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Characterization of potato virus Y isolates from tomato crops in northeast Spain

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Abstract

A collection of 39 potato virus Y (PVY) isolates from tomato, originating from several commercial crops grown in north-east Spain, was characterised by biological, serological and molecular assays. In general, no correspondence among the three different assays was observed. The results of biological characterization by inoculation of PVY isolates to *Nicotiana* spp. and pepper plants did not coincide with the differentiation provided by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies specific to PVY^{C}, $PVY^{O/C}$ or PVY^{N} strains. In some cases this was a consequence of mixed infection of different PVY strains, which selectively infected the different hosts as demonstrated by the ELISA test. Nevertheless, the majority of PVY isolates collected from the same tomato fields showed a high degree of similarity, which indicated the predominance of one-source of infection for each tomato field. Comparison of isolates based on genetic studies did not correspond well with pathological properties, because amplification by reverse transcriptase polymerase chain reaction (RT-PCR) was also selective for some PVY isolates. The sequence analysis of some PVY isolates revealed three main clusters corresponding to NTN and N potato-type strain and NP (non potato-type) strains. Tomato seems to be slightly selective with respect to PVY infection and it is possible to find a great genetic diversity of PVY isolates belonging to different groups, mixtures and probably recombinants, which can complicate the adoption of cultural measures and the development of resistance programmes to control the disease.

Introduction

Potato virus Y (PVY) is the type member of the genus *Potyvirus* in the family *Potyviridae*. PVY is one of the most important viruses affecting vegetables worldwide (Shukla et al., 1994). It is naturally transmitted by many aphid species in a non-persistent manner, causing epidemics of many solanaceous crops such as potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) (De Bokx and Huttinga, 1981).

Classification of PVY isolates has been based on different criteria. Biological classification of PVY strains is very complex since different groups have been classified depending on the host from which they were isolated (De Bokx and Huttinga, 1981). It has been suggested that tobacco and tomato can be infected by most, if not all, PVY strains, whereas strong strain-host specificity has been observed for strains isolated from potato and pepper (Morel et al., 2000). Historically, three PVY groups have been commonly described on potato based on their biological properties, serology and resistance response. The most common PVY strain (PVY^{O}), the stipple-streak strain (PVY^{C}) and the veinal necrotic strain (PVY^{N}) have been typified on the basis of symptoms

induced on selected potato lines bearing different resistance genes and tobacco cultivars (Jones, 1990). Monoclonal antibodies (MAbs) against PVY potato isolates have been produced for use in the seed potato industry and their efficiency has been tested for the differentiation of the main strain groups (Gugerli and Fries, 1983). Recombination is prevalent in the genus *Potyvirus* and especially in PVY (Revers et al., 1996; Worobey and Holmes, 1999). More recently, other variants originating from single or multiple recombinants between PVY^{O} and PVY^{N} isolates have been proposed (Glais et al., 2002), such as PVY^{NTN}. These are the tuber necrotic isolates associated with a potato disease, which caused a severe reduction in tuber quality in Eastern Europe (Beczner et al., 1984). PVY^{N}-W discovered in Poland (Chrzanowska, 1991) and PVY^{Z} found in Great Britain, overcome the hypersensitive response to PVY^{C} and PVY^{O} conferred by resistance genes (Jones, 1990). PVY tobacco isolates have been classified on the basis of symptoms developed in various *N. tabacum* cultivars susceptible or resistant to root-knot nematode (Gooding, 1985). In capsicum, PVY isolates have been classified as PVY(0) pathotype, which does not overcome $pvr2^{1}$ and $pvr2^{2}$ resistance genes; PVY(1), which overcome $pvr2^{1}$ but not $pvr2^{2}$ and PVY(1–2), which overcomes both resistance genes (Gebré-Selassié et al., 1985). Luis-Arteaga and co-workers (1997) reported the presence of a fourth patotype PVY-PRW with the ability to infect the pepper cultivar Puerto Riko Wonder. Studies of serological variability in pepper strains using monoclonal antibodies demonstrated that most PVY pepper isolates were more closely related to the PVY^{O} strain group, but no correspondence was found between pathotypes and serotypes (Soto et al., 1994).

The taxonomy of PVY isolates has also been aided by results of molecular studies. Analysis of the coat protein (CP) sequence seems to be a useful criterion for clarifying the taxonomy of the potyvirus group (Shukla et al., 1994), but no discrimination between PVY pathotypes from pepper was possible on the basis of their respective CP amino acid sequences (Llave et al., 1999). Phylogenetic tree classification based on sequence analysis of either a 5′ non-translated region or the adjacent P1 coding region gave the same clustering into three groups using a group of PVY isolates from potato, tobacco, tomato and pepper (Tordo et al., 1995). As the study of genetic variability in PVY isolates by nucleotide sequence requires considerable effort, alternative methods such as reverse transcriptase and polymerase chain reaction followed by restriction fragment length polymorphism assay (RT-PCR-RFLPs) have been implemented (Blanco-Urgoiti et al., 1996). Applying this method, these authors distributed the PVY isolates independently of their original host into three main clusters: potato PVY^{O}, potato PVY^{N} and non potato-type PVY^{NP}.

