Detection and Characterization of a Distinct Isolate of Tomato yellow fruit ring virus from Potato

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ABSTRACT

Golnaraghi, A. R., Pourrahim, R., Ahoonmanesh, A., Zamani-Zadeh, H. R., and Farzadfar, Sh. 2008. Detection and characterization of a distinct isolate of Tomato yellow fruit ring virus from potato. *Plant Dis.* 92:1280-1287.

A distinct isolate (TY-PF36) of Tomato yellow fruit ring virus (TYFRV) was obtained from potato (*Solanum tuberosum*) in Iran. Chlorosis and necrotic spots on leaves associated with leaf and stem necrosis symptoms appear on the affected plants. Of 32 plant species and cultivars mechanically inoculated, 24 were susceptible to the virus isolate. The isolate strongly reacted with TYFRV antibodies in enzyme-linked immunosorbent assay (ELISA), but not with the specific antibodies of other tospoviruses tested. TYFRV-specific N gene primers described previously, however, failed to produce DNA fragments from the total RNA extracts of the infected plants in reverse transcription–polymerase chain reaction (RT-PCR). Nucleotide sequencing of the complete N gene and partial L gene of this isolate revealed considerable differences to those reported for TYFRV with identities ranging from 83.9 to 84.2% and 84.9 to 85.4%, respectively. Two specific primers were designed for detecting TY-PF36 using RT-PCR; TY-PF36 was detected in symptomatic field samples of potato, peanut, soybean, and two weed species, *Datura metel* and *D. stramonium*.

Additional keywords: potato viruses

The genus *Tospovirus* within the family *Bunyaviridae* consists of several enveloped plant viruses that cause great losses in many economically important crops worldwide (37). For example, annual losses due to the disease caused by *Tomato spotted wilt virus* (TSWV) amount to about \$1 billion (34). Tospoviruses are characterized by quasi-spherical enveloped particles, 80 to 120 nm in diameter, and contain three single-stranded RNA segments denoted S, M, and L RNAs (7,9). Viruses from this genus are exclusively transmitted by a limited number of phytophagous thrips species in a circulative and propagative manner (27,38). To date, 14 tospovirus species have been identified on the basis of their natural mode of transmission, host range, serological rela-

tionship of the nucleocapsid (N) protein, and their N protein sequence (7,9). These viruses can induce a range of symptoms including necrosis, chlorosis, ring patterns, mottling, silvering, stunting, and local lesions, depending on virus species or strain, host plant, time of infection, and environmental conditions (10,28).

Potato (*Solanum tuberosum*) is an important field crop which is susceptible to infection by several viruses belonging to different virus genera and families (23,35). TSWV is the most important tospovirus species infecting potato in the world (1,2,8,20–22,24,29). Other tospoviruses that were found infecting this crop include *Groundnut ringspot virus* (GRSV) (17), *Impatiens necrotic spot virus* (INSV) (31), *Tomato chlorotic spot virus* (TCSV) (17,29), and a tospovirus serologically related to *Peanut bud necrosis virus* (PBNV) (19).

Several tospovirus species, including INSV, *Iris yellow spot virus* (IYSV), PBNV, and TSWV, have been reported in Iran (11,14,26). Tomato yellow fruit ring virus (TYFRV), a newly proposed species in the genus *Tospovirus*, was first reported to occur in tomato plants in the Varamin area of Tehran province (39), followed by

reports of its occurrence on ornamentals, soybeans, and weed species in certain provinces of Iran (11,13,16). Preliminary results showed that TYFRV isolates from soybean, originally mistaken for TSWV, may be categorized based on their host range (15). The thrips species *Microcephalothrips abdominalis* and *Thrips tabaci* were suggested as potential vectors for TYFRV (11), and the vectoring role of the latter was experimentally demonstrated (13).

In recent years, tospovirus-like symptoms of leaf and stem necrosis have been frequently observed in Iranian potato fields. TYFRV, originally believed to be and named TSWV, was first reported on potato in 2000 (33); the widespread occurrence of this virus in the main potato production areas of Iran was subsequently documented (32). In 2004, a field survey of tospovirus diseases in this crop was conducted in the Tehran province of Iran, and samples from plants with symptoms resembling such infections were collected. They were found to be infected with TYFRV when tested by enzyme-linked immunosorbent assay (ELISA). The presence of this virus was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) using TYFRV-N gene specific primers and sequencing of the amplified fragments (12). These primers, however, failed to produce DNA fragments of expected size from some symptomatic potato leaf samples. In host range studies, the virus showed some differences from other TYFRV isolates described previously (11–13). In the present paper, some characteristics of this virus are reported including the complete N gene sequence, partial sequence of L RNA, and the successful use of RT-PCR–based detection with a pair of specific primers. The prevalence of the virus in the main areas for potato production in Iran was also investigated.

