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Molecular and biological comparison of two Tomato yellow ring virus (TYRV) isolates: challenging the *Tospovirus* species concept

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Summary. Two strains of Tomato yellow ring virus (TYRV, genus *Tospovirus*), one from tomato (referred to as TYRV-t) and the other from soybean and potato (denoted TYRV-s), collected from different geographical regions in Iran, were compared. Their genomic S RNA segments differed in size by 55 nucleotides. Comparison of the S RNA intergenic regions revealed the absence of a stretch of 115 nucleotides within the S RNA segment of TYRV-s and, conversely, of 56 nts in that of TYRV-t, apparently a stable genetic difference as it was also found in another isolate of TYRV-s collected from potato. Sequence comparison of the N protein ORFs revealed an identity of 92% between the N proteins of both strains, and the observed strong cross-reaction of TYRV-s in DAS-ELISA with a polyclonal antiserum directed against the TYRV-t N protein confirmed this high identity. Host range analysis revealed several differences, e.g. TYRV-s, but not TYRV-t, being able to systemically infect *Nicotiana* species, and TYRV-s being localised in tomato. The observed molecular and biological differences of both viruses call into question the currently used criteria for *Tospovirus* species demarcation.

Introduction

Species of the genus *Tospovirus* (family *Bunyaviridae*) have enveloped and quasi-spherical virions, 80–120 nm in diameter, and are transmitted by thrips (Thysanoptera; *Thripidae*) in a persistent manner [10]. Currently, 13 different thrips species have been reported as vectors, the western flower thrips, *Frankliniella occidentalis*, being the most important one [12, 24, 27, 36]. The tripartite tospoviral genome consists of ambisense S and M RNA segments, while the L RNA is of negative polarity [8, 9, 19]. The genome encodes 6 mature proteins: the RNA-dependent RNA polymerase protein (RdRp or L protein) by the L RNA,

the cell-to-cell movement protein (NS_M) and the suppressor of silencing (NS_S) protein in the viral sense by the M and S RNA, and the two membrane glycoproteins (G_N and G_C) and the nucleoprotein (N) in the viral complementary sense by the M and S RNAs, respectively [2, 8, 9, 19, 20, 32]. The open reading frames (ORFs) in the M and S RNA segments are separated by large AU-rich intergenic regions (IGR), which form a stable hairpin structure and are assumed to be involved in transcription termination [34].

Serological distinction among tospovirus species is based on double-antibody-sandwich (DAS)-ELISA using the N protein, which is the least conserved tospoviral protein, as antigen [7, 35]. Indeed, the identification of new tospovirus species was initially based on ELISA and resulted in the classification of tospoviruses into serogroups [6, 7]. However, serological differentiation soon appeared insufficient as a taxonomic criterion, and nowadays the N protein sequence in combination with biological characters such as thrips vector species and host range represent the main classification criteria for the establishment of a new tospovirus species, with the N protein sequence identity threshold set at 90% [5, 11]. Based on these criteria, 16 tospovirus species have been recognized with the largest diversity being observed within the Asian continent [15]. With its worldwide distribution and wide host range tomato spotted wilt virus (TSWV) is the most prominent member of all tospoviruses.

Recently, an isolate of a new tentative tospovirus species, named Tomato yellow ring virus (TYRV), was collected from tomato in Iran, and this virus was most closely related (by N protein sequence identity) to iris yellow spot virus (IYSV) [3, 15]. In a limited field survey, mainly in Teheran province, Iran, four additional tospovirus isolates were collected from chrysanthemum, gazania, potato, and soybean during 2000–2002. Upon further analysis, the first two isolates were found to represent TYRV isolates [15], whereas preliminary RT-PCR analyses of their N gene suggested that the isolates from soybean and potato represented a different tospovirus.

Here we report the further characterization of the Iranian tospovirus isolates from soybean and potato and show that they belong to a distinct strain of TYRV. The degree of difference in molecular and biological characters between the two strains of TYRV may urge revision of the *Tospovirus* species concept.