PVY is widespread in tomato crops and has been the cause of serious yield losses in different countries (Thomas and McGrath, 1988; Abad and Jorda, 2000; Rosner et al., 2000). Tomato infected by PVY^{O} and PVY^{C} strains initially causes symptoms of crinkling on young leaves and then necrotic mottling, sometimes also veinal necrosis on the backs of leaves, but no symptoms on the fruits themselves; while the PVY^{N} strains cause severe mosaic, often with interveinal yellow spots and whitish spots on fruits (Shukla et al., 1994). Other researchers have proposed seven PVY pathotypes based on their ability to infect different tomato genotypes carrying resistance characters (Legnani et al., 1997). Although some PVY isolates from tomato have already been differentiated and characterised (Abad and Jorda, 2000; Rosner et al., 2000; Morel et al., 2000), there remains a need for a good classification of the considerable variety of PVY tomato isolates. Such a classification would be useful in order to analyse sources of resistance genes, which could open the way for genetic control of the virus**.** This paper presents the results of biological, serological and molecular analysis of a collection of tomato PVY isolates and discusses their classification in pathotypes, serological groups and CP sequence homology in relation to PVY isolates from other hosts.

Materials and methods

Virus isolates, culture and inoculation

Leaf samples showing symptoms apparently caused by PVY infection and other symptomless leaf samples were collected from individual tomato plants. Samples were collected from 25 open commercial fields located in the main tomato

producing area of Catalonia (northeast Spain) during the period 2001–2004. Field samples were identified by symptoms, year and place of collection and stored at −80 °C until use. Pepper PVY(0) and PVY(1–2) isolates were kindly provided by the Dr. M. Luis (SIA, Zaragoza); PVY(1) pepper isolate was provided by Dr. S. Castro (INIA, Madrid); and potato PVY^{O} and PVY^{N} isolates were provided by Dr. J. Legorburu (NEIKER, Vitoria). These isolates were kept replicating in *N. tabacum* cv. Xanthi under greenhouse conditions and were used as reference. Each field PVY isolate was mechanically inoculated using leaf extracts from stored infected material, previously prepared in a mortar containing 0.05 M phosphate buffer, pH 7.2, mixed with 0.2% 2-mercaptoethanol, 1% polyvinyl pyrrolidone with a molecular weight of 40,000 and activated charcoal (1 w : 20 v). Mechanical inoculation of PVY isolates was carried out by rubbing the inoculum on plant seedlings at the four-leaf stage previously dusted with carborundum. These plants were kept for at least 30 days under controlled conditions in an insect-proof glasshouse at 20–27 °C (night–day) temperature.

Serological test

Leaf extracts from each fresh original sample were tested by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) using polyclonal antisera (PA) for cucumber mosaic virus (CMV), pepino mosaic virus (PepMV), tomato mosaic virus (ToMV) and tomato spotted wilt virus (TSWV) (Loewe, Otterfing, German) according to manufacturer's instructions. The same extracts were also analysed by indirect DAS ELISA using PA for PVY (Plantprint Lts., Valencia, Spain) following manufacturer's instructions. They were also tested by indirect ELISA using PA for parietaria mottle virus (PMoV), kindly provided by P. Roggero, and following the method described by Galipienso et al. (2005). Serological characterization was achieved by DAS-ELISA using MAbs for PVY^{N}, $PVY^{O/C}$ and PVY^{C} (Adgen Lts., Scotland, UK) according to manufacturer's instructions. Samples positive to CMV, PepMV, ToMV, TSWV or PMoV PA were eliminated before storage in order to avoid mixing other infections with PVY. Absorbance of ELISA plates at 405 nm was measured at 60 min using a Titertek Multiskan (Flow) ELISA reader (Labsystems, Helsinki, Finland). The negative control involved healthy plant extract and the blanks were adjusted using the substrate solution. Samples providing optical density values greater than three times the negative controls were considered positives. They were differentiated into weak positives (+) with optical density values only three to five times greater than the negative controls and strong positives (++) with optical density values greater than five times the negative controls. The results were obtained by testing the stored tomato samples at least twice and were confirmed by testing the tomato plants that had been mechanically infected and kept under greenhouse conditions. The serological test of PVY isolates used as control was carried out by infecting *N. tabacum* cv. Xanthi plants, because some of them provided weak positive values when inoculated on tomato plants. The host plants used for biological characterisation were also analysed by ELISA with the PA and MAbs three to four weeks after mechanical inoculation of the plants in order to confirm PVY infection.

