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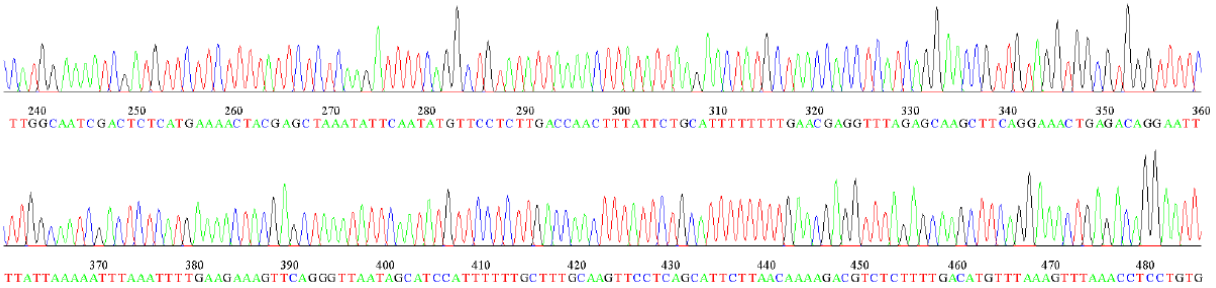
Sequence Analyses of Novel *Potato Virus Y*^{NTN} and Resistance-Breaking PVY^{O} Isolates from Sweden



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Sequence Analyses of Novel *Potato Virus* Y^{NTN} and Resistance-Breaking PVY^{O} Isolates from Sweden

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Popular Science

Potato virus Y (PVY) is a devastating potato pathogen affecting potato (*Solanum tuberosum*) production worldwide. PVY is a tuber-borne virus, and it is also spread mechanically and by aphid vectors to more than 40 species. PVY causes a wide range of symptoms on potato foliage ranging from symptomless or mild mosaic to defoliation and death. In addition, PVY causes potato tuber necrotic ringspot disease, and this greatly affects tuber marketability. Yield losses caused by PVY infection can be up to 80%, depending on infection incidence of tuber seeds, potato cultivar, PVY isolate, activity of aphid-vectors and environmental conditions. PVY displays a high genetic diversity and has the ability to evolve overtime and form new strains through crossing (recombination) between different strains and through mutations. In the last few decades, novel PVY recombinant genotypes have been described to induce severe symptoms in numerous potato cultivars. There are numerous risks associated with the emergence of new strains/variants: overcoming resistance sources, yield loss, reduction in tuber quality (due to potato tuber necrotic ringspot disease).

In Sweden, the ordinary PVY^{O} strain used to be most common, but it has been shown in a previous study to be replaced by numerous recombinant genotypes, *e.g*. the tuber necrosis-inducing PVY^{N} (PVY^{NTN}) strain, as has been reported for many European countries. In addition, the presence of resistance-breaking PVY^{O} isolates has been reported in Sweden in a previous study. Consequently, genome sequencing is required to confirm the presence of recombinant genotypes and to identify potentially new recombinants. Nearly complete genome sequence of one non-recombinant resistance-breaking PVY^{O} isolate was determined. The resistance-breaking PVY^{O} isolate was 99% identical with the ordinary PVY^{O} strain. We also determined nearly complete genome sequences of three recombinant PVY^{NTN} isolates from Sweden. The three Swedish PVY^{NTN} isolates were 99% identical with numerous PVY^{NTN} isolates from Europe. These findings confirm the presence of the PVY^{NTN} strain, which is mainly associated with potato tuber necrotic ringspot disease, in Sweden.