Materials and methods

Virus isolates and host range study

Tospovirus isolates collected in Iran, i.e. TYRV isolates from tomato, chrysanthemum, gazania, and potato (Teheran province) and one isolate from soybean (Mazandaran province), were maintained and propagated on *Nicotiana benthamiana*. To avoid formation of defective interfering (DI) isolates due to serial mechanical passages, new virus inocula were prepared from liquid nitrogen stocks every 4–5 passages [18]. TYRV and the soybean isolate were inoculated on a large range of plant species including those tested for TYRV [15] and different *Nicotiana* species and *N. tabacum* cultivars. The plants were monitored for the expression of symptoms during several weeks while being kept at 22–25 °C 12 h (light/dark) under greenhouse conditions. To confirm systemic infections in infected plants, both symptomatic

and asymptomatic leaf samples, next to uninfected leaf material as negative control, were tested by DAS-ELISA using polyclonal antiserum directed to the N protein of TYRV [7, 15].

Serological analysis

Serological analysis was performed by DAS-ELISA using polyclonal anti-N sera of TYRV [15], TSWV [7], and IYSV [3]. In this test, crude extracts from infected *N. benthamiana* and healthy plants were diluted 1:30 and were used as antigen source and negative control, respectively.

Purification of viral N protein, viral RNA, and total RNA

Systemically infected *N. benthamiana* leaves were used to purify nucleocapsids of both the TYRV-s and potato tospovirus isolates as described by de Ávila et al. [7]. Viral RNA was extracted from purified nucleocapsid preparations obtained after $CsSO_4$ gradient centrifugation by treating the extract with 1% SDS, followed by phenol/chloroform extraction and ethanol precipitation [3]. Total plant RNA was isolated from infected *N. benthamiana* plants according to Kormelink et al. [19].

Cloning of the S RNA segment and sequence analysis

For cloning, reverse transcriptase polymerase chain reaction (RT-PCR) was performed to amplify fragments representing the entire S RNA sequence of the soybean isolate. As a first step, partial sequences of the N and NSs ORFs were amplified using primer "Asian Termini" (AT; 5′dCCCGGATCC**AGAGCAATCGAGG**3′ containing (in bold) 13 terminal nucleotides conserved in all Asian tospoviral S RNA segments) in combination with a universal hairpin primer (UHP; 5′dCACTGGATCCTTTTGTTTTTGTTTTTTG) [4]. To amplify and clone the remaining sequences, specific primers were designed based on sequences obtained from S RNA-derived clones. For most of the cloning procedure, RT-PCR was carried out using Superscript RT and the Expand Long Template PCR System (Roche), with the exception of the intergenic region (IGR), which was amplified by immunocapture RT-PCR [22]. PCR products were cloned into pGEM-T Easy vector (Promega Corp. Madison, WI) and sequenced by the dideoxynucleotide chain termination method [29] using an automatic sequence machine (Applied Biosystems, Foster City, CA). Sequences obtained were analyzed using BLAST. RNA folding structures were predicted using MFold [37]. Multiple sequence alignment was carried out using Vector NTI (Invitrogen). Phylogenetic trees were constructed from ClustalW [31] data in PAUP 3.1. with a heuristic search, based on 100 replicates, using midpoint rooting [31].

The nucleotide sequence for the complete S RNA of TYRV-s has been submitted to Genbank under accession number DQ462163.

Results

RT-PCR amplification and cloning of the Iranian soybean and potato tospoviral N genes

During the previously documented identification and characterization of a new tomato-infecting tospovirus in Iran [15], denoted tomato yellow ring virus (TYRV), a primer set was designed to allow rapid amplification and cloning of the N gene of this virus [15]. This set was tested here on four recently collected tospovirus isolates from Iran, sampled from chrysanthemum, gazania, soybean, and potato, as these were suspected to also belong to this species. RT-PCR amplification of

