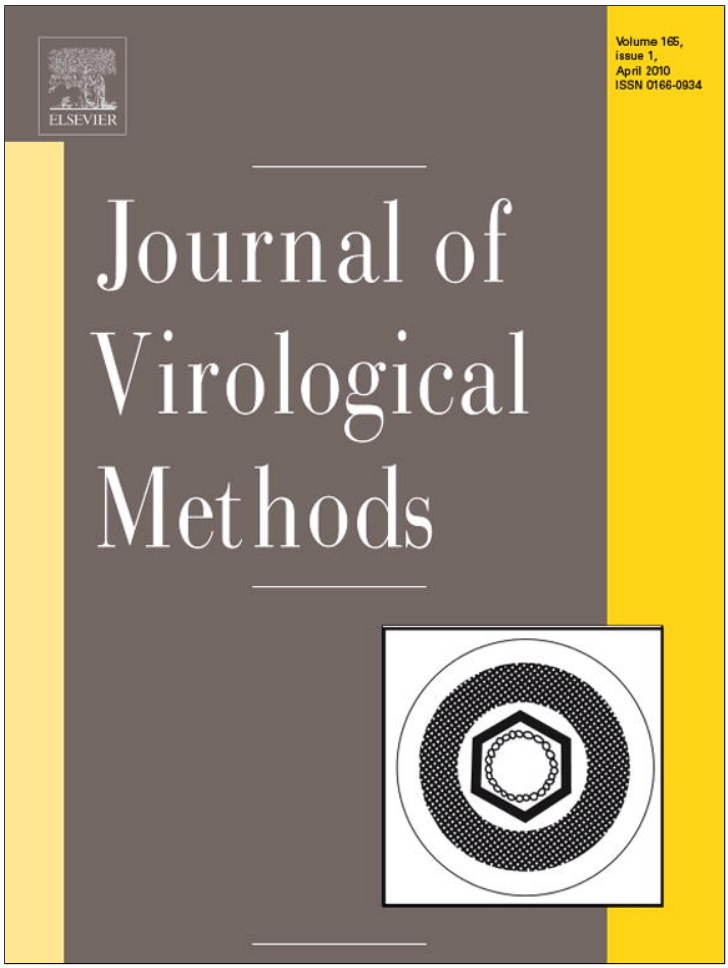

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The simultaneous differentiation of *Potato virus Y* strains including the newly described strain PVY^{NTN-NW} by multiplex PCR assay

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New recombinant strain and genotype of PVY, designated as PVY^{NTN-NW} and SYR-III, respectively, shared properties with PVY^{NTN} and PVY^{N}W has been reported recently. PVY^{NTN-NW} predominated in potato fields in Syria and was able to induce potato tuber necrotic ringspot disease (PTNRD). Due to the rapid spread of the recombinant strains of PVY which might be the case of PVY^{NTN-NW}, a specific and reliable detection method is an essential step to control this strain and minimize its spread. The shared properties of PVY^{NTN-NW} and SYR-III with PVY^{NTN} and PVY^{N}W, however, complicate their identification involving multiple detection methods. Therefore, a multiplex polymerase chain reaction (PCR), that relies on a combination of previously published and newly designed primers was developed for the detection and identification of PVY^{NTN-NW} and SYR-III in single or mixed infections with the main PVY strains, PVY^{O}, PVY^{N}, PVY^{NTN} and PVY^{N}W. In addition, the present PCR assay was able to detect the recombination points in the P1 region enabling the differentiation of the variable genotypes of the recombinant strains PVY^{NTN-NW}, PVY^{NTN} and PVY^{N}W. The reliability of this PCR assay was confirmed using a significant number of well characterized PVY isolates collected from Syria and Japan including those of PVY^{NTN-NW}, SYR-III, PVY^{O}, NA-PVY^{N}, PVY^{N}W and PVY^{NTN}.

