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Laboratory of Tropical Plant Protection, Department of International Agricultural Development, Tokyo University of Agriculture, Tokyo, Japan

Whole Genome Sequence and Characterization of a Novel Isolate of PVY Inducing Tuber Necrotic Ringspot in Potato and Leaf Mosaic in Tobacco

M. Chikh Ali[1,2], T. Maoka[3] and K. T. Natsuaki[1]

Authors' addresses: [1]Graduate School of Agriculture, Department of International Agricultural Development, Tokyo University of Agriculture, Tokyo, Japan; [2]General Organization for Seed Multiplication (GOSM), Aleppo, Syria; [3]National Agricultural Research Center for Hokkaido Region (NARCH), Sapporo, Japan (correspondence to M. Chikh Ali. E-mail: 70060002@nodai.ac.jp)

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Abstract

In a previous study on a Syrian isolate of *Potato virus Y* (PVY), namely PVY-12, a point mutation in the coat protein (CP) was detected. This mutation caused the double reactivity of this isolate to monoclonal antibodies specific to O and N serotypes. We report here the biological and molecular characteristics of PVY-12. In potato, PVY-12 behaved like a PVY^{NTN} isolate inducing potato tuber necrotic ringspot disease although it induced mosaic in tobacco like PVY^{O}. The genomic analysis grouped PVY-12 with the recombinant PVY^{NTN} isolates, which is consistent with the phenotype in potato. PVY-12 HC-Pro had the two amino acids K400 and E419 that were previously reported as determinant keys of the tobacco necrotic response. This indicates the involvement of other determinants in this phenotype yet to be determined. This is the first report on a PVY^{NTN} isolate that induces mosaic in tobacco, implying that the induction of potato tuber necrosis does not require the ability to induce the tobacco necrosis. PVY-12 genome had four recombinant points in the P1, HC-Pro/P3, 6K2/NIa and C terminal region of the CP gene identical to those of PVY^{NTN} isolates 12–94 and 34/01. The PVY-12 central genomic part flanked by nucleotide positions 2414 and 8604 had highest similarity with that of the Syrian isolate SYR-NB-16 suggesting a common origin of these isolates. This common origin was supported using the phylogenetic analysis of this region. In addition, the phylogenetic analysis of the whole genome of the reported North American $PVY^{N:O}$ and the European $PVY^{N}W$ along with other PVY isolates suggests that $PVY^{N:O}$ might have descended from $PVY^{N}W$ with the isolate SASA-207 as a nearest-known relative.

Introduction

Potato virus Y (PVY), the type-member of the genus *Potyvirus* (family *Potyviridae*) has a single-stranded positive-sense genomic RNA of *c*. 9.7 kb (Riechmann et al., 1992; Fauquet et al., 2005). Potato isolates of PVY can be differentiated into two main serotypes: the O serotype, which includes PVY^{O}, PVY^{C} and $PVY^{N}W$ (designated as $PVY^{N:O}$ in North America) and the N serotype that includes PVY^{N} and PVY^{NTN} (de Bokx and Huttinga, 1981; Van den Heuvel et al., 1994; McDonald and Singh, 1996a,b; Chrzanowska and Doroszewska, 1997; Ounouna et al., 2002). PVY has two distinguished phenotypes in tobacco, mosaic that is characteristic of isolates of PVY^{O} and PVY^{C} and veinal necrosis caused by PVY^{N}, PVY^{NTN}, $PVY^{N}W$, and $PVY^{N:O}$ (de Bokx and Huttinga, 1981; Le Romancer et al., 1994; Van den Heuvel et al., 1994; McDonald and Singh, 1996a,b; Chrzanowska and Doroszewska, 1997; Blanco-Urgoiti et al., 1998; Kerlan et al., 1999; Piche et al., 2004; Glais et al., 2005). The tobacco necrosis response is determined by two amino acids K400 and E419 of the HC-Pro cistron of PVY (Jacquot et al., 2005; Tribodet et al., 2005). However, recently reported isolates of $PVY^{N}W$ and NA-$PVY^{N/NTN}$ were unable to induce necrosis in tobacco even though these have the K/E amino acid motifs (Schubert et al., 2007).