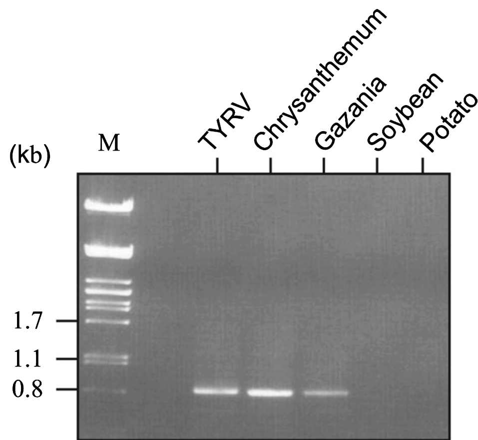

Fig. 1. RT-PCR-based differentiation of Iranian TYRV isolates. *M*, molecular size marker λ DNA × *Pst*I. Primers for amplification of the N gene were deduced from the TYRV-t S RNA sequence

total RNA from infected *N. benthamiana* resulted, for the chrysanthemum and gazania isolates, in a clear fragment co-migrating with the amplified N gene from TYRV (Fig. 1). Upon sequence analysis, these isolates were shown to be almost identical to TYRV from tomato (further referred to as TYRV-t) with only 0.7–1.5% sequence divergence in their N protein sequence (data not shown). However, no fragments could be amplified for the soybean and potato isolates, indicating that these isolates might be distinct. Also, amplification using a multiplex primer set that recognises the N genes of TSWV, INSV, GRSV, and TCSV remained negative.

N protein serology

As PCR amplification remained negative for the soybean and potato isolates, their serological relationship to TYRV-t and other tospoviruses was determined using

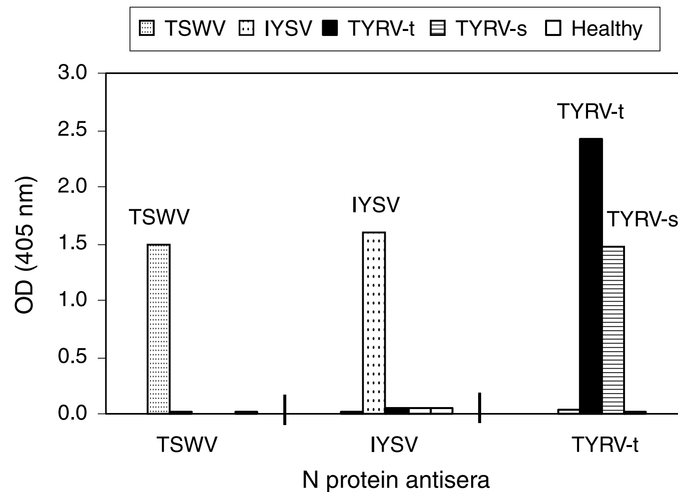

Fig. 2. Serological differentiation between TYRV-t (tomato isolate) and the soybean isolate (TYRV-s). The chart shows A405 values (y-axis) from a double-antibody-sandwich enzyme-linked-immunosorbent-assay (DAS-ELISA) performed using polyclonal anti-N sera from different tospoviruses (shown at the x-axis) and extracts from infected plants as antigen source (represented by patterned bars)

DAS-ELISA. No reaction was observed with polyclonal antisera against TSWV or IYSV, but a strong positive reaction was obtained with anti-TYRV serum (Fig. 2). Repeated analysis showed that the ELISA values were always consistently lower compared to the homologous TYRV-t signal. This provided evidence that, despite the negative RT-PCR outcome, both the soybean and potato isolates had a close taxonomic relationship to TYRV-t.

Comparative analysis of the S RNA segment

To elucidate the taxonomic position of the soybean isolate, its entire S RNA was cloned and sequenced and subsequently compared to the TYRV-t S RNA segment. As is typical for tospoviruses, the S RNA of this isolate contained, in ambisense arrangement, ORFs for the NSs and N proteins, with a predicted M_r of 50.0 and 30.0 kDa, respectively. The NSs and N proteins of the soybean isolate and TYRV-t showed 94 and 92% amino acid (aa) sequence identity, respectively. This still falls, albeit marginally, inside the range allowed within the ICTV species concept for tospoviruses [11], and in view of the marked differences, the soybean isolate was further referred to as TYRV-s (*versus* TRYV-t for the tomato isolate). TYRV-s and TYRV-t also displayed clear differences in their IGRs, the IGR of TYRV-s lacking 115 nts present in TYRV-t and, conversely, that of TYRV-t lacking 56 nts present in TYRV-s (Fig. 3). The central part of both IGRs can be folded in a secondary structure which has a longer stretch of ds-RNA (34 nts) in TYRV-s than in TYRV-t (28 nts) [15]. The 5′ and 3′-terminal untranslated regions (UTR) were of similar length (71–72 nts), with a high potential tendency to form a genomic panhandle pseudo-circle.