MATERIALS AND METHODS

Virus isolates. TYFRV cultures used in current studies were isolated in the summer of 2004 from naturally infected potato plants in Tehran province showing necrosis on leaves and stems (Fig. 1). The virus was

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color in the online edition.

Accepted for publication 21 April 2008.

doi:10.1094/PDIS-92-9-1280
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transferred to *Nicotiana rustica* by mechanical inoculation using an extract of diseased leaves prepared in inoculation buffer (0.05 M sodium phosphate buffer containing 1% 2-mercaptoethanol, pH 7.0). A virus culture established in *N. rustica* plants, named in this work as TY-PF36 isolate, was subsequently purified by two serial single-lesion inoculation passages on cowpea (*Vigna unguiculata*) and maintained in *N. rustica*. Inoculum was stored at -70°C as fresh and freeze-dried infected *N. rustica* leaves. Some well-characterized isolates of TYFRV (TY-PF12 and TY-M23) were included in this study as positive controls. The isolate TY-PF12 was obtained from potato (12), and TY-M23 is an isolate of TYFRV detected in symptomatic soybean plants (13). All virus preparations used in the studies were prepared from infected *N. rustica*.

Host range. Thirty-two plant species and cultivars were mechanically inoculated. Reactions of some weed species present in Iranian potato fields were also evaluated in this study (Table 1). Inoculated plants were kept in a greenhouse for observation of symptom development. Inoculated plants, both symptomatic and asymptomatic, were tested by ELISA using the TYFRV-specific antibodies. In host range trials, at least two plants of each species or cultivar were inoculated, and the tests were repeated two times or more if the results were negative or inconclusive.

ELISA. The isolate TY-PF36 was analyzed by double-antibody sandwich ELISA (DAS-ELISA) (6) using the antisera against different tospoviruses. Specific antisera against TYFRV and Watermelon silver mottle virus (WSMoV) were provided by S. Winter (DSMZ, Braunschweig, Germany), whereas those for GRSV, INSV, IYSV, TCSV, and TSWV were purchased from Loewe (Sauerlach, Germany) or Bioreba (Reinach, Switzerland). Absorbance at 405 nm ($A_{405\text{ nm}}$) was recorded using a microplate reader (Tecan Spectra, Austria) 60 min after the addition of the substrate. Reactions were considered positive if the readings were equal to or exceeded three times the mean value of the negative controls.

RT-PCR. As a first step, the forward and reverse degenerate primer pairs gL3637 and gL4435c, published previously for amplification of the conserved region of the L RNA of tospoviruses (5), and the specific primers TYf and TYr for the amplification of TYFRV N gene (13) were used in RT-PCR. The test also was performed using TsMCR2 and 3'T12 (30) as well as UHP and J13 primers (7), designed to amplify regions of the N gene of different tospoviruses.

In the second step, the primers TYf and TYr were used in combination with the aforementioned tospovirus N gene primers. Reverse transcription was done using 3'T12, J13, and TYr in separate reactions. PCR amplifications were done using re-

verse/forward primer pairs 3'T12/TYf, J13/TYf, TYr/TYr, TYr/J13, TYr/UHP, TYr/3'T12, and TYr/TsMCR2. The PCR program was as follows: 94°C for 1 min, 30 cycles of 94°C for 30 s, 43°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min.

Total RNA was extracted from 0.1 g of *N. rustica* leaf tissue using the RNA extraction solution (RNX plus, CinnaGen Inc., Tehran, Iran), and first-strand cDNA synthesis was performed using RevertAid M-MuLV reverse transcriptase (Fermentas, Lithuania) according to the manufacturers instructions. PCR amplifications were carried out using *Taq* DNA polymerase (CinnaGen), and thermocycling was done in a Primus thermal cycler (MWG-Biotech Co., Ebersberg, Germany). The primers used were synthesized by MWG-Biotech Co.

Cloning, sequencing, and sequence analysis. The amplified DNA fragments of approximately 0.8 and 1.2 kb for TY-PF36 isolate, obtained using gL3637/gL4435c and TYr/UHP, respectively, were excised from the gel, cleaned by Nucleospin (Macherey-Nagel, Germany), and cloned into pGEM-T Easy vector (Promega Corp., Madison, WI, USA), as suggested by the suppliers. Sequences from both strands of the cloned DNA in three independent clones were determined by the custom sequencing service of MWG-Biotech Co. using standard M13 primer pairs. The nucleotide sequence

Fig. 1. Field symptoms associated with Tomato yellow fruit ring virus (TYFRV) infections on potato: **a**, necrotic spots on leaves; **b**, leaf and stem necrosis.