Biological test

Leaf extracts from stored samples were used for PVY mechanical transmission inoculating at least three plant seedlings at the four-leaf stage. Bioassay plants included *N. glutinosa, N. tabacum* cv. Xanthi, *L. esculentum* cv. Marmande (Fitó S.A., Barcelona, Spain) and cv. Lisboa (Seminis, Almeria, Spain) carrying *Sw-5* and $Tm2^{2}$ genes, which provided resistance to TSWV and ToMV respectively and *C. annum* cv. Delfos, R-9005 and R-9016 (Fitó S.A.) and cv. Oman and Lido (Seminis). The pepper hybrids R-9005 and Oman carried the $pvr2^{2}$ gene that provided resistance to PVY (1) isolate.

Nucleic acid extraction and primer design

Field samples and the host plants used for biological characterization were used for nucleic acid extraction; 50–100 mg of fresh infected tissue were collected and nucleic acid extracts (NAE) were obtained using $TRIzol^{R}$ reagent (Invitrogen, Grand Island, USA) according to the manufacturer's instructions.

Two primer pairs were used for DNA amplification. The primer pairs PVY 1 forward and PVY 1 revers (1F/1R) were designed from a CP region (879 nt) by Blanco-Urgoiti and co-workers (1996). One more degenerate primer pair PVY 2F (5′-ACG TCM AAA ATG AGA ATG CC-3′) and PVY 2R (CAT TTG WAT GTG CGC TTC C-3′) yielding a PCR product of 510 nt was designed from conserved sequences of cDNAs corresponding to the CP region of PVY isolates from N, NTN, O and C strains using OLIGO 4.0 software. The sequences were obtained by the amplification with 1F/1R primers from our own PVY isolates (F14K and C10C) that showed a high nucleotide identity with PVY SON41 isolate and NNP strain; GenBank accession numbers were AJ439544 and AF237963, respectively. These sequences were aligned using the Clustal W programme (Thompson et al., 1994) with two sequences of PVY^{O} and PVY^{N} strains; GeneBank accession numbers were AF345650 and AY745492, respectively.

RT-PCR

First strand cDNA was synthesized by RT in a 10 μl reaction containing 2 μl of total RNA incubated at 65 °C for 5 min with 100 pmol of 1R, and 2R antisense primers. Then, 0·5 mM of each dNTPs, 10 mM DTT, 60 units of RNase H-Superscript II Reverse Transcriptase (Invitrogen, Grand Island, USA) and the buffer were mixed and incubated at 42 °C for 1 h, followed by a 5 min incubation at 65 °C to inactivate the enzyme.

The PCR was carried out using either the 1F/1R or 2F/2R primer set. The cocktail included 5 μl of the RT products, 50 pmol of each primer, 5 μl of 10X buffer (166 mM $(NH_4)_2S0_4$, 670 mM Tris–HCl at pH 8·8 and 0·1% Tween 20), 1 unit of *Taq* DNA polymerase (Invitrogen, Grand Island, USA) and equimolar concentrations of $MgCl_2$ (1.5 mM) and dNTPs to minimize the DNA polymerase errors (Eckert and Kunkel, 1990) in 50 μl of total volume. Amplification was performed in a Perkin-Elmer 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturing cycle of 4 min at 94 °C. This was followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C, and a final extension of 7 min at 72 °C. Analysis of PCR products was performed by electrophoresis in 2% agarose gels in 1X TAE buffer (40 mM Tris-acetate pH 8·3, 1 mM EDTA) and visualised on a UV-transiluminator after ethidium bromide staining.

Sequencing and phylogenetic analysis

The RT-PCR products obtained using 2F/2R primers were cleaned with a QIAquick purification kit (Qiagen Ltd., West Sussex, UK) and directly sequenced using the amplification primers and BigDye terminator v3.1 Cycle Sequencing Kit in a 3730 DNA Analyser (Applied Biosystems, Foster City, CA, USA), DNA sequencer. The nucleotide identity of resulting sequences was determined with respect to the 471 nt of PVY CP sequences obtained from the GenBank/EMBL database using the Blast programme. The nucleotide sequences were aligned with the Klustal W programme (Thompson et al., 1994) and MEGA (Kumar et al., 2001) and the nucleotide distances between pairs of sequences were estimated using the Kimura 2 – parameter method (Kimura, 1980). The topology of the phylogenetic tree was inferred using the Neighbour Joining method (Saitou and Nei, 1987) and statistical significance was calculated by applying a bootstrap analysis with 1000 replicates.

