Molecular Characterization of Recombinant Strains of *Potato virus Y* From Saudi Arabia

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Abstract

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Potato virus Y (PVY) exists as a complex of strains, many of which are recombinants. The practical importance of PVY recombinant strains has increased due to their ability to induce potato tuber necrotic ring spot disease (PTNRD) that seriously affects tuber quality. In Saudi Arabia, potato production has increased fivefold during the last three decades, reaching 460,000 tons per year. Although PVY has been reported as one of the main viruses affecting potatoes, no information is available on PVY strains circulating in the country. In August 2014, a survey was conducted in a seed potato field at Al-Jouf, Saudi Arabia. PVY-positive samples selected based on visual symptoms and serological reactivity were subjected to strain typing using multiplex RT-PCR assays and were determined to represent recombinant PVY strains. Whole genome sequences were determined for two

representative isolates, S2 and S9, through direct sequencing of a series of overlapping RT-PCR fragments for each isolate, and found to represent strains PVY-NE11 and PVY^Z (SYR-III), respectively. One of the recombinant types, SYR-III, was previously found in nearby Syria and Jordan, but the second recombinant, PVY-NE11, was found before only in the United States. Both recombinants, PVY-NE11 and SYR-III, were previously found associated with PTNRD and thought to be rare. The current identification of PVY-NE11 and SYR-III in seed potato in a new geographic region suggests that these recombinants may not be as rare as previously believed. This is the first report on the occurrence of recombinant strains of PVY in potato in Saudi Arabia, and the first report on the PVY-NE11 strain of PVY found in potato outside of the United States.

In Saudi Arabia, potato production is carried out under irrigation in two growing seasons, the spring and the fall. Potato is produced for fresh market and for processing into chips. Seed potatoes for the spring growing season ("Elite Class") are imported from several European suppliers located in the Netherlands, the United Kingdom, and France. Potato production of the spring growing season that is certified as seed potato is planted in the following fall season to produce "A Class" seed potatoes. In 2013, the main potato producing areas in Saudi Arabia were Al-Jouf, Hail, Tabuk, Qasim, Riyadh, Hofuf, and Najran (Fig. 1), with a total production area of 17,500 ha and a total production of 460,000 tons per year of which 45,500 tons were seed potatoes (FAOSTAT 2015).

Potato virus Y (PVY) is the type species of the genus *Potyvirus*, family *Potyviridae*, the second largest family of plant viruses (Adams et al. 2012). PVY is the most economically important virus infecting potato crops worldwide, affecting both tuber yield and quality all over the world (Gray et al. 2010; Karasev and Gray 2013; Scholthof et al. 2011; Singh et al. 2008). It is also associated with major diseases in other crops, like pepper and tomato (Kerlan and Moury 2008). PVY exists as a complex of strains and variants that produce a range of symptoms in different hosts (Karasev and Gray 2013; Singh et al. 2008). Potato tuber necrotic ringspot disease (PTNRD) was found to be associated predominantly with recombinant PVY strains (Beczner et al. 1984; LeRomancer et al. 1994; Lorenzen et al. 2008), although it was later demonstrated to be caused by other, non-recombinant strains as well (Gray et al. 2010). Because of PTNRD and its effect on tuber quality, recombinant strains of PVY attracted significant attention, and their incidence and geographical distribution in potato producing areas have been of concern in the last few years

(Anfoka et al. 2014; Boukhris-Bouhachem et al. 2008; Chikh Ali et al. 2010a; Djilani-Khouadja et al. 2010; Galvino-Costa et al. 2012a, b; Gray et al. 2010; Lorenzen et al. 2006a).