Table 1. Symptoms induced by the potato tospovirus isolate (TY-PF36) on indicator plants^a

Indicator plant	TY-PF36		TY-PF12		TY-M23	
	Local	Systemic	Local	Systemic	Local	Systemic
Amaranthaceae						
<i>Amaranthus</i> sp.	NL	–	NL	–	NL	–
<i>Gomphrena globosa</i>	NL	N	NL	–	NL	–
Asteraceae						
<i>Carthamus tinctorius</i>	–	–	–	–	–	–
<i>Helianthus annuus</i>	NL	–	NL	–	NL	–
Brassicaceae						
<i>Brassica rapa</i>	–	–	–	–	–	–
<i>Raphanus sativus</i>	–	–	–	–	–	–
<i>Rapistrum rugosum</i>	–	–	–	–	–	–
<i>Sisymbrium loeselii</i>	–	–	–	–	–	–
Chenopodiaceae						
<i>Chenopodium amaranticolor</i>	NL	–	NL	–	NL	–
<i>C. quinoa</i>	NL	–	NL	–	NL	–
Cucurbitaceae						
<i>Citrulus vulgaris</i>	–	–	–	–	–	–
<i>Cucumis sativus</i>	CL, NL	–	CL, NL	–	CL, NL	–
Fabaceae						
<i>Arachis hypogaea</i>	–	Cl, N	–	Cl, N	–	Cl, N
<i>Glycine max</i> cv. Hill	CL, NL	Mo, Cl, N	CL, NL	Cl, N	CL, NL	Mo, Cl, N
<i>Phaseolus aureus</i> cv. Gohar	NL	–	NL	–	NL	–
<i>P. vulgaris</i> cv. Talash	NL	–	NL	–	NL	–
<i>Pisum sativum</i>	–	–	–	–	–	–
<i>Vicia faba</i>	NL	–	NL	–	NL	–
<i>Vigna unguiculata</i> cv. Mashad	CL, NL	–	CL, NL	–	CL, NL	–
Solanaceae						
<i>Capsicum annuum</i>	–	M	–	M	–	M
<i>Datura metel</i>	NS	M, LD, SN	NS	NS, LD, Su	NS	NS, LD, Su
<i>D. stramonium</i>	NS	Mo, LD, N	NS	NS, LD, TN	NS	NS, LD, N
<i>Nicotiana benthamiana</i>	CS	M, LD, Cl, N	CS	M, LD, Cl, N	CS	M, LD, Cl, N
<i>N. glutinosa</i>	NS	TN	NS	TN	NS	TN
<i>N. rustica</i>	CL, NL	CS, LD, Su, M	CL, NL	CS, LD, StN, Su, M	CL, NL	CS, LD, Su, M
<i>N. tabacum</i> cv. Samsun	CL, NL	N	NL	–	NL	–
<i>N. tabacum</i> cv. White Burley	–	Cl, N	–	–	–	–
<i>Petunia hybrida</i>	NL	–	NL	–	NL	–
<i>Solanum lycopersicum</i>	NL	–	NS	LD, Su	NS	LD, Su
<i>Solanum melongena</i>	–	–	–	–	–	–
<i>Solanum nigrum</i>	NL	–	NL	–	NL	–
<i>Solanum tuberosum</i> cv. Agria	NL	Cl, SN	NL	Cl, N	NL	Cl, N

^a Isolates TY-PF12 and TY-M23 were used as positive control. Cl, chlorosis; CL, chlorotic lesions; CS, chlorotic spots; LD, leaf deformation; M, mosaic; Mo, mottling; N, necrosis; NL, necrotic lesions; NS, necrotic spots; SN, severe necrosis; Su, stunting; TN, top necrosis; –, no infection.

obtained was compared with other sequences available in the GenBank using Basic Local Alignment Search Tool (BLAST) (3) at the National Center for Biotechnology Information, Bethesda, MD, USA. Multiple sequence alignments were generated by the CLUSTAL W program (36). Phylogenetic trees were constructed using MegAlign ver. 5.00 from the DNASTAR package (DNASTAR, Madison, WI, USA) (4) based on N gene and deduced amino acid sequences. In this study, IYSV (AF001367) was used as outgroup.

Designing of specific primers. For specific detection of TY-PF36, a pair of primers was designed on the basis of the sequence obtained for this isolate. Upstream and downstream primers used in RT-PCR amplification were, respectively, TYNf (5'-AAACATCAGCAAAATCAAATCCC-3') and TYNr (5'-CAACTTCAGAAAAAGTAAACC-3'). These primers were designed to amplify a DNA fragment of approximately 0.67 kb, corresponding to TY-PF36 N gene. Thermocycling was done as

follows: 94°C for 1 min, 30 cycles of 94°C for 30 s, 43°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. A total of 38 TYFRV-ELISA positive field samples from potato (*Solanum tuberosum* cv. Ajax), peanut (*Arachis hypogaea*), soybean (*Glycine max* cv. Williams), *Datura metel*, and *D. stramonium* plants showing tospovirus-like symptoms, which failed to detect using TYf and TYr primers, were also tested by RT-PCR with the primer pair TYNf and TYNr.

