analysis

The partial RNA2 segment of TRSV was sequenced. The coat protein gene sequences of nucleotides and

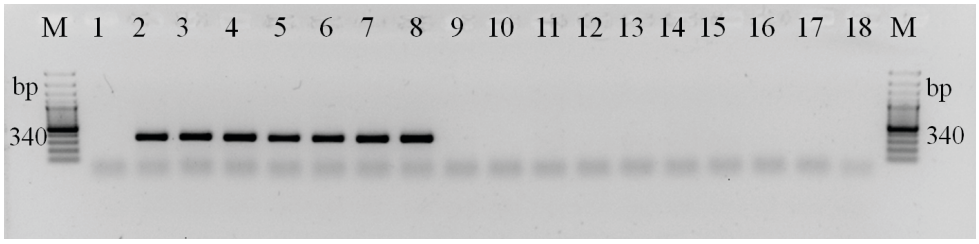

Figure 2. Detection of Nepovirus subgroup A by RT-PCR

Lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada); Lanes 1–17 (samples 1104982–1104999): Impatiens New Guinea hybrids, *Impatiens walleriana*, *Impatiens walleriana*, *Impatiens walleriana*, *Impatiens walleriana*, *Impatiens walleriana*, *Impatiens walleriana*, *Impatiens walleriana*, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids, Impatiens New Guinea hybrids; Lane 18: negative control (water control)

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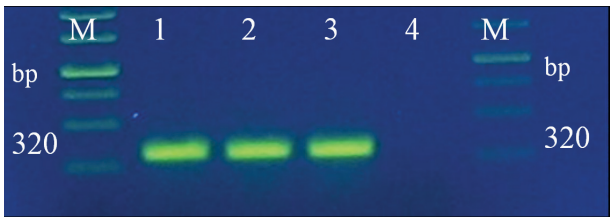

Figure 3. One-step RT-PCR for the detection of TRSV

Lane 1: sample 1104983 of *Impatiens walleriana*; lane 2: sample 1104984 of *Impatiens walleriana*; lane 3: sample 1104986 of *Impatiens walleriana*; lane 4: sample 1104982 of Impatiens New Guinea hybrids; lane M: DNA ladder 100 bp (Fermentas Int.)

amino acids were analysed. The coat protein gene sequence of TRSV from the mother plant (accession No. KP144325) and the sequences of its progeny (accession Nos KP144326, KP144327, and KP144328) have only a few sequence variations at the nucleotide level which do not cause mutations at the amino acid level. The Czech isolates showed a high sequence identity in coat protein gene both at nucleotide and amino acid level with other known TRSV sequences irrespective of plant host or geographical locations (Table 2). The coat protein gene sequence of the Czech TRSV has 6–12 amino acid differences from other known isolates. The multiple sequence alignments

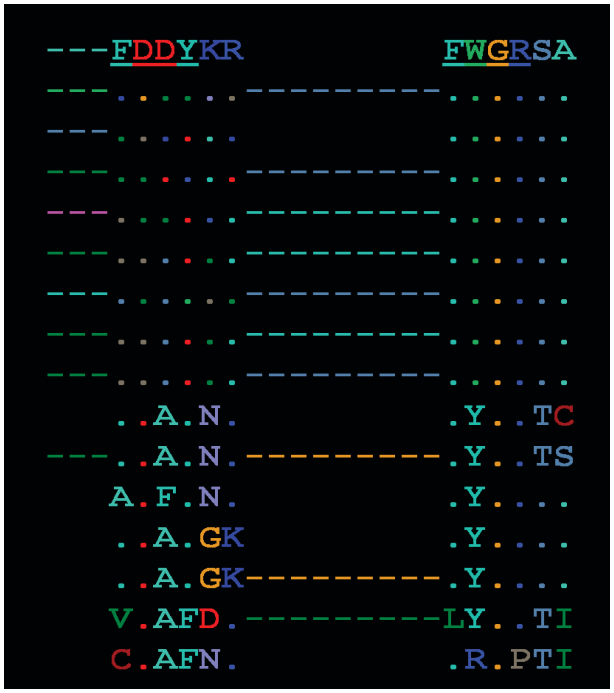

Figure 4. Multiple alignment of two nepovirus motifs of coat protein gene of nepoviruses