SSCP analysis

The single-stranded conformation polymorphism (SSCP) technique was applied for a preliminary detection of genomic differences between different PVY isolates, essentially following the procedure described by Orita et al. (1989). This technique was performed directly on a specific amplified PCR fragment of 471 nt from the CP regions, using the primers pair 2F/2R. Two μl of the PCR product was mixed with 8 μl of the denaturing solution (9.50 ml of formamide, 25 mM of EDTA and 5 mg of bromophenol blue), heated for 5–10 min at 95 °C, chilled on ice and resolved by electrophoresis on 10% polyacrylamide gels, using TBE (Tris-borate 90 mM, 10 mM EDTA, pH 8.0) and a constant voltage of 240 volts for 2.5 h. at 4 °C. The gels were stained with silver nitrate using the procedure described by Igloi (1983).

Results

Serology

Leaf samples from 61 tomato plants were positive by ELISA using specific antisera to PVY. Tomato samples with local lesions on intermediate leaves or apical mosaic always gave positive reactions in PVY ELISAs but it was also possible to detect PVY in some symptomless plants and in others with apical yellowing. Only 39 of the samples collected from 19 tomato fields were selected for the comparative study (Table 1) because a few stored samples lost their infectivity; in some samples PVY either formed part of a mixed infection together with other viruses, or the NAE could not be amplified by RT-PCR in other cases. Serological differences between field PVY tomato isolates were obtained using the generic PA and MAbs; three different PVY potato serotypes were distinguished. Three PVY field isolates were positive when using the PVY^{N} MAbs, 29 were positive with $PVY^{O/C}$ MAbs, and seven with both $PVY^{O/C}$ and PVY^{N} MAbs (Table 1). A positive reaction in ELISA using $PVY^{O/C}$ MAbs must have been due to infection by serotype PVY^{O}, because none of the PVY isolates reacted positively with the PVY^{C}-specific MAbs (data not included in Table 1). Some PVY isolates reacted weakly in ELISA but the results between MAbs or PA did not correspond, with the exception of isolate B1 that always reacted weakly with both PA and PVY^{N} MAbs. Some samples with mixed infections only reacted to one of the MAbs after mechanical transmission to *Nicotiana* spp. or pepper cultivars (data not shown). For example, field isolate B5G mechanically inoculated to *N. glutinosa* only gave positive reaction with $PVY^{O/C}$ MAbs, while it reacted with PVY^{N} MAbs when inoculated to *N. tabacum* cv Xanthi. The three PVY isolates from pepper used as control reacted with PVY PA. The PVY(1) isolate also produced a weak positive reaction to $PVY^{O/C}$ MAbs, although capsicum pathotypes are often not recognised by MAbs produced to potato PVY strains (Llave et al., 1999; Abad and Jorda, 2000). PVY^{O} and PVY^{N} isolates from potato used as control reacted positively in ELISA with the PVY PA and $PVY^{O/C}$ and PVY^{N} Mabs, respectively.

Biological characterization

Results obtained by biological characterization are shown in Table 1. Correspondence between the symptoms of infection observed in tomato plants under field conditions and those reproduced in tomato plants infected by mechanical inoculation in the glasshouse under controlled conditions were poor for both tomato genotypes assayed. The results obtained with the two tomato genotypes did not show any noticeable differences and are therefore presented together in the same column in Table 1. A few PVY isolates (PVY(1), PVY^{O} and PVY^{N}) mechanically transmitted to tomato did not induce any observable symptoms. All PVY isolates infected *N. glutinosa* and *N. tabacum* cv. Xanthi but the most severe symptoms of infection were observed in *N. glutinosa*. Some isolates (e.g. F14K, C10C and SP1) that caused veinal chlorosis, also caused stunted growth and leaf deformation in *N. glutinosa*, but only mild mosaic in *N. tabacum* cv. Xanthi. PVY isolates induced two different types of symptoms in *N. tabacum* cv. Xanthi infected plants; mild mosaic or mosaic that sometimes progressed to necrosis, stunted growth and leaf deformation.

None of the PVY isolates tested infected plants from 9005 or Oman pepper cultivars carrying the resistance gene to PVY(1) isolate, except the pepper isolate PVY(1–2) used as control that broke this type of resistance. The biological behaviour of PVY isolates was variable upon mechanical inoculation to pepper plants from 9016, Delfos or Lido cultivars that had no gene-related resistance to PVY. The results obtained with these three pepper genotypes have been grouped in the same column (Table 1) for clarity. The four isolates that were collected from tomato field number 18 induced severe symptoms in pepper, similar to the PVY pepper isolates used as control. The four PVY isolates that were collected from tomato field number 17 induced mosaic and additional yellowing symptoms in some of the inoculated pepper plants. Some PVY isolates infected pepper, as confirmed by ELISA, but did not induce symptoms of infection. These isolates caused a weak positive reaction, sometimes not in all repetitions (denoted by * in Table 1), and depended on the pepper genotype (data not shown). The other PVY isolates did not infect any of the pepper plants. In general, the PVY isolate groups collected from the