1. Introduction

Potato virus Y (PVY) is the most common and destructive virus found in most potato production area (Valkonen, 2007). It is the type species of the genus *Potyvirus*, family *Potyviridae* with a single-stranded positive-sense genomic RNA of approximately 9.7 kb (Berger et al., 2005). According to the reaction of potato cultivars carrying different resistance genes and *Nicotiana tabacum*, potato isolates of PVY are classified into five strain groups PVY^{O}, PVY^{C}, PVY^{N}, PVY^{Z} and PVY^{E} (Singh et al., 2008). According to their sequences, isolates of PVY^{N} strain group fell into two genetic subgroups which are the European (PVY^{N}) and North American (NA-PVY^{N}) (Ogawa et al., 2008). Among these strain groups and subgroups, PVY^{O} and PVY^{N} are the most frequent strains in potato whereas PVY^{C} is not common in potato fields and has less economic importance (Blanco-Urgoiti et al., 1998a; Kerlan et al., 1999). Isolate of PVY^{Z} and PVY^{E} have been reported only once in the original reports and have not been noted elsewhere (Jones, 1990; Blanco-Urgoiti et al., 1998b; Kerlan et al., 1999; Singh et al., 2008) which decrease their significance with regard to potato production.

Genomic recombination plays a significant role in PVY evolution and has led to the emergence of new genotypes/strains including the recombinant PVY^{NTN}, PVY^{N}W and PVY^{NTN-NW} (Glais et al., 2002; Chikh Ali et al., 2007, 2010). PVY^{NTN} and PVY^{N}W were first found in Hungary and Poland, respectively but within a comparatively short time frame they have become common in potato fields in most potato production area (Kerlan et al., 1999; Piche et al., 2004; Glais et al., 2005; Lorenzen et al., 2006; Crosslin et al., 2006; Schubert et al., 2007; Chikh Ali et al., 2007). Many factors might help these new strains to increase their fitness. PVY^{N}W is more infectious in potato, able to infect a larger potato cultivar range and to cause milder symptoms than PVY^{N} or even symptomless infection (Chrzanowska, 1991; Glais et al., 2005) allowing such isolates to escape from the field inspection. In the same way the recombinant PVY^{NTN}, the causal of potato tuber necrotic ringspot disease (PTNRD) was reported to be able to overcome the field resistance of potato cultivars (Van den Heuvel et al., 1994). In addition, the lack of sufficient identification methods is another important factor helped these strains to build up their population.

PVY^{N}W has two main recombination structures, "A" with a recombination point at the HC-Pro/P3 and "B" with an additional

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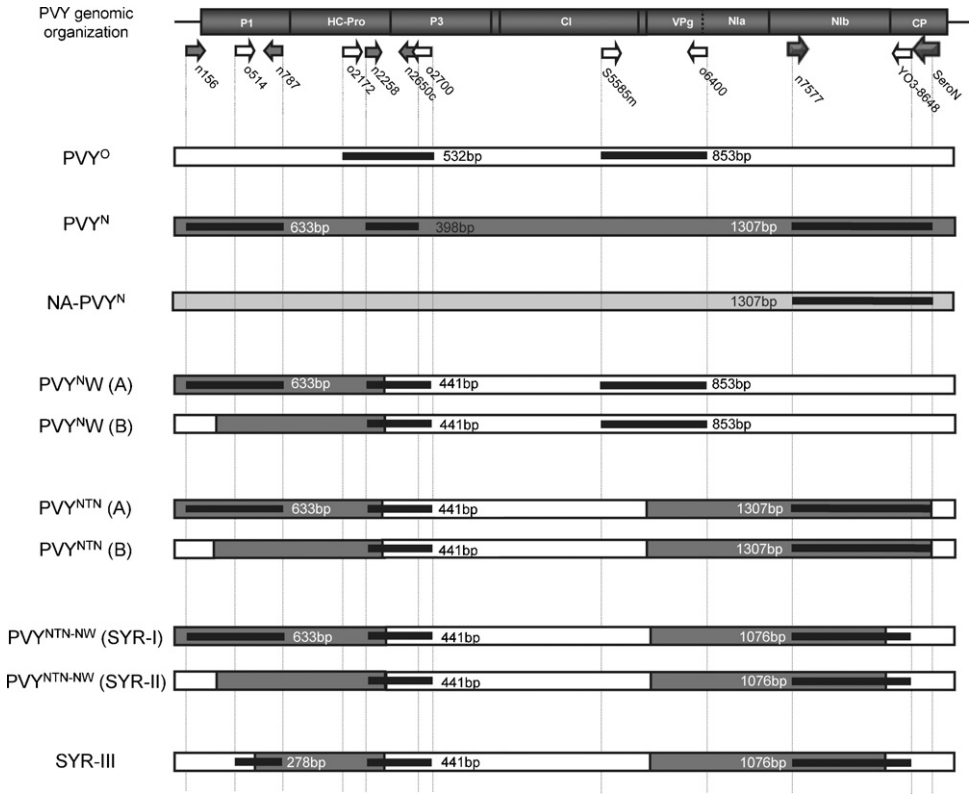

