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The detection of recombinant, tuber necrosing isolates of *Potato virus Y* (PVY^{NTN}) using a three-primer PCR based in the coat protein gene

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Abstract

A simple and reliable procedure for reverse transcription-polymerase chain reaction (RT-PCR) detection and strain differentiation of *Potato virus Y* (PVY) was developed. Three primers were designed within the coat protein (CP) and nuclear inclusion protein b (NIb) region, exploiting a single base polymorphism identified as being present in all the recombinant PVY^{NTN} isolates published. Samples infected with PVY produce a single band of 569 bp, while isolates belonging to PVY^{NTN} strain give an additional band of 334 bp. The technique was tested on a collection of well-characterised isolates of PVY from a range of strains and was found to detect all of the isolates reported as belonging to the PVY^{NTN} strain. All of the isolates detected possess a recombination event within the coat protein. Further sequence analysis revealed that all the recombinant PVY^{NTN} isolates reported thus far would be detected using this assay, whilst isolates thought to be PVY^{NTN} that do not possess the coat protein recombination event would not be detected.

Keywords: *Potato virus Y*; Three-primer PCR; Coat protein gene

1. Introduction

Potato virus Y (PVY) is one of the most damaging plant viruses economically due to the importance of its plant host species, which include potato, pepper, tomato and tobacco. In connection with its wide host range, PVY displays a high variability. Potato strains are commonly subdivided into three main groups PVY^{O}, PVY^{C} and PVY^{N} (de Bokx and Huttinga, 1981); according to systemic and local symptoms, they induce on *Nicotinia tabacum, Physalis floridana* and *Solanum tuberosum*. However, in the last few decades, new strain variants have been reported, such as the tuber necrosis strain (PVY^{NTN}).

Isolates of PVY^{NTN} are associated with potato tuber necrotic ringspot disease (PTNRD) (Beczner et al., 1984; Le Romancer and Kerlan, 1991). PTNRD was first noted in Hungary (Beczner et al., 1984) and then in Germany (Weidemann, 1985). There is evidence for its occurrence in most potato producing countries. The disease is characterised by a superficial necrosis on tubers, which occurs at harvest and often develops during storage. PVY isolates inducing necrosis on tubers belong to the PVY^{N} subgroup according to their reactions on *N. tabacum*. Molecular studies have revealed the presence of a recombination break point within the coat protein of most isolates associated with the PTNRD phenotype (Revers and Le Gall, 1996; Boonham et al., 1999, 2002a,b). However, a number of isolates have been found to be associated with PTNRD which do not have the recombination break point (Ohshima et al., 2000; Boonham et al., 2002a,b).

Several molecular methods have been developed to distinguish between PVY^{N} and PVY^{NTN} isolates. Some are based around sequence differences at the 5′ terminal end of the genome (Weidemann and Maiss, 1996; Weilguny and Singh 1998; Rosner and Maslenin, 1999), some at the 3′ end of the genome (Rosner and Maslenin, 2001), including exploitation of the coat protein recombination event (Boonham et al.,

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2002a,b), whilst others are based on variability across the entire genome (Glais et al., 1998).

In a previous study (Cerovska et al., 2001), we determined the coat protein sequence for seven NTN isolates. Comparisons between these sequences and other published PVY sequences (Robaglia et al., 1989; Thole et al., 1993) revealed further sequence polymorphism in this region of the genome. Based on this polymorphism, a new PCR method is reported that takes advantage of a single nucleotide difference observed in the coat protein-coding region of the PVY^{NTN} isolates.

2. Materials and methods

2.1. Virus isolates

Virus isolates were kindly provided by Dr C. Kerlan (INRA Rennes, France) and Dr P. Dedic (Potato Research Institute, Czech Republic). Isolates were maintained in *N. tabacum* cv. Samsun. The leaves of infected plants were harvested 2 weeks after inoculation and were tested by ELISA for the occurrence of other potato viruses using polyclonal antibodies raised against PVA, PVX, PVS. In addition, the PVY strain specific monoclonal antibodies from Adgen were also used.