PVY^{NTN} is a new variant of PVY^{N} strain with regard to the serotype and pathotype in tobacco. However, unlike PVY^{N}, PVY^{NTN} induces the potato tuber necrotic ringspot disease (PTNRD). The majority of PVY^{NTN} isolates have recombinant genomes (Boonham et al., 2002; Glais et al., 2002; Lorenzen et al., 2006; Schubert et al., 2007). Nonetheless, non-recombinant PVY^{NTN} isolates have also been reported (Nie and Singh, 2003).

$PVY^{N}W$ and $PVY^{N:O}$ are other variants of PVY which have O serotype but induce veinal necrosis in tobacco like PVY^{N} (McDonald and Singh, 1996a; Chrzanowska and Doroszewska, 1997; Blanco-Urgoiti et al., 1998; Kerlan et al., 1999). $PVY^{N}W$ and $PVY^{N:O}$ isolates have also undergone recombination events in their genomes at HC-Pro/P3 resulting in a genomic segment including 5′NTR, P1 and HC-Pro genes which belong to PVY^{N} while the rest of their genome belongs to PVY^{O} (Glais et al., 2002). Some $PVY^{N}W$ isolates had another recombination in the P1 gene (Glais et al., 2002; Schubert et al., 2007). Several isolates of $PVY^{N:O}$ have been reported to induce atypical necrosis in potato tubers (Piche et al., 2004; Lorenzen et al., 2006; Schubert et al., 2007).

More recently, new isolates of PVY with variable recombination patterns in the genome were reported from Poland and Germany (Schubert et al., 2007) and Syria (Chikh Ali et al., 2007a).

The emergence of these new variants or isolates that do not fit into the classical classification of PVY strains, and the continuous increase in their incidence (Nie and Singh, 2003; Glais et al., 2005) make it necessary to reclassify PVY strains considering these new variants or isolates. Such reclassification requires intensive studies on PVY populations in different potato production areas and characterization of the new PVY isolates. Such studies have been reported from many potato production areas in the world (Le Romancer et al., 1994; Chrzanowska and Doroszewska, 1997; Blanco-Urgoiti et al., 1998; Kerlan et al., 1999; Ohshima et al., 2000; Boonham et al., 2002; Piche et al., 2004; Baldauf et al., 2006; Lorenzen et al., 2006; Ogawa et al., 2007; Schubert et al., 2007). These studies help to estimate the dynamics of PVY populations and determine the new isolates that have new characteristics and genomic structures (for details see Schubert et al., 2007).

The PVY population in Syria is being studied and typical $PVY^{N}W$ isolates were reported in addition to new PVY isolates, provisionally referred to as PVY^{SYR} (Chikh Ali et al., 2007a). SYR-NB-16, the representative isolate of PVY^{SYR}, shared characteristics with both $PVY^{N}W$ and PVY^{NTN} due to the recombination between genomes of $PVY^{N}W$ and PVY^{NTN} ancestor isolates (Chikh Ali et al., 2007a). In another study on PVY from Syria, the serological properties of a mutant isolate of PVY, referred to as PVY-12, were reported (Chikh Ali et al., 2007b). This isolate reacted with two monoclonal antibodies specific to the N and O serotypes due to a point mutation in its CP (Chikh Ali et al., 2007b). Moreover, PVY-12 had a unique combination of biological properties as it belonged to PVY^{NTN} according to its phenotype in potato and to PVY^{O} according to its symptoms in tobacco. We report here the biological and molecular characterization of this isolate including its complete genome sequence.

Materials and Methods

Virus isolate

PVY-12 was collected from a potato plant derived from a symptomless potato tuber (Chikh Ali et al., 2007b). Secondarily infected plants showed mosaic and mild stipple streak symptoms under greenhouse conditions.