Multiple sequence alignment and phylogenetic relationship

To establish the taxonomic position of TYRV-s (and TYRV-t) within the genus *Tospovirus*, the N protein sequences were compared with those of members of established tospovirus species. This analysis showed that TYRV-s (from soybean and potato) and TYRV-t (from tomato, chrysanthemum, and gazania) are most

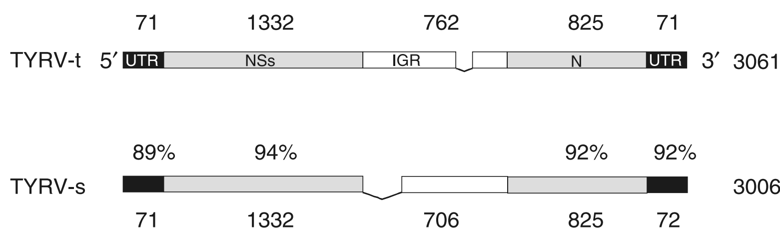

Fig. 3. Comparative analysis of the S RNA segments of TYRV-t and TYRV-s. Untranslated regions (UTR) at the termini are marked in black, open reading frames (ORFs) in gray, and the intergenic region (IGR) in white. Only major deletions within the IGR are indicated by interruption of the IGR-box. The sizes of the UTR and IGR are indicated in nucleotides above (TYRV-t) and below (TYRV-s) the S RNA. The sizes of the N and NSs ORFs are indicated in amino acids. The level of identity (%) between the UTRs and IGRs (at the nucleotide level) and between the ORFs (amino acid level) of both viruses is shown between the S RNA segments