2.2. Nucleotide sequences

For the design of primers the both sequences retrieved from GenBank were used and also sequences produced in our laboratory (isolates Lukava, Nicola, Vital, Igor, Ranka, Orleans and Tu660). For the initial design, we used 40 GenBank sequences with well-documented phenotype. The sequence positions follow the numbering according to the sequence D00441 (PVY^{N}-Fr, Robaglia et al., 1989).

2.3. Sequencing and analysis

Nucleotide sequencing of CP was performed directly on PCR products. The products were sequenced using the same primers as used for PCR amplification in conjunction with the ThermoSequenase™ kit 2.0, following manufacturers' instructions (Amersham), before being analysed using an ABI prism 377 DNA sequencer (Applied Biosystem, Inc.). The resulting data was processed using the program CHROMAS and analysed using the program LaserGene (DNAstar, Madison, WI). Multiple alignments of the selected amino acid and nucleotide sequences were obtained using the program ClustalW 1.7 (Thompson et al., 1994). Amino acid sequences were aligned using the Dayhoff PAM substitution matrix series (Dayhoff et al., 1983). Phylogenetic analysis was carried out using the Neighbour–Joining method in Clustal (for details see Cerovska et al., 2001).

2.4. IC-RT PCR for differentiation of PVY^{N} and PVY^{NTN} strains

Immunocapture RT PCR was carried out according to Romero et al. (1997) using 0.5 ml PCR tubes coated with 1 μg/ml of rabbit anti-PVY polyclonal IgG. The reverse transcription was carried out in the same tube by adding 20 μl of the reaction mixture containing 4 μl of 5 × AMV buffer (Promega), 8 μl of 2.5 mM dNTP mix (TaKaRa), 10 U RNasin (Promega), 1 μM of the reverse primer Mor2 (5′-CAA ACC ATA AGC CCA TTC ATC-3′; complementary to nt 8905–8925) and 5 U of AMV reverse transcriptase (Promega). The reaction was incubated at 42 °C for 1 h.

Reverse transcription products (2 μl) were then transferred into 48 μl of PCR mixture containing: 5 μl of 10 × complete Taq buffer (Top Bio), 4 μl of 2.5 mM dNTP (TaKaRa), primers Mor1, 2 and 3, each in 1 μM concentration (Mor1 5′-AGG AGG AAG CAC TAA GAA G-3′; corresponding to nt 8594–8611 and Mor3 5′-GCA CCA AAT CAG GAG ATT CTA CT-3′; corresponding to nt 8359–8383), 2.5 U of Taq polymerase (Fermentas) and water to a final volume of 50 μl.

The PCR amplification was carried out in a 'ProGene' cycler (Techne), using 30 cycles of the following programme: 30 s at 94 °C, 30 s at 60 °C and 1 min at 72°C. Amplification products (4 μl) were separated on a 1% agarose gel in TBE buffer containing $1 \cdot 10^{-5}$ mg/ml of ethidium bromide.

For reference, the methods described by Weilguny and Singh (1998) and Weidemann and Maiss (1996) were used for PVY^{NTN} differentiation.

3. Results

3.1. Sequence analysis and primer design

From the sequence analysis of 40 isolates taken from the public databases and seven sequences from laboratory (Cerovska et al., 2001) it was apparent that almost all known PVY^{NTN} isolates have a G at position 8611, but most other isolates (PVY^{O}, PVY^{N} and PVY^{C}) have an A at the same position. This single nucleotide polymorphism was exploited in the design of the PCR primers. The primers were designed so that two flanking primers bind to the conserved regions in the CP and NIb, respectively, giving a band of 569 bp. Internal primer Mor1 is designed to perfectly match the sequence of PVY^{NTN}, but having at least one mismatch on the 3′ end with other isolates. This primer, together with Mor2, gives an additional band of 334 bp (see Fig. 1).