Fig. 1. Genomic structure of PVY strains targeted in multiplex PCR assay with primer location (arrows) and expected products (black bar). Numbers near the black bars indicate the expected band size. Strain/variant name and the genotype (in parenthesis) are written to the left side.

recombination point in the P1 region (Glais et al., 2002). The recombinant PVY^{NTN} also has two main recombination structures, the first with 3 recombination points at the HC-Pro/P3, Vp-g and the C terminus of the CP while the second has another recombination point in the P1 region (Ogawa et al., 2008); for simplicity, these recombination structures of PVY^{NTN} will be referred to as "A" and "B", respectively. The non-recombinant PVY^{NTN} cannot be distinguished from NA-PVY^{N} according to the genetic structure (Ogawa et al., 2008).

A new recombinant strain of PVY namely, PVY^{NTN-NW} that shared a genomic structure with PVY^{NTN} and PVY^{N}W has been recently reported from Syria (Chikh Ali et al., 2010). PVY^{NTN-NW} strain was able to induce PTNRD and dominated potato fields in Syria which indicate the significance of this strain (Chikh Ali et al., 2010). PVY^{NTN-NW} has two genotypes, SYR-I and SYR-II (Chikh Ali et al., 2010). A similar recombinant genotype to SYR-I and SYR-II was reported from Syria, and was designated as SYR-III (Chikh Ali et al., 2010). The general overview of the genetic structures of PVY recombinants is presented in Fig. 1.

Due to the global trading of seed potatoes, the distribution of a new PVY strain is very likely to happen. That was very obvious in the case of PVY^{N}, PVY^{NTN} and PVY^{N}W and this could be true in the case of PVY^{NTN-NW}. In fact, two isolates with characteristics resembling those of SYR-I have been recently reported from China (Hu et al., 2009). To control this new strain and track its occurrence in potato production areas, a reliable molecular detection and identification methods is needed. The objective of the present study was to develop a multiplex polymerase chain reaction (PCR) assay for the detection and identification of PVY^{NTN-NW} and SYR-III and to differentiate them from the main PVY strains, PVY^{O}, PVY^{N}, PVY^{NTN} and PVY^{N}W in the mixed infection.

2. Materials and methods

2.1. Virus isolates

Total of seventy-nine PVY infected samples collected from Syria and Japan were tested. Among them, thirty-four were infected with isolates of PVY^{NTN}, PVY^{N}W, PVY^{NTN-NW} and SYR-III collected from Syria. These isolates were characterized previously and sequence data is available in Genbank for most of them (Table 1; Chikh Ali et al., 2010). In addition, leaf samples of ten potato plants showing virus like symptoms collected in Syria, 2009 were saved on FTA® Plantsaver Cards (Whatman®, NJ, USA) according to the manufacturer's instructions. Ten potato samples infected with PVY provided by the National Agricultural Research Center for Hokkaido Region (NARCH), Japan were tested. Three potato samples infected with PVY were kindly supplied by Dr. H. Horita, Hokkaido Chuo Station, National Center for Seeds and Seedlings, Japan. Twenty-two potato samples infected with PVY were kindly supplied by Ms. M. Taniguchi, Shiribeshi Sub-station, Hokkaido Chuo Station, National Center for Seeds and Seedlings, Japan.

2.2. Primer design

For the detection of PVY^{NTN-NW} and SYR-III in the single and mixed infection with other PVY strains, 12 primers were used in a multiplex PCR assay. Among them 4 reported by Lorenzen et al. (2006), one by Schubert et al. (2007), one by Chikh Ali et al. (2008a) and 6 were designed in this study (Table 2). Primers design was carried out based on nucleotide sequences of 58 PVY isolates available in Genbank. All primers had calculated annealing temperature within 57–58.9 °C. The quality of these primers was tested by the program FastPCR (Kalendar, 2004, available online from Institute of

Table 1
Primers used in the multiplex PCR assay.