Abstract

Potato virus Y (PVY) is a devastating potato pathogen affecting potato (*Solanum tuberosum*) production worldwide. In the last few decades, novel PVY recombinant genotypes have been described to induce severe symptoms in numerous potato cultivars. This study aimed to determine complete and partial genome sequences of some Swedish PVY isolates with novel biological properties. The complete coding sequence of one resistance-breaking PVY^{O} (O-MB) and three PVY^{NTN} isolates were determined, along with six partial genomes. Isolate O-MB shared an amino acid identity of 99% with the PVY^{O} and PVY^{Z} strains and was in phylogenetic group $PVY^{O/Z}$. O-MB differs from all studied isolates belonging to the PVY^{O} and PVY^{Z} strains by having a valine at position 90 in the HC-Pro protein, and from numerous PVY^{O} and PVY^{Z} isolates by having arginine at position 155 and glutamine at position 276 in the P3 protein. These amino acid residues may play a role in overcoming the resistance gene Ny_{tbr} in potato. In addition, the three Swedish PVY^{NTN} isolates shared an amino acid identity of 99% with isolates of the variant PVY^{NTN} (A) and were in phylogenetic group PVY^{NTN}. Three of the partial genome sequences shared an amino acid identity of 99 – 100% with PVY^{NTN} and PVY^{Z}-NTN and were in phylogenetic group PVY^{NTN}. These findings confirm the presence of the PVY^{NTN} strain in Sweden. Two of the partial genome sequences shared an amino acid identity of 100% with the PVY^{N}-W strain, and one sequence shared an amino acid identity of 99% with isolates of the PVY^{O}, PVY^{Z} and PVY^{N}-W strains. These three partial genome sequences were in phylogenetic group $PVY^{O/Z}$. Recombination analyses revealed that O-MB is a non-recombinant isolate, while the Swedish PVY^{NTN} isolates have three recombinant junctions located within the HC-Pro/P3, VPg and CP cistrons, which is similar to numerous European isolates belonging to the variant PVY^{NTN} (A). Interestingly, PVY defective RNA molecules (D-RNAs), *i.e*. PVY genomes with single-deletions of 1582 to 5149 nucleotide residues in length spanning the regions NIb to CP, CI to NIb, 6K2 to CP and CI to CP, were found for PVY for the first time. There were ten different patterns of PVY D-RNAs identified in this study. The D-RNA of NWO-KE1-2, which belongs to the PVY^{NTN} strain, was found to have an in-frame single-deletion of 2907 nucleotide residues spanning CI/ to NIb region as well as a duplication of 383 nt located within the CI cistron. It is possible that these D-RNAs could play a role in PVY pathogenicity/virulence, adaption and/or evolution.

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List of Abbreviations

6K1 and 6K2	6 kilo Dalton (kDa) proteins 1 and 2
A	Alanine
aa	Amino acid (s)
AMV	*Alfalfa mosaic virus*
AU	Adenine-Uracil
BBMV	*Broad bean mottle virus*
CI	Cytoplasmic inclusion
CMV	*Cucumber mosaic virus*
CP	Coat protein
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
EtBr	Ethidium bromide ($C_{21}H_{20}BrN_3$)
HC-Pro	Helper-component proteinase
I	Isoleucine
kb	Kilo base-pair
K	Lysine
L	Leucine
N	Asparagine
NIa	The nuclear inclusion protein a
NIb	The nuclear inclusion protein b
NTR (UTR)	Non-translated (untranslated) region
P	Proline
P1	Protein 1, the first protein encoded by the 5' region of a *Potyvirus* genome
P3	Protein 3, the third protein encoded by the 5' region of a *Potyvirus* genome
P3N-PIPO	P3 N-terminal protein-pretty interesting *Potyviridae* ORF
Q	Glutamine
R	Arginine
RDP	Recombination Detection Programme
RJs	Recombinant junctions
RT-PCR	reverse transcription-polymerase chain reaction
S	Serine
TBE	Tris-Borate-EDTA
TEV	*Tobacco etch virus*
V	Valine
VPg	Viral protein genome-linked
ZYMV	*Zucchini yellow mosaic virus*