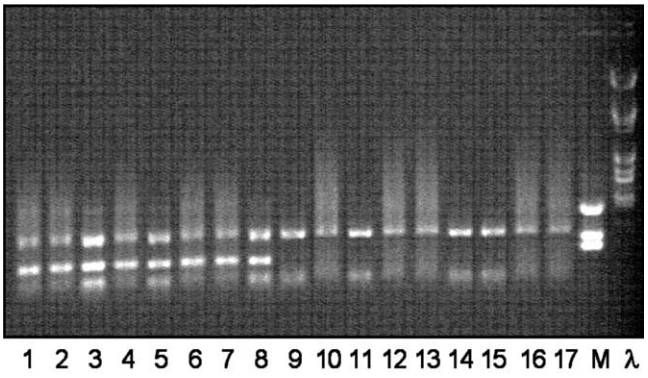

Fig. 1. Differentiation of PVY strains with RT-PCR method based on CP sequences on selected collections of isolates representing various PVY strains. Three-primer PCR was carried out under said conditions and 10 μl aliquots were suggested to electrophoresis in 1.0% TBE agarose. In individual lanes are the following isolates of PVYNTN: 1, Nicola; 2, Lukava; 3, Vital; 4, Igor; 5, Ranka, 6, PVYNTN-Lb; 7, PVYNTN-FrOrl; 8, PVYNTN-H; PVYN: 9, Tu660; 10, B203; 11, N242; 12, PVYN Irl; and PVYO: 13, Nl702; 14, PVYO-Sc; 15, Pona O; 16, KC49; 17, BI. M, marker DNA of 867, 503 and 398 bp and in the last lane DNA digested with *Eco*RI and *Hin*dIII.

Primers do not exhibit any cross-reactivity with PVA or PVX.

3.2. PCR testing

A panel of isolates representative of four PVY groups: PVY^{NTN}, PVY^{N} (non-tuber necrosing), PVY^{N} Wilga and PVY^{O} isolates was tested. PCR profiles are shown in Fig. 1 and results are summarized in Table 1. All isolates tested gave a band of 569 bp corresponding to the product of primers Mor2 and Mor3. The NTN isolates (H, Lb, FrOrl, Igor, Nicola, Vital, Ranka and Lukava) gave an additional band of 334 bp, which corresponds to DNA amplified with primers Mor1 and Mor2. The Canadian isolate Tu660 gave only one band of 569 nt. This isolate is known to induce tuber necrosis on some susceptible potato cultivars in greenhouse conditions, but is usually characterised as PVY^{N} also with other molecular techniques (McDonald and Singh, 1996; Dedic and Ptacek, 1998). Also, one isolate belonging to the Wilga group $Y^{N}N242$ was tested which does not induce tuber necrosis in potato (Kerlan, Tribodet, Glais, unpublished results) its PCR profile is the same as for the other non PTNRD isolates.

The designed assay was also compared with results obtained using two of the more commonly used molecular methods (Weidemann and Maiss, 1996; Weilguny and Singh, 1998).

3.3. In-silico PCR

Following the testing of the PCR primers on several known isolates, it was noted that many new sequences, particularly of PVY^{NTN} isolates, were present on the sequence database. In order to gauge how the primers designed in this study would react to these isolates, further sequence comparisons were made in the region of the NTN specific primer (Mor 1), Table 2. The results show that the NTN isolates (24 sequences) most commonly found in Europe are similar to each other in that that they share very high sequence homology (98–100%, Cerovska et al., 2001), they also contain a recombination in their coat protein (Revers and Le Gall, 1996; Boonham et al., 1999) and have a G at position 8611. In addition, there is a second group of isolates associated with symptoms of PTNRD (isolates 53-29

Table 1
Biological and molecular typing of 17 PVY isolates, illustrating a comparison of three PCR techniques using different selected primers