PVY have been classified into PVY^O, PVY^C, PVY^N, PVY^Z, and PVY^E strains, based on reactions in potato cultivars carrying the resistance genes *Ny_{1br}*, *Nc_{1br}*, and *Nz_{1br}*, and in tobacco (*Nicotiana tabacum* L.; Chikh-Ali et al. 2014; Jones 1990; Karasev and Gray 2013; Kerlan et al. 1999; Singh et al. 2008). In the most recent classification, PVY isolates were divided into nine strains, PVY^O, PVY^C, PVY^N, PVY^Z, PVY^E, PVY^{N-Wi}, PVY^{N-O}, PVY^{NA-N}, and PVY-NE11 (Karasev and Gray 2013). PVY^Z, PVY^E, PVY^{N-Wi}, PVY^{N-O}, and PVY-NE11 are recombinant strains with different recombinant structures and parental sequences represented most often by PVY^O and PVY^N, but sometimes by unknown parents, like PVY-NE11 (reviewed in Karasev and Gray 2013). The strain PVY^Z, which triggers the resistance gene *Nz_{1br}*, comprises several diverse recombinants (Karasev and Gray 2013) designated as PVY^Z-NTN (Kerlan et al. 2011) and PVY^{NTN-NW} (Chikh Ali et al. 2010a); PVY^{NTN-NW} recombinant, in turn, was further divided into three subtypes, SYR-I, SYR-II, and SYR-III (Chikh Ali et al. 2010a). All the recombinant types compiled into the PVY^Z strain are composed of PVY^O and PVY^N parental sequences, and have two main conserved recombinant junctions (RJs), RJ2 and RJ3; they differ, however, in the presence and positions of other recombinant junctions (see Karasev and Gray 2013). Most of the recombinants of PVY, including PVY^Z-NTN, PVY^E, PVY^{NTN-NW}, and PVY-NE11, caused PTNRD (Chikh Ali et al. 2010a; Galvino-Costa et al. 2012a; Karasev and Gray 2013; Kerlan et al. 2011; Lorenzen et al. 2008).

In the past, surveys reported PVY as one of the main viruses infecting potatoes in Saudi Arabia (Al-Saikhan et al. 2014; Al-Shahwan et al. 1997; Sabir 2012). For instance, Al-Shahwan et al. (1997) detected PVY in 20 to 63% of potato samples collected from two main potato production areas in the north of Saudi Arabia, Tabuk and Hail, from both spring and fall growing seasons. However, PVY strain composition in potato production areas has not been studied in Saudi Arabia. In neighboring countries, with similar potato production cycles, PVY population structure was studied in Syria and Jordan,

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where recombinants such as PVY^{NTN-NW} (SYR-I, -II, and -III), PVY^{NTN}, and PVY^{N-Wi} were reported as dominant (Anfoka et al. 2014; Chikh Ali et al. 2010a). In Tunisia, a nearby Mediterranean country, PVY population was reported to be dominated by PVY^{NTN} with few exceptions belonging to PVY^N and PVY^O (Boukhris-Bouhachem et al. 2008; Djilani-Khouadja et al. 2010). Here, we report on the identification and characterization of nine recombinant PVY isolates from Saudi Arabia, and, for the first time, on the occurrence of significant recombinants of PVY, namely PVY-NE11 and SYR-III, in the country.

Material and Methods

Sample collection. A 25-ha seed potato field planted with the potato cultivar Hermes was sampled in August 2014, in the beginning of the fall growing season. The field was located near Al Jouf, Saudi Arabia, and belonged to the Al Jouf Agricultural Development Co. Plants showing virus-like symptoms of mosaic and crinkling were initially tested with the general PVY immuno-strip test (Agdia, Elkhart, IN) and PVY-positive samples were printed on FTA Classic Cards (Whatman, United Kingdom) according to the manufacturer's instructions. The cards were dried at room temperature for 1 h, sealed in paper envelopes with silica gel, and shipped to the University of Idaho laboratory for further analysis.

RNA extraction, RT-PCR, and sequencing. RNA was extracted from FTA cards according to the method of Ndunguru et al. (2005). RNA pellets were dissolved in 30 μ l nuclease-free water, heated at 65°C for 5 min, and then chilled on ice for 3 min. Reverse transcription (RT) was done according to the method of Chikh-Ali et al. (2013). For strain identification, two different multiplex RT-PCR assays reported by Lorenzen et al. (2006b) and Chikh Ali et al. (2010b) were performed with a slight modification. The multiplex RT-PCR (Chikh Ali et al. 2010b) was modified by replacing the primer n7577 with a new primer (n7892: 5'-CTCAACTCCAGATGGAA CAATTGTC-3'). This resulted in two shorter PCR products, 992 and 761 bp, replacing the 1,307 and 1,076 bp products, respectively, in the original protocol (Chikh Ali et al. 2010a). Control isolates representing nine strains of PVY and additional recombinant variants were from the laboratory collection (Chikh-Ali et al. 2013).

Two of the nine samples, S2 and S9, were selected for whole genome sequencing. The whole genome sequencing was performed directly on a series of overlapping PCR fragments amplified on cDNA synthesized from the S2 and S9 samples using SYR-III-L4 and NE-11 specific primers, respectively, as described in Karasev et al. (2011). Successfully amplified PCR products were treated with Exosap-It (Affymetrix, Cleveland, OH) and submitted for Sanger sequencing to Genewiz, Inc. (South Plainfield, NJ). Individual sequence reads were assembled using the SeqMan program of the Lasergene 9 Suite (DNASTAR). The whole genome sequences for isolates S2 and S9 have been deposited in the GenBank database under the accession numbers KP793715 and KP793716, respectively.