Primer name	Polarity	Sequence (5′–3′)	Location	Reference	In silico PCR[c]
n156	Sense	GGGCAAACTCTCGTAAATTGCAG	160–179[a]	This study	PVY^{N} and PVY^{NTN}
o514	Sense	GATCCTCCATCAAAGTCTGAGC	515–536[b]	This study	PVY^{O} and $PVY^{N}W$
n787	Antisense	GTCCACTCTCTTTCGTAAACCTC	770–792[a]	This study	PVY^{N} and PVY^{NTN}
n2258	Sense	GTCGATCACGAAACGCAGACAT	2260–2281[a]	Lorenzen et al. (2006)	PVY^{N} and PVY^{NTN}
o2172	Sense	CAACTATGATGGATTTGGCGACC	2169–2191[b]	Lorenzen et al. (2006)	PVY^{O} and $PVY^{N}W$
n2650c	Antisense	TGATCCACAACTTCACCGCTAACT	2627–2650[a]	Lorenzen et al. (2006)	PVY^{N} and PVY^{NTN}
o2700	Antisense	CGTAGGGCTAAAGCTGATAGTAG	2678–2700[b]	This study	PVY^{O} and $PVY^{N}W$
S5585m	Sense	GGATCTCAAGTTGAAGGGGAC	5578–5598[b]	Lorenzen et al. (2006)	PVY^{O} and $PVY^{N}W$
o6400	Antisense	GTAACTCCTAAACAAATGGTGGTTCG	6405–6430[b]	This study	PVY^{O} and $PVY^{N}W$
n7577	Sense	ACTGCTGCACCTTTAGATACTCTA	7582–7605[a]	This study	PVY^{N}, PVY^{NTN} and NA-PVY^{N}
YO3-8648	Antisense	CTTTTCCTTTGTTCGGGTTTGAC	8635–8657[b]	Schubert et al. (2007)	PVY^{O} and $PVY^{N}W$
SeroN	Antisense	GTTTCTCCTATGTCGTATGCAAGTT	8864–8888[a]	Chikh Ali et al. (2008a)	PVY^{N}, PVY^{NTN} and NA-PVY^{N}

[a] Numbered according to N-605 (GenBank accession no. X97895).
[b] Numbered according to PVY^{O} (GenBank accession no. U09509).
[c] Performed using the program FastPCR (Kalendar, 2004, available online from Institute of Biotechnology, University of Helsinki).

Table 2
PVY isolates tested by the multiplex PCR developed in the present study.

Strain type based on previous identification	Total no. of isolates	Type isolate	Origin	Products in the multiplex PCR (bp)	Classification according to multiplex PCR
PVY^{O}[a]	7	Jisuke-3	Japan	853 + 532	PVY^{O}
NA-PVY^{N}[b]	9	Akaimo-3	Japan	1307	NA-PVY^{N}
$PVY^{N}W$[c]	8	SYR-Wi-11	Syria	853 + 441	$PVY^{N}W$ (B)
PVY^{NTN}[c]	2	PVY-27	Syria	1307 + 633 + 441	PVY^{NTN} (A)
PVY^{NTN}[c]	4	PVY-12	Syria	1307 + 441	PVY^{NTN} (B)
PVY^{NTN}[a]	18	Toyoshiro	Japan	1307 + 633 + 441	PVY^{NTN} (A)
$PVY^{NTN\text{-}NW}$ (SYR-I)[d]	2	SYR-NB-16	Syria	1076 + 633 + 441	$PVY^{NTN\text{-}NW}$ (SYR-I)
$PVY^{NTN\text{-}NW}$ (SYR-II)[d]	12	SYR-II-2-8	Syria	1076 + 441	$PVY^{NTN\text{-}NW}$ (SYR-II)
SYR-III[d]	4	SYR-III-2-4	Syria	1076 + 441 + 278	SYR-III

[a] Based on the multiplex PCR assay reported by Lorenzen et al. (2006) and multiplex assay reported by Nie and Singh (2003).
[b] No classification was available.
[c] Based on the multiplex PCR assay reported by Lorenzen et al. (2006), multiplex assay reported by Nie and Singh (2003) and sequence analysis (Chikh Ali et al., 2007, 2008a,b,c, 2010).
[d] Based on sequence analysis (Chikh Ali et al., 2010).