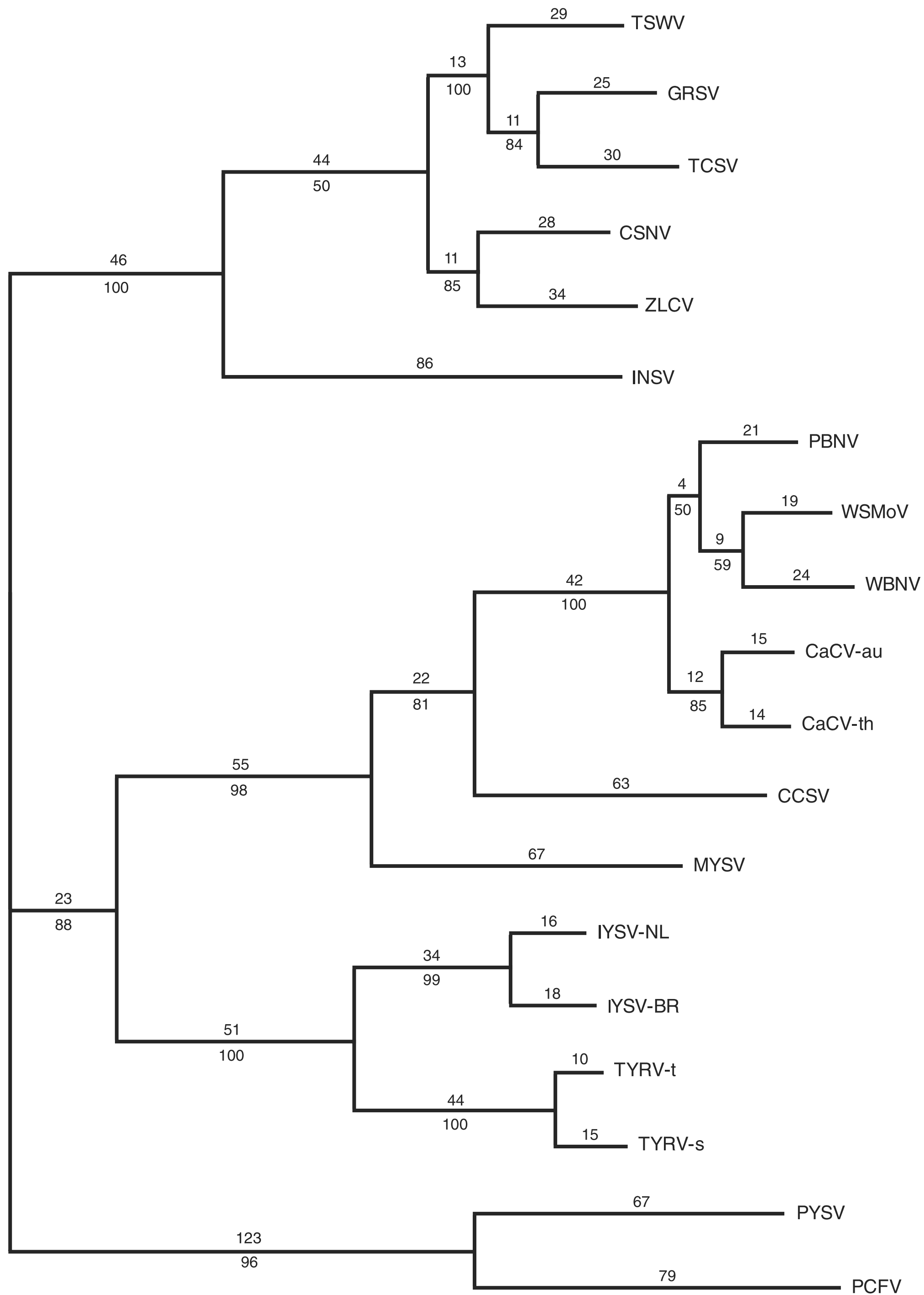

Fig. 4. Evolutionary relationship of tospovirus species. The phylogenetic tree was constructed using PAUP 3.1, with the multiple alignment of tospoviral N protein sequences from ClustalW as input data. Genbank accessions for the tospovirus N gene sequences used are listed in Table 2. The tree was based on bootstrap analysis (100 replicates) of a heuristic search, using midpoint rooting

closely related (72.4–73.1%) to IYSV-NL (Table 2). Data from a multiple sequence alignment were used as input to construct a phylogenetic tree, revealing a close clustering of TYRV-t, TYRV-s, and IYSV-NL within the large Eurasian tospovirus cluster (Fig. 4).

Comparative host range analysis

In view of the clear molecular difference between TYRV-t and TYRV-s (i.e. 8% divergence in their N protein and deletions in their IGRs), and in view of their different crop origin, it was next investigated whether the two strains would exhibit differences in their host range (Table 1). Upon testing a large number of plant species, it was found that on some hosts both TYRV isolates induced only local

Table 1. Host range symptomatology after infection with TYRV-t and TYRV-s

Plant Species	TYRV-t		TYRV-s	
	Local	Systemic	Local	Systemic
Leguminosae				
Glycine max cv. Sahar	CLL, NLL	CS, MO	CLL, NLL	CS, MO
Pisum sativum	CLL	MO, NS	CLL	–
Solanaceae				
Capsicum annum	CS	M, LD	CS	M, LD
Datura metel	CS	M, SN	CS	M, SN
D. stramonium	CS, NLL	C, LD	CS, NLL	C, LD
Lycopersicon esculentum cv. Pito Early	CS	CS, NS, TN, GR	CLL	–
Nicotiana benthamiana	CS	VC, M, MO, LD	CS	VC, M, MO, LD
N. christii	NLL	M, LD	NLL	M, LD
N. clevelandii	M	PN	M	PN
N. edwardsonii	NLL	M, LD	GS, NLL	PN
N. glutinosa	CLL	–	NLL	GS, LD
N. kawakamii	NLL	–	–	–
N. roselata	CS	MO, LN, TN	CS	MO, LN, TN
N. tabacum Av	GS, LL	–	GS	CS,VC
N. tabacum B2/7	GS	–	GS	GS, LD
N. tabacum B84/61	GS, NLL	–	–	GS, VC, NS
N. tabacum D25	–	–	NLL	M, N
N. tabacum Sr1	–	–	M, LD	M, LD
N. tabacum Samsun	LL	–	NLL	CS
N. tabacum van Hieko	LL	–	GS, NS	GS,VC
N. tabacum White burley	–	–	CS	Y
N. tabacum Xanthi	NLL	–	NLL	NS, LD
Petunia hybrida	NLL	–	NLL	–