Biological characterization

PVY-12 was maintained in *Nicotiana tabacum* cvs Samsun, Xanthi and White Burley and inoculated onto plants of six potato cultivars (Shimabara, Nishiyutaka, Pentland Crown, Desiree, Maris Bard and King Edward). Inoculated plants were grown in a non-air-conditioned greenhouse or in an incubator at 20–23°C with a 14-h photoperiod. Symptom observation started 3 days after inoculation and was carried out daily for 1 month. Harvested potato tubers were kept at room temperature and checked daily for necrotic symptoms for 6 weeks.

RNA extraction

Total RNA was extracted from infected fresh tobacco leaves (cv. Samsun) or purified virions using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Generation and sequencing of RT-PCR products

Nucleic acid sequencing was carried out according to Chikh Ali et al. (2007a). To determine the full nucleotide sequences of the 5′ and 3′ termini, a BD SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) was used according to the manufacturers' instructions. For this purpose, total RNA was extracted from purified virions. RACE PCR was carried out using two primers FY-1 (5′-GGATGATTCATCGATTAGGTGATGTTGC-3′) and RY-1 (5′-GAAACAGATTGTTGACATGTAAGTTGCC-3′) for 5′ and for 3′ termini, respectively, and universal primer mix provided by the manufacturer (Clontech).

Sequence analysis

Sequence analysis was carried out using the DNASIS software (Hitachi Software Engineering Co., Tokyo, Japan). The program SISCAN Version 2 (Gibbs et al., 2000) was used to detect the recombination events and assess their significance. The sequence identification of PVY-12 was compared with other PVY isolates available at the GenBank using a BLAST program provided by the NCBI. For the multiple alignment, the program CLUSTALX version 1.81 (Thompson et al., 1997) was used with the default parameters. Phylogeny inference was conducted using the neighbour joining (NJ) and maximum likelihood (ML) methods. The NJ trees were calculated with 1000 bootstrap replicates using the neighbour joining option in CLUSTALX. The ML analysis was performed using the program TREE-PUZZLE version 5.0 (Strimmer and von Haeseler, 1996; Strimmer et al., 1997) at 1000 puzzling steps. Cladograms were

displayed using TREEVIEW (Page, 1996). An isolate of *Pepper mottle virus* was used as an outgroup. Four trees were constructed based on genomic segments, 37–495, 650–2170, 2415–5715 and 2415–9679 (numbered according to PVY-12 genome). The PVY-12 nucleotide sequence and those of all PVY isolates with known full nucleotide sequences in the GenBank were used. Isolate names and GenBank accession numbers are shown in Fig. 2a.

Results

Biological characterization

PVY-12 induced mosaic and mottle in tobacco cvs Samsun, Xanthi and White Burley but necrosis was not observed, although this test was repeated continuously for more than 2 years. In general, potato symptoms were severe (Table 1). Mosaic and necrotic reactions were found in all cultivars with varying severity levels according to the cultivar (Table 1). Tuber necrosis developed in tubers of cvs Nishiyutaka, Pentland Crown and Desiree. Tuber symptoms appeared as superficial pink arcs and rings, often around the eyes, initially protruding from tuber surfaces which later became sunken and necrotic (Fig. 1).

Nucleotide sequencing and sequence analysis

The full genomic RNA of PVY-12 (excluding the Poly A trace) was 9698 nucleotides that encoded a polyprotein precursor of 3061 amino acids. Sequence data of PVY-12 have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (accession number AB185833).

Analysis of the full nucleotide sequence of PVY-12 using the program SISCAN with two isolates O-139 and N-605 represent PVY^{O} and PVY^{N} strains, respectively, as parental isolates revealed that PVY-12 genome has undergone four recombinant points at nucleotide positions 496, 2414, 5833 and 9178 with Z values > 3, which resulted in a genomic RNA with five heterogeneous segments, numbered from no. 1 through 5.