Biotechnology, University of Helsinki). Their specificity was tested using in silico PCR search function in the FastPCR program against all PVY isolates with available complete sequence in Genbank. Sequence of primers, location and expected products according to the target sequences are shown in Table 1 and Fig. 1.

2.3. RNA extraction and RT-PCR

Total RNA was extracted from dried (0.01 g) and/or fresh (0.05 g) potato or tobacco leaves using RNeasy Plant Mini Kit (Qiagen, MD, USA) according to the manufacturer instructions. Total RNA was dissolved using 125 μl RNase-free H_2O. For samples saved on the FTA cards, discs (4 mm in diameter) were removed from the chlorophyll-stained region of each pressed sample and placed into an RNase-free 1.5 ml microcentrifuge tube. Then, RNA was extracted from these discs according to the method of Ndunguru et al. (2005).

Reverse transcription (RT) was carried out on freshly extracted RNA (4 μl) using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer instructions using an Oligo-dT primer at 2.5 μM final concentration as a reverse primer. RT mixture was incubated at 42 °C for 60 min and transcriptase was inactivated by heating at 95 °C for 5 min, and then the mixture was cooled down at 4 °C for 5 min.

PCR was carried out using TaKaRa Ex Taq™ kit (Takara Bio Inc., Shiga, Japan). All primers were used at final concentration of 0.2 μM except for S5585m and o6400, which were used at 0.4 μM. cDNA solution (1 μl) was added to a PCR tube contained 2.5 μl 10× Ex Taq buffer, 2 μl dNTPs (2.5 mM each), primers, and 0.12 μl TaKaRa Ex Taq™ (5 U/μl). Mixture was completed by MilliQ H_2O to a 25 μl total volume. cDNA amplification was performed in a PC-320 Program Temp Control System Thermocycler (Astec, Shimen-Cho, Japan) using a PCR program consisted of 30 cycles of denaturation at 94 °C for 30 s, primer annealing for 30 s and primer extension at 72 °C for 90 s followed by a final extension at 72 °C for 5 min. Annealing temperatures were 64 °C for the first 10 cycles, 62 °C for the second 10 cycles, 60 °C for the final 10 cycles. PCR product was separated in a 2% agarose gel, stained in ethidium bromide and visualized under UV illumination.

3. Results

3.1. RT-PCR

According to in silico PCR, the primers o514, o2172, o2700, S5585m, o6400 and YO38648 annealed to sequences of the PVY^{O} strain while n156, n787, n2258 and n2650 were specific of the European subgroup of PVY^{N} (Table 1 and Fig. 1). The primer pair n7577 and SeroN annealed specifically to sequences of all PVY^{N} isolates including the NA-PVY^{N} (Table 1 and Fig. 1). The multiplex PCR assay was designed to identify isolates of $PVY^{NTN\text{-}NW}$ or SYR-III in single and mixed infections with other PVY strains. According to this PCR, the mixed infection of $PVY^{NTN\text{-}NW}$ (SYR-II) with PVY^{O} is expected to produce 4 bands of 1076, 853, 532 and 441 bp whereas the mixed infection with PVY^{N} would produce 5 bands of 1307, 1076, 633, 441 and 398 bp. The mixed infection of $PVY^{NTN\text{-}NW}$ (SYR-II) with NA-PVY^{N} and/or PVY^{NTN} would give three bands of 1307, 1076 and 441 bp since this PCR is not able to differentiate PVY^{NTN} and NA-PVY^{N} in the mixed infection. The mixed infection of $PVY^{NTN\text{-}NW}$ (SYR-II) and $PVY^{N}W$ is expected to produce three bands of 1076, 853 and 441 bp. Isolates of $PVY^{NTN\text{-}NW}$ (SYR-I) would give similar band patterns to those of $PVY^{NTN\text{-}NW}$ (SYR-II) with an additional