Surveys. During the summer of 2005, surveys were conducted in Esfahan, Hamedan, and Tehran provinces, the main areas for potato production in Iran, and a total of 451 potato leaf samples showing tospovirus-like symptoms (137, 186, and 128 samples, respectively) were collected from 13 potato fields (4, 6, and 3 fields, respectively) (Table 2). These samples were tested by ELISA using TYFRV-specific antibodies. ELISA-positive samples were subsequently tested by RT-PCR using primer pairs TYr/TYf and TYNr/TYNf in separate reactions.

RESULTS

Symptoms and host range. Of a total of 32 plant species and cultivars mechanically inoculated, 24 were susceptible to the virus isolate (Table 1). Systemic invasion of TY-PF36 was observed in *Arachis hypogaea*, *Capsicum annuum*, *Datura metel*, *D. stramonium*, *Glycine max* cv. Hill, *Gomphrena globosa*, *Nicotiana benthamiana*, *N. glutinosa*, *N. rustica*, *N. tabacum* cv. Samsun, and *N. tabacum* cv. White Burley. Local lesions were found on inoculated leaves of *Amaranthus* sp., *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Helianthus annuus*, *Petunia hybrida*, *Phaseolus aureus* cv. Gohar, *P. vulgaris* cv. Talash, *Solanum lycopersicum*, *Vicia faba*, *Vigna unguiculata* cv. Mashad, and *S. nigrum*. When healthy potato plants (*S. tuberosum* cv. Agria) were artificially inoculated with this isolate, necrotic lesions followed by chlorotic and severe necrotic symptoms developed, resembling those observed in the field (Fig. 1). In contrast to TY-PF12 and TY-M23, the isolate TY-PF36 induced systemic

Table 2. Results of enzyme-linked immunosorbent assay (ELISA) and reverse transcription–polymerase chain reaction (RT-PCR) run on symptomatic potato leaf samples taken from 13 fields in Esfahan, Hamedan, and Tehran provinces of Iran

Province	No. of fields surveyed	No of samples collected	TYFRV infection using:		
			TYFRV-Ab (ELISA)	TYr/TYf (RT-PCR)	TYNr/TYNf (RT-PCR)
Esfahan	4	137	15 (10.9) ^a 5.4–17.4 ^b (4) ^c	5 (3.6) 0.0–8.8 (3)	10 (7.3) 0.0–13.0 (3)
Hamedan	6	186	34 (18.3) 8.0–27.0 (6)	10 (5.4) 0.0–9.1 (5)	23 (12.4) 2.8–20.0 (6)
Tehran	3	128	29 (22.7) 14.9–28.6 (3)	8 (6.3) 2.1–9.5 (3)	19 (14.8) 10.6–19.0 (3)
Average	13	451	78 (17.3) 5.4–28.6 (13)	23 (5.1) 0.0–9.5 (11)	52 (11.5) 0.0–20.0 (12)

^a Number of infected samples followed by percent infection in parentheses.

^b Range of percent infection.

^c Number of infected fields.

Fig. 2. Reverse transcription–polymerase chain reaction using gL3637/gL4435c primers to amplify a DNA fragment of approximately 0.8 kb. Lane 1, 1-kb DNA ladder (Fermentas, Lithuania); lane 2, TY-PF12; lane 3, TY-M23; lane 4, TY-PF36; lane 5, healthy plant.

Fig. 3. Reverse transcription–polymerase chain reaction using the Tomato yellow fruit ring virus (TYFRV)-specific primers TYf/TYr to amplify a DNA fragment of approximately 1.2 kb. Lane 1, 1-kb DNA ladder (Fermentas, Lithuania); lane 2, TY-PF12; lane 3, TY-M23; lane 4, TY-PF36 (this study); lane 5, healthy plant.