The member indicate the amino acid positions in CP gene of TRSV

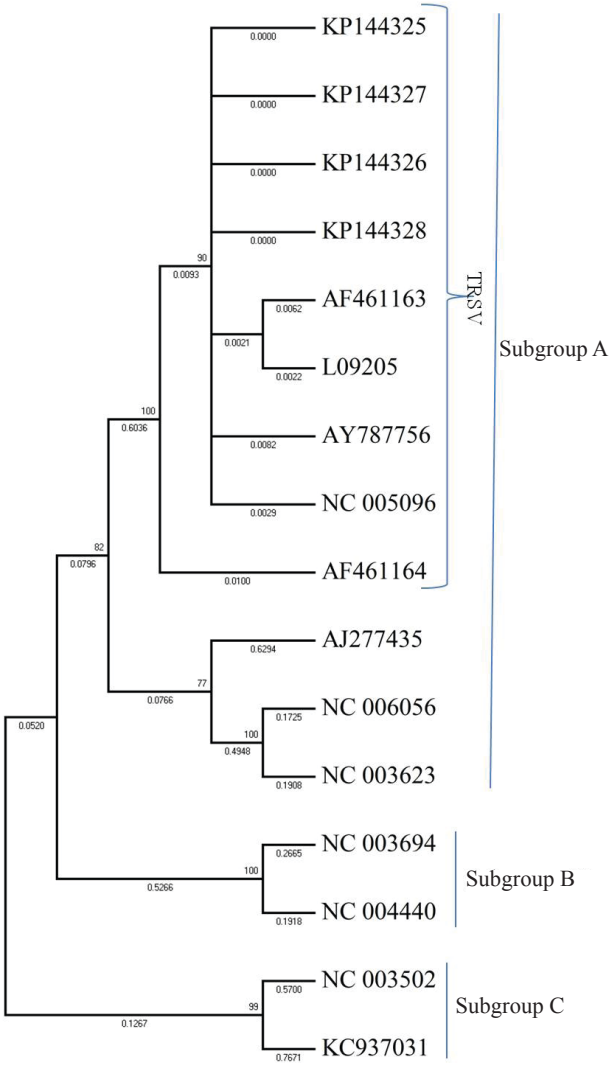

Figure 5. Phylogenetic tree reconstructed by the Neighbour-joining method based on the alignment of 16 coat protein gene sequences (amino acid) of nepoviruses (subgroups A, B, and C), including nine *Tobacco ringspot virus* isolates

The branch lengths (lower numbers) and bootstrap values > 70% are presented on branches (upper numbers)

GenBank accession numbers of sequences used are as follows: *Tobacco ringspot virus*-KP144325, KP144326, KP144327, KP144328 (Impatiens isolates), AF461163 (cherry isolate), L09205, NC_005096, AF461164 (soybean isolates), AY787756 (tobacco isolate), *Arabis mosaic virus*-NC_006056 (grapevine isolate), *Grapevine fanleaf virus*-NC_003623 (grapevine isolate), *Olive latent ringspot virus*-AJ277435 (olive isolate), *Beet ringspot virus*-NC_003694 (sugar beet isolate), *Tomato black ring virus*-NC_004440 (tomato isolate), *Blackcurrant reversion virus*-NC_003502 (blackcurrant isolate), *Cherry leaf roll virus*-KC937031 (blueberry isolate)

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Table 2. Coat protein gene sequence identity of the Czech TRSV compared with other nepoviruses

Nepovirus subgroup	Virus	Accession number	Host	Country of origin	KP144325 (CZ)		KP144326 (CZ)		KP144327 (CZ)		KP144328 (CZ)	
					nt	aa	nt	aa	nt	aa	nt	aa
Subgroup A	TRSV	KP144325					99.8	100	99.7	100	99.7	100
		KP144326	Impatiens	CZ					99.8	100	99.9	100
		KP144327									99.7	100
		AF461163	Cherry	UK	99.59	99.92	99.59	99.92	99.59	99.92	99.59	99.92
		L09205	Soybean	USA	99.55	99.96	99.55	99.96	99.55	99.96	99.54	99.96
		AF461164	Soybean	Iran	99.33	99.79	99.34	99.79	99.34	99.79	99.34	99.79
		AY787756	Tobacco	China	99.27	99.92	99.27	99.92	99.29	99.92	99.28	99.92
		NC_005096	Soybean	USA	99.62	99.96	99.61	99.96	99.60	99.96	99.62	99.96
	ArMV	NC_006056	Grapevine	Germany	94.36	92.58	94.37	92.58	94.37	92.58	94.36	92.58
	GFLV	NC_003623	Grapevine	France	94.10	92.48	94.11	92.48	94.12	92.48	94.11	92.48
	ORSV	AJ277435	Olive	Italy	94.17	92.69	94.17	92.69	94.18	92.69	94.16	92.69
Subgroup B	BRSV	NC_003694	Sugar beet	USA	93.81	92.23	93.81	92.23	93.81	92.23	93.81	92.23
	ToBRV	NC_004440	Tomato	Poland	93.85	92.52	93.87	92.52	93.86	92.52	93.87	92.52
Subgroup C	BRV	NC_003502	Blackcurrant	Finland	93.94	92.33	93.94	92.33	93.93	92.33	93.94	92.33
	CLRV	KC937031	Blueberry	New Zealand	93.80	91.93	93.81	91.93	93.80	91.93	93.81	91.93