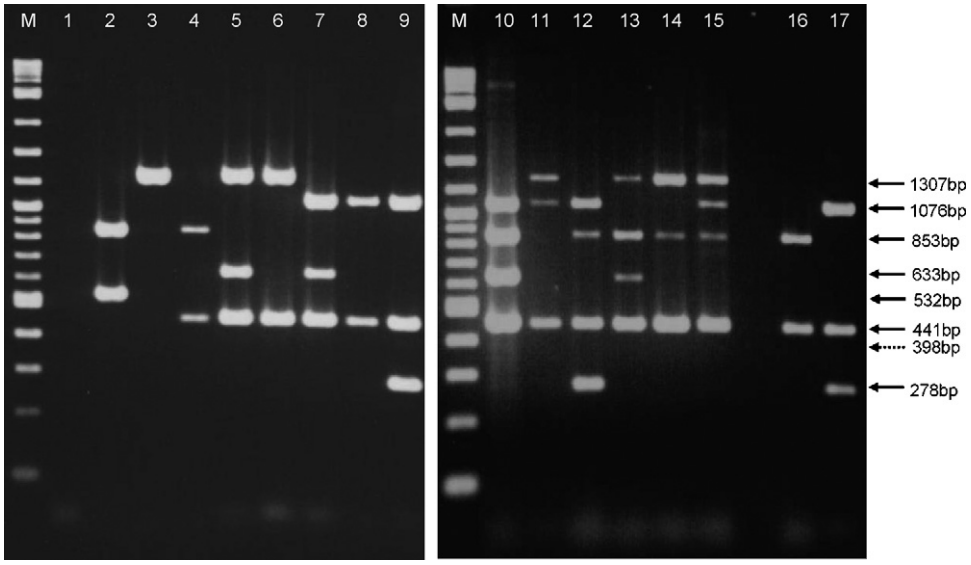

Fig. 2. Results of the multiplex PCR assay after separation in agarose gel by electrophoresis. Lane M, a 100 bp DNA ladder; lane 1, healthy potato control; lane 2, PVY^{O}; lane 3, NA-PVY^{N}; lane 4, $PVY^{N}W$ (B); lane 5, PVY^{NTN} (A); lane 6, PVY^{NTN} (B); lane 7, PVY^{NTN-NW} (SYR-I); lane 8, PVY^{NTN-NW} (SYR-II); lane 9, SYR-III; lane 10, natural mixed infection with PVY^{NTN-NW} and $PVY^{N}W$ detected in a potato leaf sample; lane 11, natural mixed infection with PVY^{NTN-NW} (SYR-II) and PVY^{NTN} (B) (or/and NA-PVY^{N}) detected in a potato leaf sample; lane 12, natural mixed infection with SYR-III and $PVY^{N}W$ (B) detected from an FTA card; lane 13, natural mixed infection with PVY^{NTN} (or/and NA-PVY^{N}) and $PVY^{N}W$, of which at least one belong to the A genotype, detected from an FTA card; 14, natural mixed infection with PVY^{NTN} (B) (or/and NA-PVY^{N}) and $PVY^{N}W$ (B) detected from an FTA card; lane 15, artificial mixed infection with PVY^{NTN-NW} (SYR-II), PVY^{NTN} (B) and $PVY^{N}W$ (B); lane 16, $PVY^{N}W$ (B) detected from an FTA card; lane 17, SYR-III detected from an FTA card. The dotted arrow indicates an expected band for PVY^{N} that was not tested in this study and proposed based on in silico PCR.

Table 3
Comparison of the identification of PVY mixed infections using Lorenzen et al. (2006) PCR assay and the multiplex PCR assay developed in the present study.

Sample name	Origin	PCR assay (Lorenzen et al., 2006)[a]	Products in the multiplex PCR (bp)	Classification according to multiplex PCR
FaY-1	Syria	PVY^{NTN}	1307 + 1076 + 441	PVY^{NTN-NW} (SYR-II) and PVY^{NTN} (B)[b]
S7	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 441 + 278	SYR-III and $PVY^{N}W$ (B)
5	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 633 + 441	PVY^{NTN-NW} and $PVY^{N}W$
8	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 633 + 441	PVY^{NTN-NW} and $PVY^{N}W$
MaY-1	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 441	PVY^{NTN-NW} (SYR-II) and $PVY^{N}W$ (B)
1-7	Syria	PVY^{NTN} and $PVY^{N}W$	1307 + 1076 + 853 + 633 + 441 + 278	SYR-III and PVY^{NTN}[b] and $PVY^{N}W$
09-1-13	Syria	PVY^{NTN} and $PVY^{N}W$	1307 + 853 + 633 + 441	PVY^{NTN}[b] and $PVY^{N}W$
09-9-1	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 441 + 278	SYR-III and $PVY^{N}W$ (B)
09-9-4	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 441 + 278	SYR-III and $PVY^{N}W$ (B)
09-9-7	Syria	PVY^{NTN} and $PVY^{N}W$	1076 + 853 + 441 + 278	SYR-III and $PVY^{N}W$ (B)
09-9-9	Syria	PVY^{NTN} and $PVY^{N}W$	1307 + 853 + 633 + 441	PVY^{NTN}[b] and $PVY^{N}W$
09-10-2	Syria	PVY^{NTN} and $PVY^{N}W$	1307 + 853 + 441	PVY^{NTN} (B)[b] and $PVY^{N}W$ (B)
Inkanohitomi-42	Japan	nt	1307 + 853 + 633 + 532 + 441	PVY^{NTN}[b] and PVY^{O}