Fig. 4. Reverse transcription–polymerase chain reaction of total RNA extracted from TY-PF36 infected plants using TYr as reverse primer in combination with 3'T12, J13, TsMCR2, TYf, and UHP as forward primers. Lane 1, 1-kb DNA ladder (Fermentas, Lithuania); lane 2, TYr/TYf; lane 4, TYr/J13; lane 6, TYr/UHP; lane 8, TYr/3'T12; lane 10, TYr/TsMCR2; lanes 3, 5, 7, 9, and 11, similar reactions for healthy plant.

necrosis on *G. globosa* and severe necrosis on *D. metel*, produced local and systemic symptoms on *N. tabacum* cvs. Samsun and White Burley, and locally infected *S. ly-*

copersicum. TY-PF36 also caused more severe symptoms (severe necrosis) on potato plants (Table 1). Infections were confirmed by positive reaction to TYFRV-

Table 3. Reaction ($A_{405\text{ nm}}$) of the potato *Tospovirus* isolate (TY-PF36) with different tospovirus antibodies in double-antibody sandwich enzyme-linked immunosorbent assay formats^a

Antibodies	Negative control	Positive control	TY-PF36
GRSV-L	0.014	0.690	0.013
INSV-L	0.014	0.801	0.017
IYSV-L	0.015	1.015	0.014
TCSV-L	0.018	0.710	0.017
TSWV-L	0.017	0.988	0.019
TSWV-B	0.021	0.973	0.020
TYFRV-D	0.019	1.112	0.890
WSMoV-D	0.025	0.504	0.052

^a The average of $A_{405\text{ nm}}$ values 60 min after adding substrate is indicated. The highest absorbance value recorded for this isolate is shown in bold.

specific antibodies in ELISA. Similar to TY-PF12 and TY-M23 isolates, no local or systemic infections were produced on *Brassica rapa*, *Carthamus tinctorius*, *Citrus vulgaris*, *Pisum sativum*, *Raphanus sativus*, *Rapistrum rugosum*, *Solanum melongena*, and *Sysimbrium loeselii* when inoculated with TY-PF36, as determined by symptom expression, ELISA, and bioassay on *V. unguiculata*.

Serology. Positive reactions were recorded in DAS-ELISA of extracts prepared from TY-PF36-infected *N. rustica* leaves using TYFRV-specific antibodies. TY-PF36 extracted from infected *N. rustica* did not react with specific antibodies prepared to GRSV, INSV, IYSV, TCSV, TSWV, and WSMoV (Table 3).

RT-PCR using gL3637 and gL4435c primers. A DNA fragment of ca. 0.8 kb was amplified by RT-PCR from total RNA extracted from a TY-PF36 infected plant using gL3637/gL4435c primer pair designed on the basis of the conserved regions of the L gene of tospoviruses. Similar fragments were also produced for the isolates TY-M23 and TY-PF12. No products were obtained with RNAs from healthy leaf tissue (Fig. 2). The amplified DNA of TY-PF36 was cloned and sequenced (GenBank accession no. EU126932) and compared with those in the GenBank using BLAST. The results confirmed that this sequence corresponded to a region of the TYFRV L RNA. Computer analyses of this sequence revealed 84.9, 85.1, and 85.4% nucleotide identity with TYFRV isolates from soybean, TY-M23 (EU130947); tomato (AJ493271), named in this paper TY-Tom isolate; and potato, TY-PF12 (EU130948), respectively. The deduced amino sequence obtained for TY-PF36 RdRp gene shared 98.8% identity with the other sequences studied.

RT-PCR cloning of the N gene. No DNA products were obtained from TY-PF36 infected or healthy plants in RT-PCR using the primers TYf and TYr specific for TYFRV N gene (13). However, DNA fragments with the expected size were