1. Introduction

1.1. *Potato virus Y* (PVY) distribution, host range and transmission

Potato virus Y (PVY) is one of the most important plant viruses with a worldwide distribution (Kehoe and Jones, 2016) and infecting a wide range of host plants comprising 495 species in 72 genera from 31 families, including numerous economically important solanaceous crop plants, *e.g*. potato (*Solanum tuberosum*), pepper (*Capsicum sp.*), tomato (*S. lycopersicum*) and tobacco (*Nicotiana tabacum*) (Kerlan, 2006). PVY infection may result in huge losses in potato crops in terms of tuber yield and quality. The infection in potato may be symptomless, but can also lead to plant death or the induction of potato tuber necrotic ringspot disease. PVY is a tuber-borne virus and it is also transmitted by more than 40 aphid species in a non-persistent manner (Quenouille *et al*., 2013). The green peach aphid (*Myzus persicae*) is considered to be the most efficient vector (Nanayakkara *et al*., 2012), although the bird-cherry oat aphid (*Rhopalosiphum padi*) is considered to be the most significant vector in Sweden, due to its presence in massive numbers (Sigvald, 1985; 1989).

1.2. PVY genome

PVY belongs to the genus *Potyvirus* in the family *Potyviridae*. PVY virions are flexuous and filamentous with a length of about 730 – 740 nm and a diameter of 11 nm. The 5'-end of the PVY genome is covalently linked to a genome-linked viral protein (VPg) through a tyrosine residue and the 3'-end contains a poly(A)-tail. As in other potyviruses, the PVY genome is a positive-sense and single-stranded RNA (ssRNA) molecule of about 9.7 kb that is translated into a single polyprotein of about 3062 amino acid (aa) residues. Subsequently, the polyprotein is cleaved by three viral proteases into ten multifunctional proteins. In addition, the potyviral genome contains a short open reading frame (ORF) P3N-PIPO, which is generated by +2 frameshifting of the P3 cistron (Quenouille *et al*., 2013) (Fig. 1). P3N-PIPO encodes a putative protein of variable length among PVY isolates, approximately 76 aa (Cuevas *et al*., 2012). Protein 1 (P1) is one of the potyviral proteases and the most variable protein among potyviruses. The helper-component proteinase (HC-Pro) is a silencing suppressor protein that supresses RNA silencing, the plant defence mechanism, and also is one of the potyviral proteases and has many other functions, *e.g*. in virus multiplication, symptom development, aphid transmission, and cell-to-cell and systemic movement. Protein 3 (P3) is suggested to be involved in pathogenicity, systemic infection, virus multiplication and movement. The functions of the 6 kDa protein 1 (6K1) are unknown. The cylindrical inclusion (CI) is responsible for the formation of pinwheel-shaped inclusion bodies. The 6 kDa protein 2 (6K2) is involved in virus replication. The VPg has numerous functions. One of the VPg functions is its essential role in virus replication and translation. The nuclear inclusion protein a (NIa) acts as the major protease in viral polyprotein cleavage. The nuclear inclusion protein b (NIb) is the viral RNA-dependent RNA polymerase (RdRp). The coat protein (CP) is indispensable for virion assembly, aphid transmission, as well as cell-to-cell and systemic movement (Quenouille *et al*., 2013) (Fig. 1).