[a] Multiplex PCR assay reported by Lorenzen et al. (2006) was performed according to Chikh Ali et al. (2008b).
[b] Or/and NA-PVY^{N} since PVY^{NTN} is indistinguishable from NA-PVY^{N} in the mixed infection using multiplex PCR.

band of 633 bp whereas SYR-III is expected to produce an additional band of 278 bp compared to PVY^{NTN-NW} (SYR-II).

The present multiplex PCR identified all isolates tested in agreement with their classification and could reveal more details on these isolates compared to PCR assays those published previously (Table 2). PVY^{NTN-NW} isolates of the genotype SYR-I (2 isolates) produced bands of 1076, 633 and 441 bp while PVY^{NTN-NW} isolates of the genotype SYR-II (12 isolates) produced two bands of 1076 and 441 bp (Fig. 2 and Table 2). Isolates of SYR-III (4 isolates) produced three bands of 1076, 441 and 278 bp (Fig. 2 and Table 2). PVY^{O} isolates (7 isolates) produced expected bands of 853 and 532 bp (Fig. 2). Isolates of NA-PVY^{N} (9 isolates) produced a band of 1307 bp (Fig. 2). Isolates of PVY^{NTN} genotype A (20 isolates) produced bands of 1307, 633 and 441 bp while those of the B genotype (4 isolates) produced two bands of 1307 and 441 bp (Fig. 2 and Table 2). Isolates of $PVY^{N}W$ genotype B (8 isolates) gave bands of 853 and 441 bp (Fig. 2 and Table 2). No isolates of the genotype A of $PVY^{N}W$ were detected in the isolate set tested. Thirteen samples were mixed infected with variable PVY strains (Fig. 2 and Table 3). Among them, mixed infections of PVY^{NTN-NW} or SYR-III with other recombinant strains were identified. For instance, the sample 5 was identified as PVY^{NTN} and $PVY^{N}W$ according to the PCR assay reported by Lorenzen et al. (2006) but it produced bands of 1076, 853, 633 and 441 bp in the multiplex PCR indicating mixed infection with PVY^{NTN-NW} and $PVY^{N}W$ (Table 3 and Fig. 2, lane 10). The sample FaY-1 was identified as PVY^{NTN} according to the PCR assay reported by Lorenzen et al. (2006) but it produced bands of 1307, 1076 and 441 bp in the present PCR indicating mixed infection with PVY^{NTN-NW} (SYR-II) (Table 3 and Fig. 2, lane 11). A mixed infection with PVY^{NTN} and $PVY^{N}W$ was identified in the sample S7 using the PCR reported previously (Lorenzen et al., 2006) whereas it was identified as mixed infection with SYR-III and $PVY^{N}W$ (B) using the present multiplex PCR (Table 3 and Fig. 2, lane 12). Moreover, the multiplex PCR assay developed in the present study could detect natural (Table 3) and artificial (Fig. 2, lane 15) triple infections with PVY^{NTN-NW} or SYR-III with PVY^{NTN} and $PVY^{N}W$. The multiplex PCR could detect and identify PVY isolates from total RNA eluted from FTA cards for both single and mixed infected samples (Fig. 2).

4. Discussion

The method for PVY detection and identification has continued to improve following the development of new virus detection technologies and the emergence of new PVY strains and variants.