A

TY-Tom	:	-----*	20	*	40	*	60	TYf	*	80	*	100	:	49
TY-M23	:	-----						TTTTGTTTTTGTGTTTTGGTTTTTGTTTT-TTGA					:	11
TY-PF12	:	-----											:	11
TY-PF36	:	TATTTTTTATTTTGTGTTTTGTGTTTTCTTTATATGTATATTTATTTAACTATTAAACACTCTTATGTATAATTGAACACACATTTAAG.AA.A.AAAC											:	106
												tTttTtTggtt		
TY-Tom	:	..T.	120	*	140	*	160	*	180	*	200	*	:	155
TY-M23	:	..T.			A..C..G.				C..CT.				G..	116
TY-PF12	:	..T.			T.				G..G.				G..	116
TY-PF36	:	A..ACAG.			A..T.G.A..C..A..G..C.A..G.A..C..C..TAC.								A..GG.	212
		tT			catataACTTATTtAAACACcCtTgAgTAtAtTT				AACACA				AaTtAAaTgT	
					AtA				AA				aaaACTtAAACACACTGTATAAACTATAT	
					GCACACA				AAAA					
TY-Tom	:		220	*	240	*	260	*	280	*	300	*	3	259
TY-M23	:													220
TY-PF12	:													220
TY-PF36	:	T.GG.....C.....A..A.T.....T.....A.....T..C.C.....A.ACG.....A												318
		CTGAAATaAaAtATT			TAAAtCATATATAgTAATaAa				ACACACAATGACTAGAgtTACTTTAGTAATCTTAAATAcTAtAtAATACTgAgT				ATAGCAGAAAATg	
TY-Tom	:	...TT..T.	340	*	360	*	380	*	400	*	420	*	:	364
TY-M23	:	T.....					A..T.....						:	325
TY-PF12	:	T.....											:	325
TY-PF36	:	...T...CTT.C...T.T...G...G.TA..C...T..A...G...G...C..T...G.T											:	424
		TTT			a		AA		caGtAAAAGCAcTcATTAAAAATGCATCaTCTTTTTGGAAgAtGGtTTgGG				TTtGAAGACCCAGCACCAtTgTCATCAACAATtTtCCAAAAGc	
TY-Tom	:		440	*	460	*	480	*	500	*	520	*	:	470
TY-M23	:												:	431
TY-PF12	:												:	431
TY-PF36	:	G.....C.....T..G.....CT.....G.....G.....G.....G.....T											:	530
		aCTTTCAAGAGCTTTAATtTGcTCATTGAAcTtAtTCAATGAAGCAGCACCAGCAGT							CCTGGAGTAcAGTCATT				AGaATTTTTATGGTTTCTTCAAAAATGTC	
TY-Tom	:		540	TYNf	*	560	*	580	*	600	*	620	*	576
TY-M23	:					T.....A.....						T.....C.....C.	:	537
TY-PF12	:												:	537
TY-PF36	:	AAACATCAGCAAAATCAAATCCC.....A.....T..T..T.....C.....A.....G..T...A.....C.....											:	636
		TTGGA			ACccCAGtgAAAgTgAATtCtTTAGTtGCCATaAc				CgGGCgATCTTGACAgCTGtTCATAAGTTGAAATTTgCTTAT				CccAAAGcTTctTtTtTa	
TY-Tom	:		640	*	660	*	680	*	700	*	720	*	740	682
TY-M23	:												:	643
TY-PF12	:												:	643
TY-PF36	:	A.....G.....CA.....T.....GAA.....A..A..C.A..C.....T.....G.....G.....											:	742
		CATTCTGgAAATAGCCaATGGAATGCAgtTGACAGCAAAcTTATCAAGACTTGCCATCAaggGGAGAGGgCctCctAgTGTtA							CATgACTCTaGCTGTaGTTGC					
TY-Tom	:		760	*	780	*	800	*	820	*	840	*	:	788
TY-M23	:												:	749
TY-PF12	:												:	749
TY-PF36	:	C..T.A..C.....C.....C..C.....G.....C.....C.....AGCCA...T.....C											:	848
		ATCAAAATtTaGgGCTaGGTTTtAAACCATATGCGtTtACCATtGGAAGcTcACAAAGtTtTCATACATTTTCTGtTGCTCgcttgCATTtCCACAGCAATCAGt												
TY-Tom	:		860	*	880	*	900	*	920	*	940	*	:	894
TY-M23	:												:	855
TY-PF12	:												:	855
TY-PF36	:	C..T.....G.....C.....C.....A.....C.....C.....T.....C.....T.....											:	954
		TCCATGAACATtTTgGCTCTtATgAGCctTCCAACCTTCTGAATGTCCAATCATCTTGACCAGC							CTCACAGTtGCACT				GGaACAATaATAGaTtGcCTTGGA	
TY-Tom	:		960	*	980	*	1000	*	1020	*	1040	*	1060	1000
TY-M23	:												:	961
TY-PF12	:												:	961
TY-PF36	:	T..A.....T.....C.....C.....A.....T.....C.....T.....G.....											:	1060
		ATTTAAAcTgGcCTGCTTTtACCATTtTGTAAATaCCTGCTCTGTCTTAAATTTGTGTAAACGTTgTTGAACGTcATTtTTCATGGcTTTGTAGCCAAACAAA												
TY-Tom	:		1080	*	1100	*	1120	*	1140	*	1160	*	:	1106
TY-M23	:												:	1067
TY-PF12	:												:	1067
TY-PF36	:	C.....C.....C.....C.....G.....G.....G.....T.....T.....T.....C.....											:	1166
		CTCtTTGAAGTTGAATCCTGCAGtTTctTCAGctTcAATCACTACATCTGCTTcCACCACgCAAGAAGCTTCTCAATgTTCTCTTTGcTcActCgTGCGGTAGAC												
TY-Tom	:		1180	*	TYNr	1200	*	1220	TYr	*	1240	*	:	1179
TY-M23	:								AATGTGTTATACCTCGATTGCTCT				:	1116
TY-PF12	:												:	1116
TY-PF36	:								GGTTTACTTTTTCTGAAGTTG.				:	1216
		ATGGTGTTTACTTAGGAAGCaGcTTACTTTgTg							GAAGTGGTTTGAATTT					