The complete nucleotide sequence of PVY-12 shared highest identity with those of PVY^{NTN} isolates 12–94 (99.03%) and 34/01 (99.02%). PVY-12 segment no.1 had highest similarity of 99.19% with that of Isol5 and Gr99. The segment no. 2 showed highest similarity with that of a PVY^{NTN} isolate, v942490 (99.37% identical) followed by 99.32% identity with SYR-NB-16, Ditta, and 423-3. In the segment no. 3, PVY-12 genome had a highest similarity with SYR-NB-16 (99.6%) followed by PB312 (99.4%). PVY-12 shared a similarity of 99.4% with v942490, PB312, 423-3 in segment no 4. The segment no. 5 shared identity of 99.62% with PB312. As PVY-12 and SYR-NB-16 showed highest similarity in the segment no. 3, homology search was conducted for the homologous genomic segment from 2414 through 8604 (Chikh Ali et al.,

Table 1
Foliage and tuber symptoms caused by *Potato virus Y*-12 in six potato cultivars

	Desiree	Pentland Crown	Maris Bard	Nishiyutaka	Shimabara	King Edward
Foliage Symptoms	Mm, Sns, Lld	M, Sns	Sm, Tn, Lld, Ss, Sns	Vn, Sns	M, Sns	Mm, Ss, Lld, Sns
Tuber Symptoms	N	N	Sl	N	Sl	Sl

Mm, mild mosaic; Sm, severe mosaic; M, mosaic; Sns, systemic necrotic spots; Vn, necrotic streak on leaf veins and petioles; Lld, lower leaf dropping; Tn, necrotic spots on upper leaves; Ss, stipple streak, N, necrotic arcs and rings on the tubers; Sl, symptom-less.

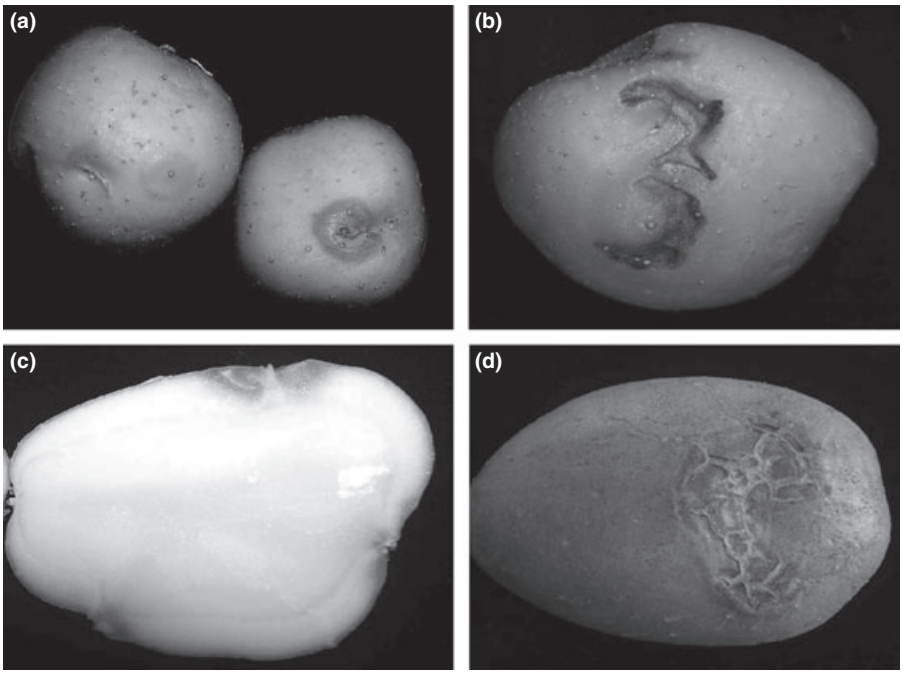

Fig. 1 Tuber necrosis caused by *Potato virus Y* -12; (a,b) tubers of cv. Nishiyutaka showing necrotic ringspots and arcs; (c) cross section in a tuber of cv. Nishiyutaka with superficial necrosis; (d) tuber of cv. Desiree showing necrotic arcs