C Chlorosis; *CLL* chlorotic local lesion; *CS* chlorotic spots; *GS* green spots; *LD* leaf deformation; *LN* leaf necrosis; *M* mosaic; *MO* mottling; *N* necrosis; *NLL* necrotic local lesion; *NS* necrotic spots; *PD* plant death; *PN* plant necrosis; *TN* top necrosis; *SN* stem necrosis; *VC* veinal chlorosis; *Y* yellowing; – no symptoms

Table 2. Tospoviral N protein sequence identities (%)

Virus[a]	TSWV	TCSV	GRSV	INSV	CSNV	ZLCV	PBNV	WSMoV	WBNV	MYSV	PCFV	PYSV	CCSV	CaCV-au	CaCV-th	IYSV-NL	IYSV-BR	TYRV-t	TYRV-s
TSWV	100	77	78	53	75	72	25	28	26	26	18	19	24	29	29	30	31	30	30
TCSV	–	100	81	52	72	71	27	27	26	27	19	20	23	28	28	29	29	31	30
GRSV	–	–	100	52	73	75	27	29	28	27	19	19	25	29	29	29	30	31	31
INSV	–	–	–	100	53	50	27	27	26	24	21	21	23	27	28	26	26	29	27
CSNV	–	–	–	–	100	80	26	28	24	29	20	20	24	28	27	31	29	29	29
ZLCV	–	–	–	–	–	100	26	26	26	26	19	19	24	27	27	29	28	29	30
PBNV	–	–	–	–	–	–	100	86	85	60	21	20	66	84	85	42	44	40	43
WSMoV	–	–	–	–	–	–	–	100	86	58	20	20	65	86	86	41	44	39	43
WBNV	–	–	–	–	–	–	–	–	100	58	19	20	66	82	82	42	43	39	42
MYSV	–	–	–	–	–	–	–	–	–	100	19	19	59	59	60	47	49	45	46
PCFV	–	–	–	–	–	–	–	–	–	–	100	59	18	20	21	18	18	17	18
PYSV	–	–	–	–	–	–	–	–	–	–	–	100	17	20	22	18	21	20	19
CCSV	–	–	–	–	–	–	–	–	–	–	–	–	100	64	64	45	44	44	45
CaCV-au	–	–	–	–	–	–	–	–	–	–	–	–	–	100	92	43	44	40	43
CaCV-th	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	45	45	44	44
IYSV-NL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	91	75	74
IYSV-BR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	74	72
TYRV-t	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	92
TYRV-s	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100

[a]The tospovirus species refered are the following: TSWV(D00645); TCSV(S54325); GRSV(S54327); INSV(S400057); CSNV(AF067068); ZLCV(AF067069); PBNV(U27809); WSMoV(Z46419); WBNV(AF045067); MYSV(AF067151); PCFV(AF080526); PYSV(AF013994); CCSV(AY867502); CaCV-au (AY036058); CaCV-th (Thai-Tospovirus, AF134400); IYSV-NL(AF001387); IYSVBR(AF067070); TYRV-t (AY686718); TYRV-s in this study

The identities (%) of the N protein have been calculated from the sequence data using the Vector NTI program (gap opening penalty 10 and gap extension penalty 0.1)

symptoms, whereas on others, systemic symptoms were observed. Symptoms varied from only necrotic lesions up to plant necrosis. In total, 10 out of 22 plant species tested became systemically infected by TYRV-s but not by TYRV-t. On the other hand, TYRV-t induced mosaics and systemic necrotic spots on *Pisum sativum*, while this plant did not become infected with TYRV-s. Furthermore, cross-inoculations on tomato and soybean cultivars revealed that TYRV-t was able to systemically infect tomato and soybean, whereas TYRV-s only infected soybean (Table 1). These results showed that TYRV-s and TYRV-t exhibit striking differences in their host range and symptomatology.