TRSV – *Tobacco ringspot virus*; ArMV – *Arabis mosaic virus*; GFLV – *Grapevine fanleaf virus*; ORSV – *Olive latent ringspot viru*; BRSV – *Beet ringspot virus*; ToBRV – *Tomato black ring virus*; BRV – *Blackcurrant reversion virus*; CLRV – *Cherry leaf roll virus*; nt – nucleotide; aa – amino acid; CZ – Czech Republic

have shown that the recorded divergence of TRSV was by 10% lower than of other nepoviruses, subgroup A, B, or C (the results are not shown). The phylogenetic analysis also supported this finding. The Czech TRSV isolates were grouped together in a separate clade with all TRSVs, and other nepoviruses formed clades with their respective subgroup (Figure 5). Two amino acid motifs, the N-terminal Phe-Asp-Ala-Tyr (FDAY) and the C-terminal Phe-Tyr-Gyl-Arg (FYGR), are found in the Czech isolate of TRSV. All TRSV CP genes in these two motifs are equally found at positions 80 for Phe-Asp-Ala-Tyr (FDDY) and 497 for Phe-Trp-Gyl-Arg (FWGR), respectively (Figure 4).

DISCUSSION

Identification of a causal agent of virus diseases is always a great challenge for plant pathologist, particularly in the case of a disease in non-cultivated host. There are several diagnostic tools like symptom observation (including biological indexing in herbaceous plants), electron microscopy, serological and molecular assays, which may be used for the identification of certain viral agents. In the case of identification of unknown viruses a combination of different assays is needed. In this paper we described the identification of the quarantine viral pathogen *Tobacco ringspot virus* in an ornamental host, *Impatiens walleriana*. The symptoms in mother plant and herbaceous hosts like *Nicotiana benthamiana*, *N. occidentalis* P1, and *Chenopodium quinoa* indicate that the causal agent of the disease may have a viral origin. However, the subsequent ELISA tests for detection of the suspected viruses known to infect *Impatiens* plants such as TSWV, INSV, AMV, BBWV 1, CMV, TMV ToMV, and TRSV were unsuccessful. Using a nepovirus-group specific primer-based RT-PCR (Wei & Clover 2008) we identified the causal agent of the disease as a nepovirus belonging to the subgroup A. Sequence analysis of the PCR fragment confirmed that the disease has been caused by TRSV. This is the first report of the emergence of TRSV in the Czech Republic.

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TRSV is a quarantine pathogen in Europe and elsewhere in the world (EPPO/CABI 1992) having a wide range of crop and non-crop hosts (Demski & Kuhn 1989).Therefore, the finding of the incidence of the virus is of a high phytosanitary concern in the Czech Republic. The persistent mode of transmission by nematodes (Wang & Gergerich 1998), seed and pollen transmission ability (Zadeh & Foster 2004) together with a wide host range of distinct plant species (Brunt *et al*. 1996) make TRSV an easily spread pathogen in agro-ecosystem. Furthermore, recent finding showed that TRSV could replicate and produce virions even in honeybees, *Apis mellifera,* and contribute together with common bee viruses to the declination of host population (Li *et al*. 2014).

Beside the detection of TRSV by RT-PCR, ELISA, and confirmation of the detection by sequencing, we also analysed the coat protein gene sequences of the Czech isolate of the virus. The coat protein gene of TRSV from the Czech Republic has a high sequence identity both at amino acid and nucleotide level with sequences from the UK (cherry isolate), the USA (soybean isolate), China (tobacco isolate), and Iran (soybean isolate) (Table 2). The sequence identity reaches over 99% both at nucleotide and amino acid level while compared with TRSV isolates from abroad. The sequence identity with other viruses from nepovirus group A like ArMV, GFLV, and ORSV reaches over 90% as well as with the viruses from subgroup B (BRSV, ToBRV) or subgroup C (BRV, CLRV). Our results correspond with earlier reported sequence analysis of coat protein gene (Zadeh & Foster 2001) or RNA-dependent RNA polymerase (RdRp) gene sequences (Fuchs *et al*. 2010) of TRSV. A similar range of sequence identity was also recorded in other nepoviruses, such as *Cherry leaf roll virus* – CLRV (von Bargen *et al*. 2012). The typical nepovirus conserved motifs in N-terminal FDAYXR and the C-terminal FYGRXS (Le Gall *et al*. 1995) occur equally in all TRSV coat protein sequences at positions 80 (N-ter) like FDDYKR and 497 (C-ter) like FWGRSA. The C-terminal motif is involved in stabilizing the quaternary structure of the capsid protein, whereas the N-terminal motif is located on the surface of the CP (Chandrasekar & Johnson 1998).

For first time we reported here the emergence of quarantine pathogen *Tobacco ringspot virus* (TRSV) in the Czech Republic. The sequence of the coat protein gene of the virus from *Impatiens* plant was analyzed. A one step RT-PCR assay was described here for the routine detection and monitoring of incidence of this quarantine virus.

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