Table 1. Biological and serological characteristics of different field PVY isolates from tomato determined by inoculation in test plant and enzyme-linked immunosorbent assay (ELISA)

Isolate	FN	Symptoms					ELISA		
		Field	Ng	Nx	Le	Ca	PA	O/C	N
B1	1	a	vce	w	a	0	+	–	+
B3	1	a	sd	w	a	0	++	+	++
C12M	2	y	vcsd	w	mn	a*	++	+	–
C10C	2	y	vcsd	w	msd	0	+	++	–
D3D	3	a	vc	w	m	0	++	++	–
F2	4	a	sd	sd	ms	0	++	++	–
P15D	5	a	vc	nsd	m	a	++	–	++
T10D	6	a	sd	mnd	w	0	++	++	+
B5G	7	a	sd	sd	a	0	++	++	++
B29N	7	y	sd	nsd	mn	0	++	+	++
E8C	8	y	vcsd	nsd	ys	a*	++	++	+
G25L	9	y	sd	w	mnd	a*	++	+	–
MI18S	10	n	vc	vc	ms	a*	++	++	–
GII 29L	11	n	sd	w	sd	0	++	+	–
A2	12	m	sd	w	ms	0	+	++	–
MI3Q	13	m	vc	sd	m	a	++	+	+
SP1	14	mn	vcsd	w	ms	a	++	–	++
D12E	15	y	vc	w	mnd	a*	+	++	–
D20F	15	y	nsd	w	m	0	+	++	–
D8P	15	n	nsd	msd	md	a	++	++	–
D24I	15	n	vcsd	w	mn	a*	++	++	–
D11H	15	m	vc	w	mnd	a*	++	++	–
D17G	15	m	nsd	msd	m	a*	++	++	–
P2B	16	y	vcsd	w	md	a*	++	+	–
P2G	16	y	vcsd	w	mn	a	++	+	–
P14F	16	n	vcsd	w	mn	a*	++	+	–
P28B	16	n	vcsd	w	mn	a	++	+	–
JB T15	17	y	vcsd	sd	md	m*	+	++	–
JB T23	17	y	vcsd	vcs	ys	m*	++	++	++
JB T25	17	n	vc	w	md	m*	++	++	–
JB T9	17	m	sd	w	m	m	++	+	–
F23B	18	n	vcsd	w	mnd	nsd	++	++	–
F15E	18	n	vcsd	ms	mnd	nsd	++	++	–
F14K	18	n	vcsd	w	mnd	nsd	++	++	–
F10F	18	n	vcsd	ms	mnd	nsd	++	++	–
R24E	19	n	vc	w	ms	a*	++	+	–
R10I	19	n	vc	w	ms	a*	++	+	–
R19T	19	n	sd	sd	m	a*	++	++	–
R10H	19	m	vcsd	w	nsd	a*	++	+	–
PVY(0)			vcd	vcs	m	nd	++	–	–
PVY(1)			vc	s	a	nsd	++	+	–
PVY(1–2)			sd	w	0	nsd	+	–	–
PVY^{O}			vcsd	m	a	0	+	++	–
PVY^{N}			nsd	nsd	a	0	+	–	++

FN, Field number. Hosts: Ng, *Nicotiana glutinosa*; Nx, *Nicotiana tabacum* cv Xanthi; Le, *Lycopersicon esculentum*; Ca, *Capsicum annum*. Symptoms: a, symptomless; d, deformation; m, mosaic; n, necrosis; s, stunted; vc, veinal chlorosis; w, mild mosaic; y, yellowing. 0, no infection; *, no infection in some repetition. ELISA: PA, polyclonal antiserum; O/C and N, monoclonal antibody to the corresponding potato group; –, negative reaction; +, weak positive reaction; ++, strong positive reaction.

same tomato field (see field number in Table 1) showed a high degree of internal similarity, but revealed differences between groups of PVY isolates.

RT-PCR, sequence and SSCP analysis

PCR products of 854 and 471 nucleotides were obtained by RT-PCR using 1F/1R, and 2F/2R

respectively. It was not possible to amplify DNA from all the PVY isolates by RT-PCR and with all the primer pairs used (Table 2).