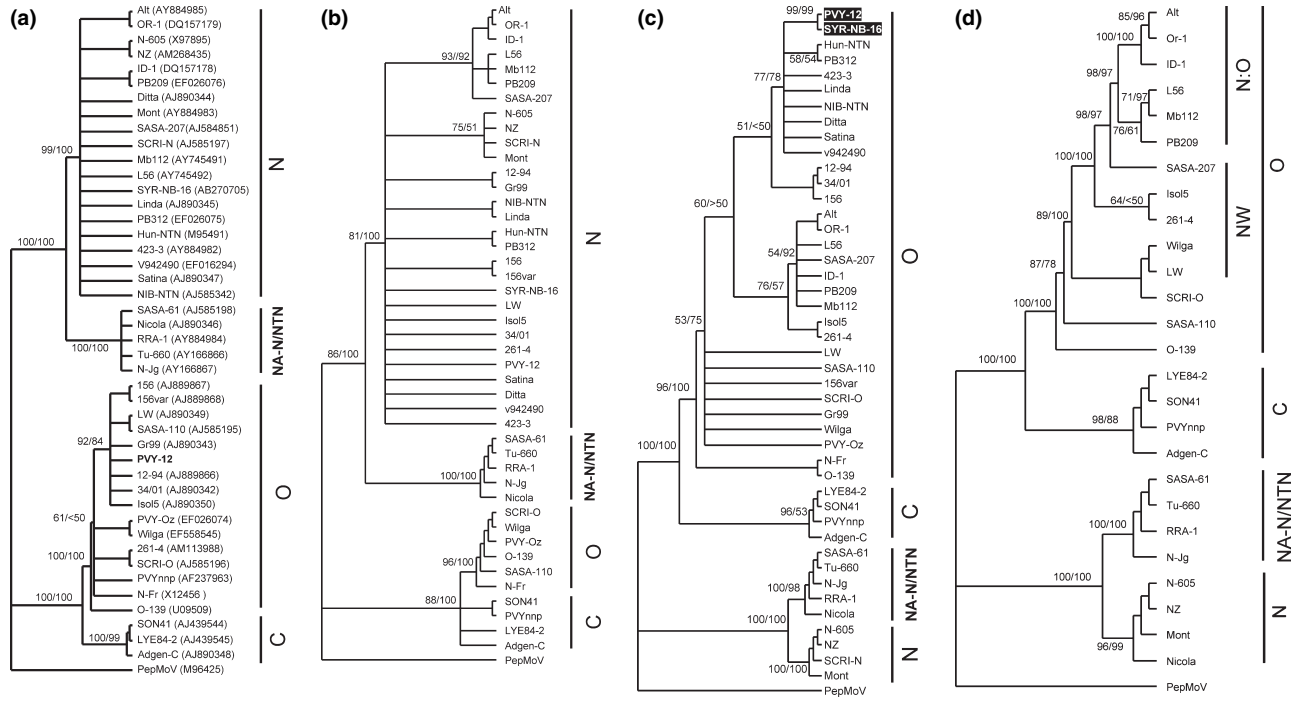

Fig. 2 Maximum likelihood cladograms of nucleotide sequences of *Potato virus Y* (PVY) isolates. Phylogenetic trees were constructed based on: (a) genomic segment from 37 to 495 of PVY isolates; (b) genomic segment from 650 to 2170; (c) genomic segment from 2415 to 5715; (d) genomic segment from 2415 to 9679 (numbered according to PVY-12 genome). Numbers at internal nodes indicate the percentage of supporting puzzling steps in the maximum likelihood and bootstrap values, respectively. Values higher than 50% were only shown. Numbers between brackets indicate the accession number of the corresponding isolate

2007a) of PVY-12 genome. PVY-12 central genomic part (2414–8604) shared highest similarity of 99.58% with that of SYR-NB-16 followed by those of the PVY^{NTN} isolates, PB312 (99.39%), v942490 (99.37%), 423-3 (99. 35%), NTN-Hun (99.34%), NIB-NTN (99.24%), Linda (99.18%), Satina (99.16%), 12–94 (99.08) and 34/01 (99%). No other sequences with similarities higher than 99% were found in the GenBank.