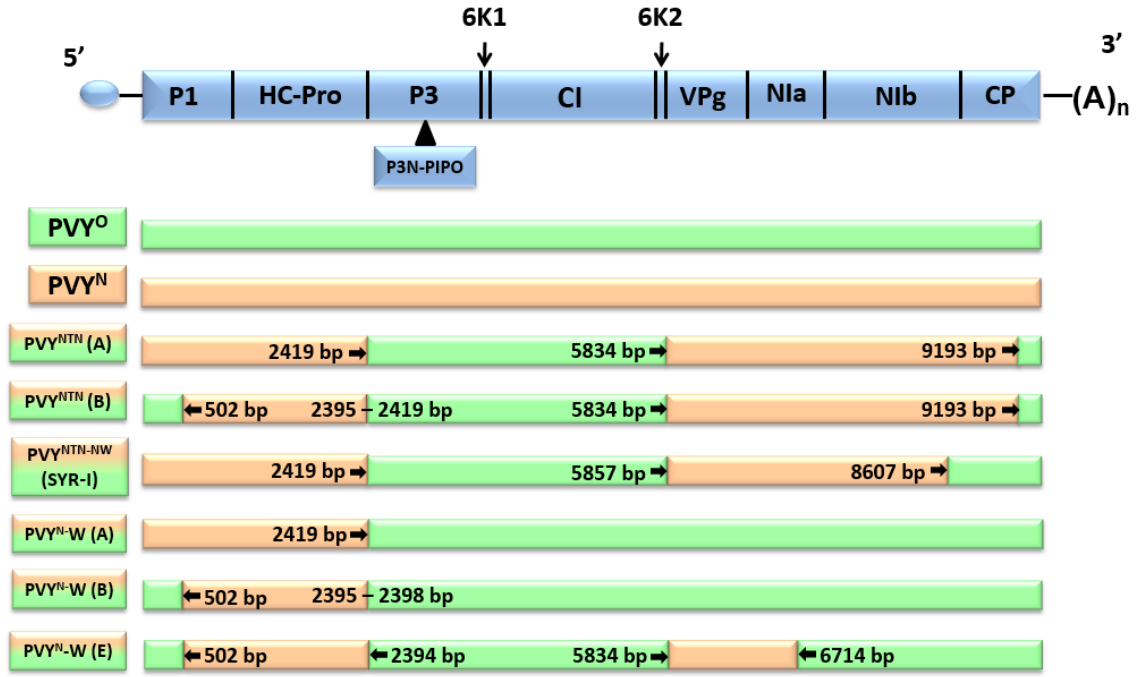

Figure 1. Genome structure of PVY strains and variants. The genome contains one major open reading frame (ORF), which is processed into ten mature proteins. In addition, a small ORF, P3N-PIPO is expressed in fusion with the P3 cistron. Light green colour represents the PVY^{O} or PVY^{Z} strains and light orange colour represents the PVY^{N} strain. Positions of recombinant junctions (RJs) between PVY^{O} and PVY^{N} are marked (Hu *et al*., 2009).

1.3. PVY genetic diversity and classification of strain groups

PVY exists in a complex of strain groups and variants. PVY classification is based on: i) serology – determined by antibody recognition of the CP. ii) hypersensitive response (HR) in test host plants and different potato cultivars carrying certain resistance genes – determined by one or several genes. iii) sequence and phylogenetic analyses, including number and positions of recombinant junctions (RJs). Serological classification and biological properties of PVY strains are not always correlating, because there are numerous PVY strains/variants, which have similar serological classification and different biological properties, such as the strains inducing tobacco veinal necrosis (PVY^{N}), North American NA-PVY^{N}, PVY^{N} tuber necrosis (PVY^{NTN}), PVY^{E} and PVY^{Z}-NTN. There are six non-recombinant strain groups: stipple streak (PVY^{C}), PVY^{N}, North American NA-PVY^{N}, ordinary (PVY^{O}), PVY^{D} and PVY^{Z}. In addition, there are six recombinant strains: PVY^{NTN}, PVY^{E}, PVY-NE11, PVY^{N}-W (called $PVY^{N:O}$ in North America), PVY^{NTN-NW} and PVY^{Z}-NTN (Karasev and Gray, 2013; Kehoe and Jones, 2016). In addition, there are various variants within a PVY strain that were formed by different mutations and/or recombination events. Inoculation with the PVY^{O} strain and its variant PVY^{O}-O5 triggers HR in potato cultivars possessing the dominant resistance genes Ny_{tbr}. In addition, inoculation with the strains PVY^{Z} and PVY^{Z}-NTN triggers HR in potato cultivars possessing the dominant resistance gene Nz_{tbr} (Karasev *et al*., 2011; Kerlan *et al*., 2011; Karasev and Gray, 2013). On the other hand, the strains PVY^{C}, PVY^{D}, PVY^{N}, NA-PVY^{N}, PVY^{NTN}, PVY^{N}-W, PVY^{NTN-NW} and PVY^{E} overcome the resistance genes Ny_{tbr} and Nz_{tbr}. Inoculation with the

recently identified PVY^D strain triggered HR in numerous potato cultivars. The majority of these cultivars do not possess any of the previously mentioned resistance genes. Hence, it is suggested that a hypothetical gene, *Nd_{tbr}*, elicits HR against the PVY^D strain (Kehoe and Jones, 2016). Furthermore, wild and cultivated potato genotypes possessing the dominant PVY-specific *Ry* genes are assumed to have an extreme resistance (ER) to all PVY strains, with ER being epistatic to HR (Valkonen, 2015).