(continued on next page)

Fig. 5. Multiple sequence alignment of **A**, N gene and **B**, N protein sequences from TY-PF36 and other isolates of Tomato yellow fruit ring virus (TYFRV). Sources of the sequences referred are as follows: TY-Tom (AJ493270), TY-M23 (EF157855), TY-PF12 (DQ419916), and TY-PF36 (EU126931) studied in this work.

present in RT-PCR of the RNAs extracted from TY-M23 and TY-PF12 infected plants (Fig. 3). The primer pairs TsMCR2/3'T12 (30) and UHP/J13 (7) also failed to amplify DNA fragments corresponding to the TY-PF36 N gene. In the second step, vari-

ous primer/primer combinations were used. DNA fragments of approximately 1.0 to 1.2 kb were amplified in RT-PCR using the following reverse/forward primer pairs: TYr/TYr, TYr/J13, TYr/UHP, TYr/3'T12, and TYr/TsMCR2, which were not pro-

duced in the control healthy total RNA extract. In similar reactions of healthy controls, smaller fragments (*ca.* 0.3 to 0.9 bp) also were seen using the above primer pairs (Fig. 4, arrowheads). The amplified DNA product obtained using TYr and

Fig. 5. (continued from previous page)

Fig. 6. Phylogenetic trees constructed by CLUSTAL W program showing the genetic relationship among **A**, N gene and **B**, N protein sequences of Tomato yellow fruit ring virus (TYFRV) isolates. Sources of the sequences referred are as follows: TY-Tom (AJ493270), TY-M23 (EF157855), TY-PF12 (DQ419916), TY-PF36 (EU126931) studied in this work, and *Iris yellow spot virus* (IYSV, AF001367).

UHP primers (Fig. 4, lane 6: approximately 1.2 kb) was cloned and its sequence was determined (GenBank accession no. EU126931). BLAST search results showed that the sequence shared the highest identity with TYFRV, and confirmed that this sequence contained the complete TYFRV N gene. Similar results were also obtained in RT-PCR cloning using the remaining primer pairs (data not shown). The number of nucleotides (825 nt) and amino acids (274 aa) obtained for N gene of TY-PF36 are exactly the same as the corresponding region in the other TYFRV isolates (Fig. 5). Multiple nucleotide sequence alignment using the CLUSTAL W program showed that the TY-PF36 N gene sequence shared 83.9, 83.9, and 84.2%

nucleotide identity with TY-PF12 (DQ419916), TY-M23 (EF157855), and TY-Tom (AJ493270), respectively (Fig. 5A). The deduced N protein amino acid sequence of TY-PF36 shared 92.3% identity with TY-PF12, 92.3% with TY-M23, and 92.0% with TY-Tom. Long stretches of conserved amino acid sequence were found among the TYFRV isolates studied. The deduced amino acid sequence of the TY-PF36 N gene had 21 amino acid substitutions, including A_{29,115,209,230}, C₁₆₈, D_{207,210}, G₂₀₅, K_{10,75}, L₁₅₇, N₇, S_{131,139,182}, T_{43,249,257,267}, and V_{78,114}, relative to the other three isolates (Fig. 5B).

The phylogenetic tree related to N gene and the deduced amino acid sequence revealed a clustering of TY-PF12, TY-M23,

and TY-Tom isolates in one branch. Interestingly, the TY-PF36 isolate studied in this work was located in a separate branch (Fig. 6).

RT-PCR using specific primers. The forward and reverse primer pair TYNf and TYNr were designed and tested in RT-PCR for specific detection of TY-PF36. A band with the expected size of ca. 0.67 kb was obtained for this isolate. These primers failed to produce visible products using total RNA extracted from healthy as well as TY-M23 and TY-PF12 infected plants (Fig. 7). The results of RT-PCR analysis also showed the presence of the virus in all 38 symptomatic field samples of *Arachis hypogaea*, *Datura metel*, *D. stramonium*, *Glycine max*

cv. Williams, and *Solanum tuberosum* cv. Ajax.

Surveys. ELISAs indicated that 78 symptomatic samples (17.3%) collected from 13 separated fields in all provinces surveyed reacted positively to TYFRV antibodies. The highest incidence of TYFRV infection was recorded in Tehran (22.7%), followed by Hamedan (18.3%) and Esfahan (10.9%). However, the virus was detected by RT-PCR in 5.1 and 11.5% of the samples using TYr/TYf and TYNr/TYNf primer pairs, respectively (Table 2). Moreover, the expected DNA fragments for each of the primer pairs used were not obtained for one and two potato leaf samples from Hamedan and Tehran provinces, respectively.