In the phylogenetic tree of genomic segment no. 1 (nucleotide position 37–495), PVY-12 sub-clustered with the O cluster along with isolates of PVY^{NTN} and $PVY^{N}W$ having a genomic breakpoint in the P1 gene (Fig. 2a; Schubert et al., 2007). In the phylogenetic tree of the nucleotide sequence 650–2170, PVY-12 fell into the N cluster along with the recombinant PVY^{NTN}, $PVY^{N}W$ and $PVY^{N:O}$ (Fig. 2b). PVY-12 and SYR-NB-16 grouped tightly in the O cluster along with the recombinant PVY^{NTN}, $PVY^{N}W$ and $PVY^{N:O}$ in the phylogenetic tree based on the genomic segment 2415–5715 (Fig. 2c). In the phylogenetic tree of the nucleotide sequence 2415–9679 of PVY isolates with no reported recombinant point within this segment, four main clusters were identified, O, C, N and NA-N/NTN (Fig. 2d). $PVY^{N:O}$ isolates reported from North America made a significant sub-cluster within the O cluster (Fig. 2d).

The putative HC-Pro of PVY-12 had the two amino acids K/400 and E/419 that were reported as determinant keys of tobacco necrotic response for PVY^{N} strain group (data not shown).

Discussion

According to its ability to induce PTNRD in potato, PVY-12 should be classified as a PVY^{NTN} isolate. On potato foliage, PVY-12 evoked severe symptoms which make isolates of the same type noticeable during the field inspection.

The PVY-12 genome had identical recombinant structure and shared highest similarity (>99%) with those of PVY^{NTN} isolates 12–94 and 34/01 (Schubert et al., 2007).

The recombinant point in PVY-12 P1 gene was previously reported for the isolate PVY^{N}WI-P (GenBank accession number AF248500) that represented a subgroup of $PVY^{N}W$ (Glais et al., 2002). Recently, this genomic breakpoint has been reported in many isolates of $PVY^{N}W$ and PVY^{NTN} including Isol5, 12–94, 34/01 and Gr99 (Schubert et al., 2007). Genomic segment no. 1 of PVY-12 shared highest identity with those of Isol5 and Gr99 (99.19%) and moreover they grouped significantly in a sub-cluster of the PVY^{O} cluster of the phylogenetic tree of the first 496 nucleotides (Fig. 2a), which suggests a common origin.

PVY-12 genomic segments nos. 2–5 (comprising of 9202 nt) showed highest homology with those of the recombinant PVY^{NTN} isolates particularly v942490, SYR-NB-16 and PB312. Interestingly, the central genomic part of PVY-12 that is flanked by nucleotide positions 2414 (the second recombinant point of PVY-12 genome) and 8604 (the third recombinant point of SYR-NB-16 genome; Chikh Ali et al., 2007a) shared

the highest similarity with that of SYR-NB-16 (99.6%) among all PVY isolates. This high similarity suggests a common origin of this genomic part for these isolates. The common origin of these isolates was supported by the phylogenetic analysis of that part of their genomes. In the phylogenetic tree of the first portion of this central genomic part (2415–5715), PVY-12 was closely related to SYR-NB-16 in which they grouped significantly in a single sub-cluster in the O cluster along with the recombinant PVY^{NTN}, $PVY^{N}W$ and $PVY^{N:O}$ isolates (Fig. 2c). The same was found for the second portion of this central genomic part flanked by the third recombinant point of PVY-12 genome and the third recombination of SYR-NB-16 genome (nucleotide position 5833 through 8604) (Chikh Ali et al., 2007a; data not shown). Considering that PVY-12 and SYR-NB-16 were collected at the same time from the same region, the conclusion of a common origin seems to be reasonable.