Isolate	Country of origin	Indexing on tobacco	PTNRD*	Moravec PCR	Weilguny PCR	Weidemann PCR
Nicola	Czech Rep.	N	+	+	+	+
Lukava	Czech Rep.	N	+	+	+	+
Vital	Czech Rep.	N	+	+	+	+
Igor	Slovenia	N	+	+	+	+
Ranka	Slovenia	N	+	+	+	+
PVY^{NTN}-Lb	Lebanon	N	+	+	+	+
PVY^{NTN} FrOrl	France	N	+	+	+	+
PVY^{NTN}-H	France	N	+	+	+	+
Tu660	Canada	N	?	−	−	−
PVY^{N} B203	France	N	−	−	−	n.t.
$PVY^{N}N242$	France	N	−	−	−	−
$PVY^{N}Irl$	Ireland	O	−	−	−	−
$PVY^{O}Nl702$	Netherlands	O	−	−	−	n.t.
$PVY^{O}Sc$	Scotland	O	−	−	−	−
Pona O	Czech Rep.	O	−	−	−	−
KC 49	Czech Rep.	O	−	−	−	−
BI	Czech Rep.	O	−	−	−	n.t.

PTNRD, typical PTNRD symptoms were observed on original tubers in natural conditions; ELISA 4C1: $+++A_{405} = 0.6–3.0$; $++ A_{405} = 0.6–0.1$ (1 h after adding substrate); −, no reaction observed; n.t., not tested.

Table 2
List of all sequences of PVY available in public databases

GenBank Accession No.	Isolate	PTNRD	Origin	Sequence at primer Mor1 position
	Mor1 Primer			AGGAGGAAGCACTAAGAAG
X68223[a]	EURH-NTN	+	?	.....
AJ390303	V97005	−	United Kingdom	.....
X97895	N-605	−	?	.....A...
AB025417	TNK	+	Japan	.....A
AF325928	53-47	+	Denmark	.....A
AJ390298	53-29	+	Denmark	.....A
AJ390309	TU619	+/−	USA	.....A
AF228630	TU660	+/−	USA	.....A
M22470[b]	N27-NZ	−	New Zeland	.....A
AJ390305	O-DES	−	United Kingdom	.....G.....A
AB025416	TND-6	+	Japan	.....T.....A
AB025415	TNN2	+	Japan	.....T.....A
AJ390287	V951218	−	United Kingdom	.....T.....A
S74813	T-13	−	Japan	.....T.....A
E03317	Hataya	−	Japan	.....T.....A
Z70238	WILGA	W	Poland	.....A.....GC.....A
AJ303094	K16.94	Pepper	Tunisia	T.....G....G.....A
AJ303093	Si15	Pepper	Sicily	T.....G....G.....A
U10378	nnp	Pepper	Hungary	T.....G....G.....A
X68225	MSNR	Pepper	?	.....A..AT.G.....A
AJ303095	Tu12.3	Pepper	Turkey	T..G.AG....G.....A
AJ303097	P21-82	Pepper	Spain	T....AG.A..G.....A
AJ005639	P21_82	Pepper	?	T....AG.A..A.....A
AJ303096	PN-82	Pepper	Spain	T....AG.AA.G.....A
X68224	NSNR	Pepper	?	T....AG.AT.G.....A
U09509	PVY-O	−	?	.....AC.....A
AJ223595	O854	−	?	.....GC.....A
AJ390306	PMB21	−	United Kingdom	.....GC.....A
AJ390292	V951204	−	United Kingdom	.....GC.....A
AJ223594	O803	−	Switzerland?	.....G.....GA
AJ223593	O768	−	Switzerland?	.....A..G.....A
AF118153	eggplantO	−	India	.....AC.....A
D00441	Fr	−	France	.....AC.....A
AF255659	O-BR	−	Brazil	.....AC....GA
X68226	CAPF-O	−	?	.....A..AC.....A
AJ390301	O-GOV	−	United Kingdom	.....A.....G.....GA
Z70239	Lipinski	−	Poland	.....A.....GC.....A
D12539	O	−	Japan	.....G...A.....GA
X14136	PVYo	−	Argentina?	.....A..AC.....A
AJ390297	NN-UK-O	−	United Kingdom	T....A..A..G.....A
AY061994	O-India	−	India	.....A..A..G..G...A
U25672	CH_2	−	China	..T..A.....AC.....A
X54058	CH_1	−	China	..T....GA..AC.....A
AF345650	O-Delhi	−	India	——..C.–..G.G...A
AF012026	Isolate 27	−	?	.....G....GC.....
AF012028	Isolate 30	−	?	.....G....GC.....A
AF012027	Isolate 28	−	?	T.....G.A..G.....A
AF012029	Isolate 45	−	?	T....TG.A..A.....A
AJ390302	PVY-C-CM	−	United Kingdom	.....G....GC.....A
AJ390307	O-Tom	−	Portugal	T....A..A..GC.....A
X68221	Chilean O	−		.....G....GC.....
X68222	POT_US	−	USA	.....AC.....A
S74810	Y-36	−	Japan	.....A..G.....A