1.4. PVY evolution

Genetic diversity plays a crucial role in PVY evolution. PVY has the ability to evolve over time and forms new strains/variants through mutations and through recombination between different strains (Karasev and Gray, 2013). Mutation can play an important role in PVY evolution. For example, it has been suggested that a tuber-necrosis-inducing NA-PVY^N isolate has evolved from a non-inducing NA-PVY^N isolate by mutations (Nie and Singh, 2003). In a similar manner, we assumed that the PVY^Z strain and resistance-breaking PVY^O isolates have evolved from the ordinary PVY^O strain by mutations. Through recombination, it is possible for PVY to evolve even faster than with mutations. Recombination has numerous benefits for PVY and other viruses, *e.g*. repair of deleterious mutations, acquisition of new genetic determinants, emergence of new strains/variants with higher fitness compared with the parental genotypes and maintenance of genetic variation. There are two types of RNA recombination: homologous and non-homologous. Homologous recombination results from crossover of two templates sharing considerable sequence identity, while non-homologous recombination results from crossover of highly variable sequence templates (Simon-Loriere and Holmes, 2012). The recombinant strains PVY^{NTN} and PVY^N-W and their variants have emerged through different events of homologous recombination between the PVY^O and PVY^N strains, and subsequently these recombinant strains have recombined again and generated the recombinant strain PVY^{NTN-NW} (Chikh Ali *et al*., 2010a). Genomic sequences resulting from non-homologous recombination between an RNA molecule of the CP cistron and 3'-end of PVY and a host retro-transposable element have been reported in numerous grapevine (*Vitis vinifera*) varieties (Tanne and Sela, 2005). Virus genes integrated into the host genome are referred to as non-retroviral integrated RNA viruses (NIRVs; Tromas *et al*., 2014) and NIRVs may be formed as a result of non-homologous recombination. Recombination detection software programmes are commonly used for detecting evidences of recombination in PVY genotypes and results are verified by inferring phylogenetic relationships.

Recombination may lead to generation of subviral RNA molecules: defective (D) RNAs, defective interfering (DI) RNAs (Pathak and Nagy, 2009). D-RNAs and DI-RNAs are derived from the genome of the (parental) helper RNA virus, while chimeric RNAs are derived from the helper RNA virus along with other sources. D-RNAs and DI-RNAs have partially or completely deleted genes required for replication, encapsidation or movement, but contain the necessary *cis*-acting elements for replication. Consequently, D-RNAs and DI-RNAs require the proteins of their helper virus. DI-RNA is distinguished from D-RNA by it interference with viral infection and symptom induction through affecting the multiplication rate of its helper virus (Graves *et al.*, 1996; Simon *et al*., 2004; Pathak and Nagy, 2009). There are different

models for the mechanisms of generation of subviral RNA molecules. Template switching or replicase jumping during regular replication of the viral genome is the most acceptable model for genomic RNA-RNA recombination and for formation of subviral RNA molecules, as being supported by biochemical assays. Moreover, *cis*-acting elements may play a role in guiding template-switching events (Pathak and Nagy, 2009).