Based on the nucleotide sequence analysis, PVY-12 belonged to the recombinant PVY^{NTN} which is in agreement with the phenotype in potato. The highest similarity between PVY-12 and 12–94 and 34/01 seems to result from the similar genomic recombinant pattern rather than being closely related, as this highest overall similarity was not supported by the identity of the five genomic segments to which other isolates were more closely related to PVY-12 than 12–94 and 34/01. Thus, the parallel emergence of PVY-12 on one side and 12–94 and 34/01 on the other side cannot be excluded.

Recombination was not the only cause of PVY-12 evolution. The PVY-12 genome was subjected to mutation that resulted in novel characteristics. Unlike the recombinant PVY^{NTN}, PVY-12 reacted with PVY^{N} and PVY^{O} monoclonal antibodies due to a point mutation in the CP gene (Chikh Ali et al., 2007b). The same might be true for the pathotype in tobacco. While recombinant isolates of PVY^{NTN} induce veinal necrosis, PVY-12 induced mosaic in tobacco. This phenotype in tobacco has raised a question about the factor(s) that altered the tobacco necrotic reaction to PVY-12, as an isolate of PVY^{NTN} even though its HC-Pro had the amino acid motifs K400/E419. The same was reported for two isolates, LW and SASA-61, which belong to $PVY^{N}W$ and NA-$PVY^{N/NTN}$, respectively (Schubert et al., 2007). The recombinant genomic structure of PVY-12 is unlikely to affect this phenotype, because isolates of PVY^{NTN}, such as 12–94 and 34/01, with similar genomic structure to PVY-12 were reported (Schubert et al., 2007). Thus, the occurrence of one point mutation or more in PVY-12 genome that might have abolished its ability to induce tobacco veinal necrosis seems to be sensible. When the full genome and polyprotein of PVY-12, LW and SASA-61 were compared with those of the necrotic isolates, there were no consistent differences between these three isolates and others that could be found (data not shown), which means that the phenotypes of PVY-12, LW and SASA-61 in tobacco were altered by different factors and/or in different mechanisms. This is in agreement with the results of Bukovinszki et al. (2007) who reported that the symptom determinants may vary between different strains of the same virus species in a particular host plant.

When the PVY-12 genome was compared with all available PVY isolates of the N pathotype in tobacco (with known full nucleotide sequences), 14 nucleotide substitutions were unique to PVY-12 genome. Eleven were within the encoded region, and among them four substitutions at nucleotide positions 1826, 4971, 5560 and 7136 altered amino acid changes from M to V, P to H, A to V, and T to S, respectively. If the HC-Pro solely determines the tobacco necrotic response, the point mutation that replaced A1826 with G1826 of PVY-12 genome and resulted in one amino acid substitution from M/273 to V/273 of the HC-Pro would be the factor that abolished the ability of PVY-12 to induce the tobacco necrotic response. However, this conclusion is speculative and requires further confirmatory evidence. Moreover, amplification error during PCR cannot be excluded.

In the phylogenetic analysis, $PVY^{N:O}$ isolates reported from North America were closely related and always grouped together which support their common origin as was suggested by Lorenzen et al. (2006). However, our phylogenetic analysis, particularly based on nucleotide sequences from 2415 to 9679 of their genomes, suggests that the North American $PVY^{N:O}$ might have descended from the European $PVY^{N}W$ isolates with the isolate SASA-207 (from UK) as a nearest known sister (Fig. 2d).

In conclusion, PVY-12 is a mutant variant of the recombinant PVY^{NTN} which might have passed a selective pressure that caused these mutations. The continuous propagation in tobacco might be one of these evolutionary inducers. According to PVY-12 phenotypes in tobacco and potato, the induction of tuber necrosis does not require the ability to induce necrosis in tobacco. In addition, according to the phylogenetic analysis of the whole genome, the North American $PVY^{N:O}$ seems to have evolved from the European $PVY^{N}W$.

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