DISCUSSION

In the present work, some characteristics of a distinct isolate of TYFRV, causing severe leaf and stem necrosis on potatoes and sometimes the death of the whole plant, were studied. The isolate TY-PF36 was detected with the TYFRV-specific antibodies in DAS-ELISA. A panel of tospovirus-specific antibodies did not react with this isolate, indicating that it was, indeed, an isolate of TYFRV (40).

The experimental host range of this virus isolate showed some similarities; however, differential host response was observed for TY-PF36 compared to previously reported isolates of TYFRV (11–13) (Table 1). These differences, especially on *N. tabacum* cvs. Samsun and White Burley as well as *S. lycopersicum*, were consistent and stable through more than 20 generations by mechanical inoculation, suggesting that TY-PF36 is a distinct strain of TYFRV. Similar observations have been previously reported for other tospoviruses (18,25). Such biologically distinct isolates may facilitate studying on the genetic determinants for host range and disease severity of TYFRV in the future.

Fig. 7. Reverse transcription–polymerase chain reaction using the TY-PF36 specific primers TYNf/TYNr to amplify a DNA fragment of approximately 0.67 kb. Lane 1, 1-kb DNA ladder (Fermentas, Lithuania); lane 2, TY-PF36 (this study); lane 3, TY-M23; lane 4, TY-PF12; lane 5, healthy plant.

This isolate was further analyzed for its N gene, which encodes N protein, an important taxonomic descriptor for tospoviruses (7,9). No DNA products were obtained in RT-PCR reactions where the primer TYf was used as the forward primer. This was mainly due to considerable mismatches (23 nt) that occurred between the forward primer TYf (38 nt in length) and the corresponding region on TY-PF36 (Fig. 5A). TY-PF36 N gene was successfully amplified using primer pair TYr/UHP and verified by sequencing. Based on the results, the N protein sequence of the potato isolate (TY-PF36) showed 92.0 to 92.3% amino acid identity to other isolates of TYFRV, marginally within the range allowed by ICTV for species determination in the genus *Tospovirus* (9). The phylogenetic analysis showed a clustering of TY-PF36 in one branch separate from TY-PF12, another TYFRV isolate from potato. Also, TY-M23, TY-PF12, and TY-Tom (obtained from soybean, potato, and tomato, respectively) were all clustered in another branch. This seems to indicate that TYFRV isolates probably are not clustered based on differences in the natural host source. Further studies should be done to confirm this hypothesis.

The data indicated that the isolate studied differs significantly in biological characters, specifically host range and symptomatology, from the other characterized isolates of TYFRV (11–13). Moreover, results obtained in this study revealed sequence variations among RdRp and N genes of TYFRV isolates. Interestingly, some TYFRV ELISA-positive samples were negative in RT-PCR when TYr/TYf and TYNf/TYNr primer pairs were used. It would be interesting to further investigate the biological, serological, and molecular characteristics of different isolates of TYFRV from various parts of Iran and from its neighboring countries. The molecular and biological variation found among TYFRV isolates seems to indicate the necessity of revision of criteria used for *Tospovirus* species demarcation.

The identification of a distinct isolate of TYFRV on potato adds new concern to the production of healthy potato plants since the virus is transmitted through potato tubers (12) and by thrips vectors (13). Similar isolates to TY-PF36 were frequently found in Iranian potato fields (Table 2), which seem to be more virulent on potato plants than the isolates studied (12). This indicates the potential importance of such isolates to cause higher yield loss on this crop. It is worth noting that the primers designed for specific detection of TY-PF36 (TYNf and TYNr) in RT-PCR detected similar isolates in other crops such as peanut and soybean, as well as in some weed species of *Datura metel* and *D. stramonium*. This could have epidemiological significance for TYFRV infection of potato crops.

Factors influencing the composition and structure of TYFRV isolates and their role in epidemics in potato crops should be studied for optimizing quality control checks in the seed potato production process.

ACKNOWLEDGMENTS

This work was supported by the Plant Virology Department of PPDRI (Tehran) and Science and Research Branch of Islamic Azad University (Tehran). The authors wish to thank S. Winter (DSMZ, Germany) for providing some of the antisera and positive controls used in this study. We are also grateful to K. Ohshima (Laboratory of Plant Virology, Faculty of Agriculture, Saga University, Japan) and Alice-Kazuko Inoue-Nagata (Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Hortaliças, Brasília, Brazil) for their kindly advice. This research was part of the Ph.D. thesis of the first author and was carried out under a project (no. 28648) of Iranian Nanotechnology Initiative entitled “Use of microarray technique for detection of important viruses of potato”.

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