Most RNA and DNA viruses tend to have D- and DI-nucleic acids (Hull, 2002). Effective translation is suggested to be an important feature for the production and maintenance of defective molecules (Tromas *et al*., 2014). There are two groups of D-RNAs and DI-RNAs: single-deletion and multiple-deletion groups. Single-deletion D-RNAs and DI-RNAs have been reported for numerous viruses from different families, such as *Alfalfa mosaic virus* (AMV), *Broad bean mottle virus* (BBMV) and *Cucumber mosaic virus* (CMV) from family *Bromoviridae; Citrus tristeza virus* (CTV) from family *Closteroviridae* (Hull, 2002); PVY from family *Potyviridae* (Youssef, 2017). Multiple-deletion D-RNAs and DI-RNAs have been reported for members of the family *Tombusviridae*, *e.g*. *Tomato bushy stunt virus* (TBSV) (Hull, 2002). PVY D-RNAs were detected by multiplex-PCR and Sanger sequencing (Youssef, 2017) and they may potentially play a role in virus pathogenicity/virulence and/or adaptation.

1.5. PVY population structure in Sweden

A few decades ago, the PVY^{O} strain was the most prevalent one in Sweden (Sigvald, 1985; 1989), but it has been replaced by a variety of recombinants, including the PVY^{NTN} strain (Youssef, 2017). The recombinant PVY^{NTN} strain has been shown to be the most prevalent strain in numerous European countries, *e.g.* the Netherlands, Scotland and Belgium (van der Vlugt *et al.*, 2008; Davie, 2014; Kamangar *et al*., 2014). PVY recombinant strains, *e.g.* PVY^{NTN}, are mainly associated with foliage necrosis in potato, as well as potato tuber necrotic ringspot disease, which significantly affects tuber yield and quality (Le Romancer *et al*., 1994; Kerlan *et al*., 2011; Karasev and Gray, 2013; 2013b; Kogovšek *et al*., 2016; Youssef, 2017).

In a recent M.Sc. thesis by Youssef (2017), 42 potato samples were collected from 13 locations in Sweden and tested by RT-PCR (Glais *et al*., 2005; Chikh Ali *et al*., 2010b; 2013). All of the PCR-tested samples were positive for PVY, with 38 samples containing the variant PVY^{NTN} (A), either as a single infection or in mixed infections with other genotypes, and with 12 of the samples containing the PVY^{O} strain in mixed infections. Sequence analysis of cloned PCR fragments of numerous Swedish PVY-infected samples confirmed the presence of PVY^{N} genotypes, *e.g*. PVY^{NTN}. Inoculation experiments showed that none of the tested sample extracts, which contained the PVY^{O} strain in mixed infections, triggered HR in potato cv. Désirée containing the resistance genes Ny_{tbr} and Nd_{tbr} against the ordinary PVY^{O} and PVY^{D} strains, respectively. These results indicate the absence of the ordinary PVY^{O} and PVY^{D} strains from the tested samples. There are numerous genetic determinants within and outside the HC-Pro cistron responsible for the induction of HR in plants carrying the Ny_{tbr} gene and subsequent systemic resistance (Moury *et al*., 2011). Tian and Valkonen (2013) identified the genetic determinants responsible for overcoming resistance based on the Ny_{tbr} gene to be the amino acid residues 227 to 327 of the HC-Pro protein. Hence, PVY recombinants with HC-Pro of the PVY^{N} strain are capable of overcoming the resistance gene Ny_{tbr}. This study aimed to determine

the complete coding sequence for a few resistance-breaking PVY^{O} isolates and to screen for the genetic determinants responsible for overcoming resistance by the *Ny_{tbr}* gene.

Foliage necrosis was mainly associated with PVY samples containing the PVY^{NTN} strain, as single or mixed infections with other recombinant genotypes (Youssef, 2017). PVY^{NTN} isolates induce veinal and/or leaf necrosis in inoculated and systemically infected potato leaves, due to the enhanced accumulation of reactive oxygen species-associated metabolites (Kogovšek *et al*., 2016). Inoculation with extract of sample NTN-2 was associated with foliage necrosis in potato cv. Désirée, whereas inoculation with extract of sample NTN-5, which originated from the same tuber source, was not associated with any forms of foliage necrosis (Youssef, 2017). Therefore, determination of the complete coding sequences of NTN-2 and NTN-5 was carried out for screening of genetic determinants responsible for the induction of foliage necrosis in cv. Désirée. In addition, we hypothesized that maybe there are new PVY recombinants present in the PVY population in Sweden and that these recombinants are able to induce novel symptoms in potato cultivars. Consequently, complete and partial genome sequencing of some Swedish PVY isolates, especially those associated with severe symptoms in potato, was carried out to confirm the presence of recombinant genotypes, as well as to identify potentially new recombinant patterns.