Several detection and identification methods of the new PVY strains and variants particularly those of PVY^{NTN} and PVY^{N}W have been developed. The majority of these emerged PCR assays targeted the recombination points of the recombinant strains to differentiate them from each other and from the classical non-recombinant strains (Boonham et al., 2002; Moravec et al., 2003; Nie and Singh, 2003; Glais et al., 2005; Lorenzen et al., 2006; Schubert et al., 2007; Rigotti and Gugerli, 2007). These methods have been used by potato virus researchers and agencies involved in PVY control. The rapid evolution of PVY and the continuous emergence of the recombinant PVY strains, however, make it necessary to update these assays to be able to deal with the new strains.

In the present article, a multiplex PCR assay was developed to detect and identify the new strain PVY^{NTN-NW} and the genotype SYR-III and differentiate them from the main strain groups PVY^{O} and PVY^{N} (including the European and North American subgroups) along with the recombinant strains PVY^{NTN} and PVY^{N}W in a single test. The reliability of this PCR assay was confirmed using a significant number of well characterized PVY isolates collected from Syria and Japan. In addition, isolates were identified in fresh, dried or FTA pressed samples. The multiplex PCR was able to identify natural and artificial mixed infections of PVY^{NTN-NW} or SYR-III with PVY^{NTN} and PVY^{N}W (Fig. 2) and variable combination of mixed infection with other strains, though it was not able always to identify the genotype of each strain in such mixed infections (Fig. 2). For instance, in the mixed infection with isolates of PVY^{NTN} and PVY^{N}W of which one belongs to the A genotype and the other to the B genotype, the multiplex PCR is unable to identify the genotype of these strains. In addition, the multiplex PCR assay would not distinguish isolates of PVY^{NTN} and NA-PVY^{N} in the mixed infection. Owing to the absence of PVY^{N} of the European type from the present sample set, the reliability of the multiplex PCR assay to detect this strain is yet to be tested. The in silico PCR, however, showed that this strain would be identified as well.

The main novel characteristic of this PCR was its ability to detect and identify isolates of the new PVY strain PVY^{NTN-NW} in both single and mixed infections with other PVY strains. Due to the shared properties of PVY^{NTN-NW} with PVY^{NTN} and PVY^{N}W (Chikh Ali et al., 2010), the identification of this strain is complicated and would give a false identification using some previous detection methods. For instance, they would be classified as PVY^{NTN} using the PCR assay reported by Lorenzen et al. (2006), whereas they would not be identified as PVY^{NTN} based on the assays reported by Boonham et al. (2002), Moravec et al. (2003) and Nie and Singh (2003) according to the in silico PCR. Furthermore, in the PCR assay reported by Rigotti and Gugerli (2007), isolates of PVY^{NTN-NW} of the SYR-I genotype would be misidentified as PVY^{N}W of the A genotype whereas PVY^{NTN-NW} of the SYR-II would be misidentified as PVY^{N}W of the B genotype. All these factors would affect the accurate identification of PVY^{NTN-NW} and increase the necessity of the present PCR assay as a robust identification method of PVY^{NTN-NW}. In comparison with the PCR assay reported by Lorenzen et al. (2006), the present multiplex PCR was able to detect recombination points in the P1 region enabling the differentiation of the different genotypes of PVY^{NTN-NW}, PVY^{NTN} and PVY^{N}W. Though no biological function or effect was linked to the recombinant structure of the P1 coding region of recombinant PVY strains so far, one cannot exclude a possible evolution value of this recombination point. According to the results of the present study on Syrian PVY isolates of PVY^{NTN-NW}, PVY^{NTN} and PVY^{N}W isolates having a recombinant P1 region were more frequent which would suggest a low selection pressure or increased fitness under the Syrian conditions and the same could be true for other regions. The present multiplex PCR is a powerful tool to study the ratio between PVY strains with recombination point in the P1 region to those lacking such a feature and consequently would enhance studied on the possible role of this recombination point in PVY epidemiology. Further, the primers used in this PCR assay cover almost the whole genome of PVY allowing the rapid and easy characterization of PVY isolates and study of the structure of PVY population in potato production area.

In conclusion, a new multiplex PCR assay was developed to detect and identify isolates of PVY^{NTN-NW} and SYR-III and to differentiate them from the most frequent and significant PVY strains in potato production area worldwide. It would be useful for PVY control and for studying the composition, characterization and epidemiology of PVY.

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