Sequence analysis. Sequence analysis was carried out using the SeqMan Pro software (DNA Star; DNASTAR, Madison, WI). For the multiple alignment, the program CLUSTAL X ver. 1.81 (Thompson et al. 1997) was used with the default parameters. Sequence identity was checked using the BLAST program provided by the National Center for Biotechnology Information (NCBI). Alignments were conducted based on the whole genome of representative isolates of PVY strains and variants: PVY^N, PVY^{NA-N}, PVY^{N-Wi}, PVY^{N-O}, PVY^{NTN}, PVY-NE11, PVY^{NTN-NW} (SYR-I, -II, and -III), PVY^O, and PVY^C. The aligned sequences were checked for recombination, using RDP (Martin and Rybicki 2000), GENECONV (Sawyer 1999), BOOTSCAN (Salminen et al. 1995), MAXCHI (Maynard-Smith 1992), CHIMAERA (Posada and Crandall 2001), 3Seq (Boni et al. 2007), and SiScan (Gibbs et al. 2000) methods in RDP4 Beta3 software (Martin et al. 2005). The analyses in RDP4 Beta3 package were done using default settings and a Bonferroni-corrected *P* value cut off of 0.05. Only recombination points detected by more than four methods in the RDP4 Beta3 program were taken into consideration. Phylogeny inference was conducted using the neighbor joining (NJ) method implemented in the program MEGA version 4 (Tamura et al.

2007) with 1,000 bootstrap replicates. Branches with bootstrap values less than 70% were collapsed.

Results

A seed field of potato cv. Hermes was visually assessed for mosaic symptoms, and estimated to have ca. 3 to 5% of symptomatic plants. Fifteen leaf samples were collected exhibiting various degrees of mosaic and crinkling, and subjected to the general PVY testing using the PVY-specific immunostrips (Agdia). Nine samples were found PVY-positive, producing two distinct bands on the test strip, and these samples were applied to the FTA Classic Cards (Whatman) for further characterization to strain.

RT-PCR assay. When all nine PVY-positive samples were subjected to the multiplex RT-PCR assay developed by Lorenzen et al. (2006b), six samples produced two bands of 181 and 452 bp, one sample produced a single band of 328 bp, and two samples produced a single band of 181 bp, suggesting PVY^{NTN} isolates in six samples and PVY^{NA-N} in one (summarized in Table 1). In the multiplex PCR assay of Chikh Ali et al. (2010b), all samples produced PVY-specific bands (Fig. 2): three samples produced a band of 441 bp, two samples produced three bands of 278, 441, and 761 bp, and three samples produced two bands of 278 and 441 bp. A single 633-bp band was amplified from one sample (summarized in Table 1). Hence, all nine samples were determined to contain recombinant isolates of PVY—five were identified as SYR-III type, one as a NE-11 type, and three as possible SYR, N-Wi, or NTN type based on the inconclusive band pattern revealed in the Chikh-Ali et al. (2013) assay.

Sequencing and sequence analysis. Two samples, S2 (typed as SYR-III) and S9 (typed as PVY-NE11) were selected for whole genome sequencing as representative isolates. The sequences of their nearly complete genomes were determined by direct sequencing of overlapping RT-PCR fragments, and were found to be 9,511 nt for S2 and 9,536 nt for S9, excluding poly(A) and the extreme 5'-terminal sequences. Both genomes coded for an open reading frame of 3,061 amino acids. S2 shared the highest identity (99.4%) with the isolate SYR-III-L4 (AB461454) reported from Syria, and 98.4% with SYR-II-DrH (AB461453) also reported from Syria (Chikh Ali

Fig. 1. Map of Saudi Arabia showing the location of Al-Jouf (marked with a rectangle) where PVY isolates were collected. Red circles with corresponding names refer to main potato production areas in Saudi Arabia.

Table 1. Strain identification of *Potato virus Y* (PVY) isolates collected from a potato field in Saudi Arabia using multiplex RT-PCR assays^a

Sample	PCR ^b amplicons	ID-Lorenzen ^b	PCR ^c amplicons	ID-Chikh-Ali ^c	Final ID	Recombination sites (nucleotide position)
S1	181/452	NTN	278/441	SYR-III?	SYR-III	P1(686), P3 (2415), VPg (5833), NIb (8426)
S2	181/452	NTN	278/441/761	SYR-III	SYR-III	
S3	181/452	NTN	441	?	NTN/SYR	
S4	181/452	NTN	278/441	SYR-III?	SYR-III	
S5	181/452	NTN	278/441/761	SYR-III	SYR-III	
S6	181	?	441	?	?	HC-Pro (2009), P3 (2489)
S7	181/452	NTN	278/441	SYR-III?	SYR-III	
S8	181	?	441	?	?	
S9	328	NA-N	633	NE11	NE11	
NE-11	328	NA-N	633	NE11	NE11	
SYR-III-1	181/452	NTN	278/441/761	SYR-III	SYR-III	P1(686), P3 (2415), VPg (5833), NIb (8426)

^a NE-11 and SYR-III-1 are reference isolates of PVY strains PVY-NE11 and SYR-III, respectively, which are maintained with PVY isolate collection of the University of Idaho.