Fourteen PVY isolates with representative biological and serological differences were selected, amplified by RT-PCR using the 2F/2R primer pair, and the 471 nt PCR products of the CP region were sequenced. Nucleotide sequence analyses showed a high degree of identity among strains SON 41, NNP pepper strain, and NTN and N potato strains (Table 2). A dendrogram (Figure 1) was generated from the estimated genetic distances between the selected PVY isolates and the sequence of NTN and N potato strains, PVY(0) and NNP pepper strains and SON 41 pepper isolate from GenBank database using the Kimura 2-parameter method (Kimura, 1980). The phylogenetic tree distributed the PVY isolates in three

Table 2. Reverse transcriptase polymerase chain reaction (RT-PCR) of different field PVY isolates from tomato and from pepper and sequence analysis of amplified nucleic acid fragments

Isolate	RT-PCR		GeneBank			
	1F/1R	2F/2R	This study accession no.	% identity	Reference isolate	Accession no.
B1	–	–				
B3		+*	AJ890321	99.58	N	AJ584851
C12M	–	–				
C10C	+	+*	AJ890333	99.36	SON 41	AJ439544
D3D	–	+*	AJ890324	98.72	NNP	AF237963
F2	–	+				
P15D	–	+*	AJ890328	99.79	NTN	AJ390289
T10D	+	+*	AJ890323	98.29	NNP	AF237963
B5G	+	–				
B29N		–				
E8C	+	+*	AJ890329	98.09	NTN	AJ390289
G25L		–				
MI18S		+				
GII 29L	–	+				
A2	–	+*	AJ890332	96.79	SON 41	AJ439544
MI3Q	+	+				
SP1		+*	AJ890327	99.79	NTN	AJ390289
D12E		+				
D20F	–	+				
D8P	–	+				
D24I		+				
D11H		+				
D17G	–	+				
P2B		+				
P2G		+				
P14F	–	–				
P28B	+	–				
JB T15		+*	AJ890334	98.93	SON 41	AJ439544
JB T23		+*	AJ890330	100	NTN	AJ390289
JB T25		+				
JB T9	+	+				
F23B	+	+*	AJ890331	99.15	SON 41	AJ439544
F15E	–	–				
F14K	+	+*	AJ890326	98.72	NNP	AF237963
F10F	+	+*	AJ890325	98.72	NNP	AF237963
R24E	–	+*	AJ890322	96.79	NNP	AF237963
R10I	–	+				
R19T	+					
R10H	–	–				
PVY(0)		+*		99.58	PN-82	AJ303096

RT-PCR: 1F/1R and 2F/2R primers pairs used; (+), positive amplification; (–), negative amplification; (*), DNA amplicons selected for sequence analysis.

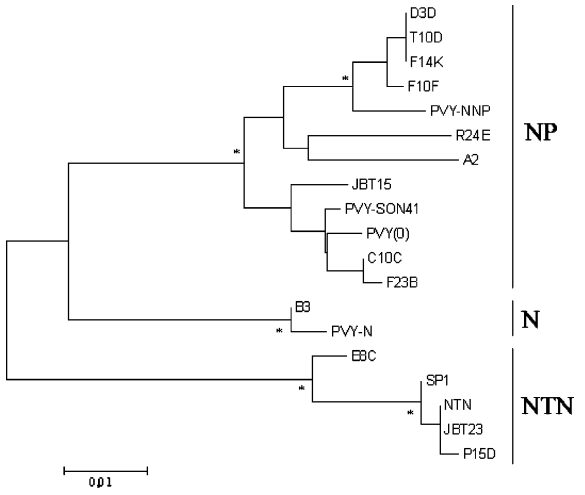

Figure 1. Phylogenetic tree of PVY isolates from tomato based on genetic distances calculated from CP nucleotide sequence amplified with the primers (2F/2R) and using the PVY strains NNP (AF237963), SON 41 (AJ439544), N (AJ584851) and NTN (AJ390289) as reference. *$p > 97\%$.

main clusters corresponding to NTN and N potato-type strains and another cluster corresponding to NP non potato-type strains. The last of these clusters formed by the most diverse sequence included nine PVY tomato isolates. The NTN potato-type group included four isolates and only the B3 isolate belonged to the N potato-type group. The nucleotide identities ranged between 95.7 and 100% for NP group isolates and 97.8–99.7% for NTN group isolates. The nucleotide identities between different groups were about 90% for NP and NTN groups, 92% for N and NTN groups and 94% for N and NP groups. The genetic distances between clusters are shown in Table 3 and the total calculated genetic diversity between PVY isolates was 0.060 ± 0.008.

Only DNA amplified with 2F/2R was used for SSCP analysis because 1F/1R only amplified DNA from a few PVY isolates. Only three patterns of double DNA bands with differences in electrophoretic mobility were produced from the PCR products for all PVY isolates assayed confirming their distribution in three clusters obtained by sequencing analysis (Figure 2). The lane 1 pattern corresponded to PVY isolates belonging to the NTN group, the lane 2 pattern corresponded to the B3 isolate belonging to the N group and lane 3 and 4 patterns corresponded to PVY isolates belonging to the NP group.

Table 3. Genetic diversity among groups of tomato PVY population and standard error

PVY group*	NP	N
NP		
N	0.066 ± 0.011	
NTN	0.096 ± 0.014	0.082 ± 0.014

(*) Three main clusters corresponding to NTN and N potato-type strains and NP non potato-type strains.