Discussion

Limited reports have appeared on the occurrence and distribution of plant viruses in Iran, a few of these describing the presence of tospoviruses such as TSWV, INSV, and PBNV [1, 12–14, 30]. However, large-scale surveys on tospovirus infections from that area are scarce, and so is information on the presence of hitherto unidentified new tospoviruses. Hence, the economical impact of tospoviral diseases in Iran (and the further Middle-East area) remains unclear. During the last few years we have investigated the identity of five tospovirus isolates from tomato, chrysanthemum, gazania, soybean, and potato collected from different regions in Iran. These analyses identified the presence of a virus belonging to a new tospovirus species, denoted Tomato yellow ring virus (TYRV), in tomato from the Varamin area [15], a virus also referred to as tomato yellow fruit ring virus (TYFRV) or tomato Varamin virus (ToVV) by others [12, genbank AJ493270]. For this tospovirus, *Thrips tabaci* and *Microcephallothrips abdominalis* were reported as potential vectors [12].

From our earlier work [15] it is evident that this virus also occurs in chrysanthemum (Varamin) and gazania (Teheran) plants as confirmed by RT-PCR cloning and nucleotide sequence analysis of the N gene. Whereas the N gene from the chrysanthemum and gazania isolates could readily be amplified by TYRV-t N-gene-specific primers, similar analysis of the soybean and potato isolates rendered negative results. DAS-ELISA, on the other hand, confirmed a clear serological relationship of the latter two isolates with TYRV, so to elucidate their taxonomic relationship to TYRV, the nucleotide sequence of the S RNA was determined. Comparison of the soybean isolate to TYRV did reveal 92% identity between the N genes, which is just above the threshold value of 90% used for species demarcation, and thereby confirmed the close relationship between TYRV and the soybean isolate. The soybean isolate thus may be best regarded as a distinct strain of TYRV, even though the presumed TYRV-specific primers allowed the soybean isolate to escape RT-PCR detection, and host range analysis of both revealed some clear differences (Table 1). In view of their differences, the strain found in soybean and potato was further referred to as TYRV-s, and the originally sequenced TYRV isolate from tomato (and now also found in gazania and chrysanthemum) was denoted TYRV-t. Further comparative analysis of the S RNA sequences from TYRV and the soybean isolate revealed two clear differences in the IGR. Sequence

analysis of the corresponding IGR and the N genes from the potato isolate of TYRV-s revealed almost (99%) completely identical sequences to the soybean isolate, demonstrating that the difference in IGR sequence is a genetically stable difference between TYRV-t on one hand and TYRV-s on the other.

Differences between the IGRs of (closely) related tospoviruses have been reported earlier [16, 17, 25]. In one study [28] on genome reassortants from closely related viruses, a correlation was observed between sequences within the IGR and competitiveness of S RNA segments during mixed infections, and in another, the S RNA was suggested to be involved in symptom differences [23]. Whether differences in the IGR would contribute to the observed host range differences between TYRV-t and TYRV-s remains to be investigated.

Whereas the N gene of TYRV-t and TYRV-s diverge up to 8% at the amino acid level, and even more (9%) when TYRV-s is compared to the gazania isolate of TYRV-t, sequence comparison of the N proteins of 52 TSWV isolates and 47 PBNV isolates deposited in Genbank revealed an intraspecies diversity worldwide of only 4% for TSWV and 5% for PBNV (data not shown). A similarly large divergence as found for TYRV can be observed only for two other tospoviruses (Table 2, Fig. 4), i.e. for IYSV [3, 26] and for CaCV [21; the thai isolate sequence data are accessible from Genbank only). However, for these viruses, only N-gene-derived data are available.

Apparently, the diversity within TYRV, even though only isolates originating from a single country are considered, is significantly greater than the intraspecies diversity observed for TSWV and PBNV, and this molecular divergence coincides with differences in biological characters, specifically host range and symptomatology. It will be of great interest to search for further variants of this virus in Iran and its neighbouring countries, which may deliver further data that will challenge the current ICTV-authorized tospovirus species demarcation criteria. Until that time, the isolates described here can best be referred to as strain TYRV-t (in tomato, gazania and chrysanthemum) and strain TYRV-s (in soybean and potato).

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