^b According to Lorenzen et al. (2006b).

^c According to Chikh Ali et al. (2010b and 2013).

Fig. 2. RT-PCR assays for the identification of PVY isolates collected from a potato field from Saudi Arabia. Nine individual PVY-positive samples selected based on immunostrip reactivity are labeled S1 through S9. **A**, results of multiplex RT-PCR assay according to Lorenzen et al. (2006b). **B**, results of multiplex RT-PCR assay according to Chikh Ali et al. (2010b). Positions and sizes of the characteristic PCR products are indicated; M indicates the lane for size markers.

et al. 2010a). Isolate S9 shared the highest identity (99.8%) with the isolate NE-11 reported from the United States (DQ157180; Lorenzen et al. 2008) and with the *Kalimeris indica* isolate ME-162 reported from China (JQ971975; Wang et al. 2012) followed by 98.4% identity to the isolate ID20 reported from the United States (HQ912867; Karasev et al. 2011).

In the recombination analysis, S2 and S9 had identical recombination structures with isolates SYR-III-L4 and NE-11, respectively (Chikh Ali et al. 2010a; Lorenzen et al. 2008; see Fig. 3). The first recombination event in S2 (686-2415, Fig. 3) was detected by *P* values using RDP (1.679×10^{-120}), GENECONV (2.876×10^{-113}), BootScan (1.195×10^{-119}), MaxChi (M; 1.026×10^{-27}), Chimaera (1.274×10^{-24}), 3SEquation (1.264×10^{-215}), and SiScan (5.361×10^{-49}). The second recombination event in S2 (5833-8426; Fig. 3) was detected by *P* values using RDP (2.938×10^{-129}), GENECONV (4.907×10^{-115}), BootScan (6.852×10^{-126}), MaxChi (9.682×10^{-48}), Chimaera (7.517×10^{-40}), 3SEquation (3.346×10^{-227}), and SiScan (1.218×10^{-54}). S9 had a single recombination event (2009-2489; Fig. 3), which was detected by *P* values using RDP (1.232×10^{-10}), GENECONV (7.649×10^{-08}), MaxChi (6.003×10^{-06}), 3SEquation (8.426×10^{-12}), and SiScan (1.029×10^{-03}). The genome of S2 had O and N sequences identified as parents, while S9 had N and NA-N as parents for the first 2,489 nt of the genome and the rest of the genome originated from an unknown parent. When subjected to phylogenetic analysis together with representatives from major recombinant and non-recombinant lineages of PVY strains, S2 grouped closely with SYR-III-L4 while S9 was grouped closely to NE-11 and was placed in an “NE-11” subcluster at an intermediate position between N and NA-N (Fig. 4).

Discussion

Potato production in Saudi Arabia has increased from about 90,000 tons in 1990 (Al-Shahwan et al. 1997) to about 460,000 tons in 2013 (FAOSTAT 2015). With the continuous import of seed potatoes and the dramatic increase of potato production, the importance of potato virus diseases is also expected to increase. Several surveys reported PVY as the main virus infecting potatoes in Saudi Arabia (Al-Saikhhan et al. 2014; Al-Shahwan et al. 1997; Sabir 2012), yet no identification or characterization of PVY strains in the country

have been reported. Several partial sequences of the capsid protein (CP) area were reported for a few PVY isolates (Al-Saikhhan et al. 2014; Sabir 2012), but these were not sufficient to determine the strain type due to the complex recombination structure of PVY strains (Karasev and Gray 2013). In the current study, two PVY recombinant types, SYR-III and PVY-NE11, were reported for the first time from Saudi Arabia. SYR-III is a recombinant of PVY with four RJs (Fig. 3) and shared properties with PVY^{NTN} and PVY^{N-Wi} (Chikh Ali et al. 2010a). Importantly, it was able to induce PTNRD in susceptible potato cultivars (like PVY^{NTN}), but unlike PVY^{NTN}, was found to have O-type serology and thus could have been erroneously typed as PVY^O. SYR-III is one of the most common PVY recombinants in Syria (Chikh Ali et al. 2010a) and was recently found in Jordan (Anfoka et al. 2014). When S2 sequence was subjected to a BLASTN search through the GenBank database, an additional closely related (98%) unpublished sequence KC903166 was found, representing a partial, 1.7-kb sequence from a tomato PVY isolate collected in 2009 in Iraq. The current report is adding Saudi Arabia to the geographic regions where SYR-III is found.