Discussion

Three different symptom types: apical mosaic, necrotic local lesion on intermediate leaves, and apical yellowing, were clearly distinguished in field tomato plants. Both mosaic and necrosis symptoms were always associated with PVY serological detection, but yellowing was only associated with it in some cases. Yellowing symptoms in tomato have been associated with infections of some PVY^N strains (Abad and Jorda, 2000); however, the results of our study demonstrated that tomato plants showing yellowing symptoms could not be associated with any specific PVY strain. Furthermore, yellowing symptoms in tomato plants that were negative in ELISA for PVY, could probably be associated with other causes, such as iron or nitrogen deficiency (Maynard, 1991). PVY was also detected by ELISA and isolated from tomato plants without apparent symptoms of virus infection. This could have been due to recent PVY infection, or be a consequence of late infection in old tomato plants, which sometimes remain symptomless under field conditions. The lack of

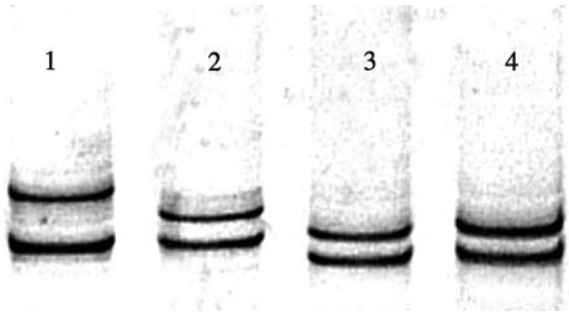

Figure 2. SSCP electrophoretic gels showing DNA amplified from PVY isolates by RT-PCR using the primer set 2F/2R. Lane 1, E8C belonging to NTN group; lane 2, B3 belonging to N group; lane 3, T10D and lane 4, C10C, both belonging to NP group.

correspondence between the symptoms observed in the field and the symptoms produced in young tomato plants 15 to 30 days after mechanical inoculations in a glasshouse can probably be attributed to differences in plant age and environmental conditions.

In the present study, there was no evident relationship between field PVY isolates causing necrotic, yellowing or mosaic symptoms in tomato plants and serological reaction with MAbs specific for PVY^{O} or PVY^{N} serotypes. This absence of such a relationship was also found in biological and serological characterization of different potyviruses by Jordan and Hammond (1991). Other researchers also reported similar findings for tomato PVY isolates. Thus, PVY isolates from tomato collected in France were distributed in PVY^{O} and PVY^{N} potato groups and mainly the non-potato group (Marchoux et al., 2000). However, these authors did not find any particular association between their arbitrary classification of pathotypes and symptom type. In the same way, Abad and Jorda (2000) distinguished different pathotypes by inoculating tomato plants with PVY isolates belonging to the same PVY^{N} group.

Some authors have considered the tobacco plant a differential host for pepper or potato isolates based on symptoms. The pepper isolate group could be the cause of mild mosaic symptoms in tobacco plants and PVY isolates belonging to PVY^{O} and PVY^{N} potato isolate group could be the cause of strong mosaic as suggested by Romero et al. (2001). In this study most PVY tomato isolates induced mild mosaic in *N. tabacum* cv. Xanthi and only a few isolates induced strong mosaic and vein necrosis; however, there was no correspondence between this biological association and the differentiation by ELISA using MAbs. The lack of correspondence was also evident from the infectivity results using different genotypes of pepper plants susceptible to PVY. Some PVY isolates such as C10C, D3D and A2 positives to $PVY^{O/C}$ MAbs did not infect any pepper plants. In contrast, other isolates such as the PVY isolate group collected from tomato fields numbers 17 and 18, and positives to $PVY^{O/C}$ MAbs, induced characteristic symptoms on these pepper cultivars. PVY isolate group collected from field number 19 provided an intermediate situation inducing only mild symptoms in some repetitions. In the same way, some PVY isolates such as P15D and SP1 only reacted positively to PVY^{N} MAbs and infected pepper plants, but the B1 PVY isolate belonging to the same serotype was not detected by ELISA in any of the inoculated pepper plants.