GenBank accession number, name of isolate, ability to induce tuber necrosis, country of origin and sequence corresponding to primer Mor1 is stated. The polymorphic nucleotide is highlighted in bold; and (.) indicates a nucleotide which matches the sequence of Mor1.

[a] The following accessions all match sequence X68223 and have a PTNRD causing phenotype AJ390308, AJ390290, AJ390289, AJ390288, AJ390296, AJ390294, AJ390291, AJ133454, AF228632, AJ390293, AF321554, X79305, X92078, M95491, AJ390300, X54611, AF228634, AF325927, AF230362, AF228635, AF228633, AF228631, AF255660.

[b] The following accessions all match sequence M22470 and do not have a PTNRD causing phenotype D12570, AJ223592, AJ390304, AJ390286, AJ390285, AJ390295, X54636, Z70237, U09508, U91747, U06789, AJ390299, AF126258.

and 53-47 from Denmark, Tu619 and Tu660 from Canada and TNK, TND-6, TNN2 from Japan) these isolates are more distantly related to other PVY^{NTN} isolates (95–97%), do not contain a recombination in the coat protein and Mor1 primer has a mismatch with their sequence in last nucleotide, so these isolates would not be reported as NTN using the described assay.

In addition to these none recombinant isolates, several other isolates on the database would also give false positive results. Three PVY^{N} isolates (AJ390303, X97895 and U91747) and 2 C isolates (X68221 and AF012026), none of which have been reported as being associated with PTNRD, all have a G at the polymorphic site 8611, these isolates would be reported as false positives.

4. Discussion

The primer sequences for the PCR method reported above were based on sequence polymorphism observed between published sequences of various PVY isolates and well characterised isolates from our own laboratory. The method has been tested on a range of well-characterised PVY isolates, including those associated with PTNRD under natural conditions (see Table 1). In the comparative testing, the assay was shown to give comparable results to two of the more commonly used molecular methods (Weidemann and Maiss, 1996; Weilguny and Singh, 1998).

Recently, many more isolates have been sequenced and the sequencing effort has been concentrated on NTN isolates. Therefore, the reliability of our three-primer assay was further verified by comparison of newly sequenced isolates with specific primer Mor1, in silico. Following these comparisons, it was clear that the three-primer PCR would detect all of the recombinant PVY^{NTN} isolates due to the presence of a G nucleotide at position 8611, however the assay would be unable to detect the non-recombinant isolates (seven isolates in total from Canada, Denmark and Japan) also thought to belong to the PVY^{NTN} group. In this respect, the assay performs in the same way as a previously published assay, which detects the recombination event directly (Boonham et al., 2002a,b). In addition, three other PVY^{N} isolates and two isolates of PVY^{C} do possess the G nucleotide at position 8611, these would also be detected by the above mentioned assay, but none of these isolates are thus far recorded as being associated with PTNRD.

Thus, the three-primer method developed is able to detect PVY infection effectively and distinguish most of PVY^{NTN} isolates from other PVY strains and in comparisons is thought to give equivalent results to other assays published. The assay is based on the identification of a G nucleotide at position 8611, which is present in all recombinant isolates of PVY^{NTN}. The results also support the recent hypothesis (Nie and Singh, 2001; Boonham et al., 2002a,b) that isolates associated with PTNRD symptoms cannot be easily classified into single strain, but their 'genotype' is more complex. The method reported is very simple and should broaden the number of methods available for the detection of polymorphisms linked to PVY^{NTN}. By combining all the available assays for PVY^{NTN} detection it would be able to study how many of each type of tuber necrosing isolates is present in a population, which is commonest and perhaps which one is more aggressive.

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