The origin of this S2 (SYR-III recombinant) isolate is unclear: Al-Jouf is separated from Syria by a desert, and no potato trade existed between the two countries for at least the last 10 years. There is also no potato seed trade with Jordan or Iraq, and thus, neither Jordan nor Iraq would be likely sources of the SYR-III recombinant of PVY. Considering the fact that all these countries are importing seed potatoes from other regions, SYR-III might have been introduced via imported seed tubers, which would imply a much wider distribution than had been reported so far. In the particular case of Saudi Arabia, it was established that seed potatoes were sourced from Europe. Hence it is plausible to assume that strains found in the present study were introduced from Europe given the lack of trade with neighboring countries.

The second Saudi isolate sequenced, S9, was identified as a PVY-NE11 recombinant using multiplex RT-PCR and sequence analysis of the whole genome. Since it was first reported in the United States (Lorenzen et al. 2008), the incidence of PVY-NE11 had been increasing (Gray et al. 2010; Karasev et al. 2011; Karasev et al. unpublished data). Outside of the United States, a PVY isolate was described from *Kalimeris indica* in China with sequence sharing high identity to the

Fig. 3. Recombinant structure of S2 and S9 isolates of PVY as determined based on nucleotide sequence analysis. Rectangles designate virus genomes, and shading corresponds to the relatedness to the parental sequences, PVY^O, PVY^N, PVY^{NA-N}, and “unknown,” indicated below the diagram. The PVY genome organization is presented at the top; individual cistrons are listed, with vertical lines corresponding to polyprotein cleavage sites. The two sequenced isolates, S2 and S9, are highlighted in bold; the two reference recombinant structures, SYR-III and NE-11, are italicized.

isolate NE-11, though it was not originally identified as a PVY-NE11 strain (GenBank accession number JQ971975; Wang et al. 2012). Thus, S9 represents the only isolate of PVY-NE11 strain found outside of the United States or China, and the first PVY-NE11 isolate found in potato anywhere outside the United States. The current finding of an NE-11 strain in Saudi Arabia also suggests that PVY-NE11 might have a wider distribution than was reported so far. PVY-NE11 has two RJs resulting in a recombinant genome with the first 2,000 nt belonging to the PVY^N strain followed by a stretch of about 696 nt of the PVY^{NA-N} strain, and the rest of the genome coming from an unknown parent of an intermediate evolutionary position between N

and NA-N (Lorenzen et al. 2008; Fig. 2A and B). PVY-NE11 is also able to induce PTNRD (Gray et al. 2010; Lorenzen et al. 2008; Piche et al. 2004; our unpublished data). Although dry samples on the FTA cards would not allow probing the serological properties of Saudi isolates S9 (PVY-NE11) and S2 (SYR-III), the sequence analysis of the S2 and S9 genomes enabled us to predict the serotypes of these two isolates such that S2 would have a O-serotype and S9 would have an N-serotype (Chikh-Ali et al. 2010a; Nikolaeva et al. 2012).

In conclusion, this is the first report on PVY strain typing in Saudi Arabia that revealed the existence of two significant recombinants, SYR-III and PVY-NE11. The finding of PVY-NE11 and SYR-III

Fig. 4. Phylogenetic analysis of nucleotide sequences of the whole genomes for representative PVY isolates conducted with the neighbor joining (NJ) algorithm. Only nodes with bootstrap values higher than 70% were retained. Brackets designate individual strains and variants of PVY according to Karasev and Gray (2013); red brackets indicate clades where S2 and S9 sequences are placed. Red colored font designates the S2 and S9 sequences paired with the closest reference sequences. Isolate name was followed by a corresponding accession number in the GenBank database.

in a new geographic area of the Middle East suggests a broader distribution of these PVY strains than thought before (Chikh Ali et al. 2010a; Lorenzen et al. 2008). It indicates that potato producers in Saudi Arabia need to be aware of the presence of these two strains in their potato production system, since both of these strains of PVY have been previously implicated in induction of PTNRD in susceptible cultivars. It also suggests that potato seed certification agencies in countries importing seed potato should be more vigilant even toward reputable suppliers of potato seed that may inadvertently introduce PVY recombinants novel to the area.

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