One reason for the discrepancies between biological and serological results of the field strains could be the presence of mixed infections by different PVY strains that may even be of the same serotype. Mixed infections could lead to erroneous conclusions in a bioassay, because some of the strains present in mixed infections could be selected, as occurred in our study. This could have occurred when mixed strains were mechanically inoculated into plants other than tomato, and the strains differed in fitness. *Nicotiana* spp. and pepper plants could both be selective hosts for some PVY strains from mixed infections, as demonstrated by serological assays. Co-infections of different PVY isolates seems frequent in tomato crops, as demonstrated by Abad and Jorda (2000) in a similar study carried out on the Islas Canarias (Spain). These researchers suggested that mixed detections using $PVY^{O/C}$ and PVY^{N} MAbs were probably due to a mixture of PVY^{N} and PVY^{C} strains based upon their biological behaviour and RT-PCR data. However, our negative results obtained by ELISA using PVY^{C} MAbs with all the PVY isolates studied did not confirm the presence of PVY^{C} strains. This was consistent with the notion that PVY^{C} is not as widespread as the PVY^{O} and PVY^{N} strain group (Kerlan et al., 1999). In contrast, some sequences of our PVY isolates, positive in ELISA to $PVY^{O/C}$, showed greater homology with PVY-NNP, an isolate that according to Fanigliulo et al. (2004) reacted with a MAbs specific for PVY^{C}. However, as suggested by the same authors, this PVY isolate originated from a recombination event involving a PVY^{O} isolate and another parental virus, which could probably be a PVY isolate belonging to the NP group. This group of PVY isolates is not infectious in potato and includes all of the PVY isolates from pepper, which are also closely related to PVY^{O} strain group as suggested by Soto et al. (1994). Finally, we must take into account the existence of commercial MAbs specific to PVY^{C} that are unreliable for detecting standard PVY^{C} isolates (Kerlan et al., 1999).

Although differences in SSCP pattern do not necessarily imply great differences in nucleotide sequences (Rubio et al., 1996), the SSCP proved to

be a very useful technique for screening PCR products before sequencing them for phylogenetic analysis. It allowed the three groups of PVY isolates with nucleotide similarities lower than 94% to be distinguished. Nevertheless, the biological behaviour of different PVY isolates could not be understood or explained using alternative comparative techniques based on genetic studies. The CP gene is the one most frequently used for studies of genetic diversity in potyviruses (Shukla et al., 1994), but its sequence does not always correspond well with characteristics based on pathological properties, as reported by other authors (Blanco-Urgoiti et al., 1998; Romero et al., 2001). The three main clusters clearly distinguished in this work were previously reported by other authors in similar studies on PVY isolates from tomato (Abad and Jorda, 2000; Marchoux et al., 2000) but no good association with biological or serological results could be established. MAbs sometimes are unable to discriminate between PVY^{O}, PVY^{C} and PVY^{N} strains (Blanco-Urgoiti et al., 1998; Boonham and Barker, 1998). This could be due to the presence of recombination events as suggested by Kerlan et al. (1999), which are very frequent in the CP encoding region of PVY isolates (Revers at al., 1996). Sequence analysis of amplified DNA fragments of some PVY tomato isolates having high homology to the PVY^{NTN} group only were positive to PVY^{N} MAbs; although, PVY^{NTN} was proposed as a recombinant between PVY^{O} and PVY^{N} (Glais et al., 2002). However, there was no evidence of this relationship with the PVY isolates assayed and the techniques applied in this study.

Finally, DNA amplification by the RT-PCR technique could also be biased toward specific genetic sequences. Preliminary DNA amplification assays achieved with a few PVY isolates using two pairs of primers, one designed from CP region and another from P1 proteinase region based on SON41 Gen Bank accession number AJ23954, provided negative results for DNA amplification for some of them (data not shown). As a result some PVY isolates were excluded from this comparative study for this reason, despite the design of two pairs of primers including one that was degenerate and designed from sequences of our own PVY isolates.

All our results suggest that tomato does not seem to be a host selective for PVY infection as does potato or pepper. Furthermore, it is possible to find PVY isolates, mixtures and probably recombinants from these other hosts as well as from natural weeds. For this reason, it is very difficult to group the PVY isolates from tomato on the basis of the previous classification of pepper and potato PVY isolates. The genetic diversity found in this study could be expected for viruses like PVY that exhibit frequent recombination events (Revers et al., 1996; Worobey and Holmes, 1999), but was considered very high if we take into account the fact that the PVY isolates were collected from a small area and were selected by amplifying their RNA using a single pair of primers. New groups could be established by applying separate criteria based on biological, serological or genetic characteristics. This could not be done following a general criterion, because the results obtained from biological and serological assays and PCR would be biased and cause erroneous conclusions. These could be due to the presence of mixed infections or deficiencies in primer design which need to be robust and able to detect the majority of isolates. Although there were also differences between groups of isolates, some PVY isolates collected from the same tomato field showed a high degree of similarity. This indicated the likelihood of a single source of infection in the majority of tomato fields but a different source in each field. The great diversity of PVY isolates that may be found in tomato, the difficulties inherent in grouping them, and the variety of plant species that could act as foci for infection represent a major problem for the development of resistance strategies and/or the adoption of general cultural practices for control of PVY disease in this crop. The search for mechanisms providing a broader spectrum of resistance, as for example genes that appear to confer resistance to unrelated viruses affecting vector behaviour, seems to be one way to avoid PVY infection.

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