2. Materials and Methods

2.1. Virus sources

The sequenced PVY isolates originated from potato samples NTN-2, NTN-5, NWO-KE1, NNW-KE2, NNW-KE3, NNW-KE6, NNW-KE266, NNW-KE278, NWO-L and NWO-MB, which were collected from different potato growing regions in Sweden during 2008 – 2015. D-RNA NWO-KE1-5 from sample NWO-KE1, which came from a PVY genome with a deletion of 2756 bp, was included in this study. NWO-KE1-5 was sequenced in a previous M.Sc. thesis (Youssef, 2017). In addition, one potato sample (NW-DeI) from the Netherlands was included. Details of their place of origin, original host plants and foliage and/or tuber symptoms were previously described by Youssef (2017). PVY-infected potato plants were maintained under greenhouse conditions of 23 °C ± 2 and 16 h photoperiod by growing sprouted tubers from PVY-infected mother plants.

2.2. Sequencing

2.2.1. RNA extraction, cDNA synthesis and PCR conditions

RNA extraction and cDNA synthesis were done according to Youssef (2017). The primer pairs 5'NTR/HR-4063 and HF/3'NTR were utilized for amplifying the PVY genome as two fragments (Glais *et al.*, 1998). The primer pair 5'NTR/HR-4063 generates a product of 4063 bp covering genomic positions 1 – 4063 (5'-UTR to CI cistron) of the PVY genome, whereas the primer pair HF/3'NTR generates a product of 5606 bp covering genomic positions 4034 – 9706 (CI cistron to 3'-UTR). The primer pair F-3739 (5'-AAC ATC ATC AGT GCT AGA TAC A-3') and R-4592 (5'- CAT TCT CAA TTA TGT TGG TTG C-3') was used to obtain

an overlap of 854 bp (genomic positions 3760 – 4569) between the PCR fragments generated using the primer pairs 5'NTR/HR-4063 and HF/3'NTR (Fig. 2). No amplification product was obtained for the PVY-infected potato samples NWO-L and NWO-MB using the primer pair 5'NTR/HR. Instead, the forward primer P1-F (Gao *et al*., 2014), together with the reverse primer 4299-R (5'-TTA ACT TGA CTG GCT GCT GTG TTG TG-3') were used (Fig. 2). The primer pair P1-F/4299-R generates a fragment of 4137 bp covering genomic positions 188 – 4324 (P1 to CI cistrons) (Fig. 2). The primer pairs 5'NTR/HR-4063 and HF/3'NTR were obtained from TAG Copenhagen A/S and the primer pair P1-F/4299-R was obtained from Invitrogen. All primers were used at a final concentration of 0.2 µM. The 50 µl PCR mix using Phusion High-Fidelity DNA Polymerase contained 5 µl cDNA and a final concentration of 0.02 U/µl Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 1 mM $MgCl_2$, 0.5x HF buffer, 0.5x GC buffer, 3% DMSO and 0.2 mM dNTPs (Thermo Scientific). Gradient PCR assays (1), (2), (3) and (4) were run employing the primer pairs 5'NTR/HR, HF/3'NTR, F-3739/R-4592 and P1-F/4299-R, respectively. Conditions for gradient PCR assays were as follows: initial denaturation at 98 °C for 30 s, followed by 33 cycles of denaturation at 98 °C for 10 s, annealing for 30 s at 52 – 61 °C for PCR (1), and 60 – 65 °C for PCR (2), (3) and (4), extension at 72°C for 150 s and final extension for 10 min at 72°C.

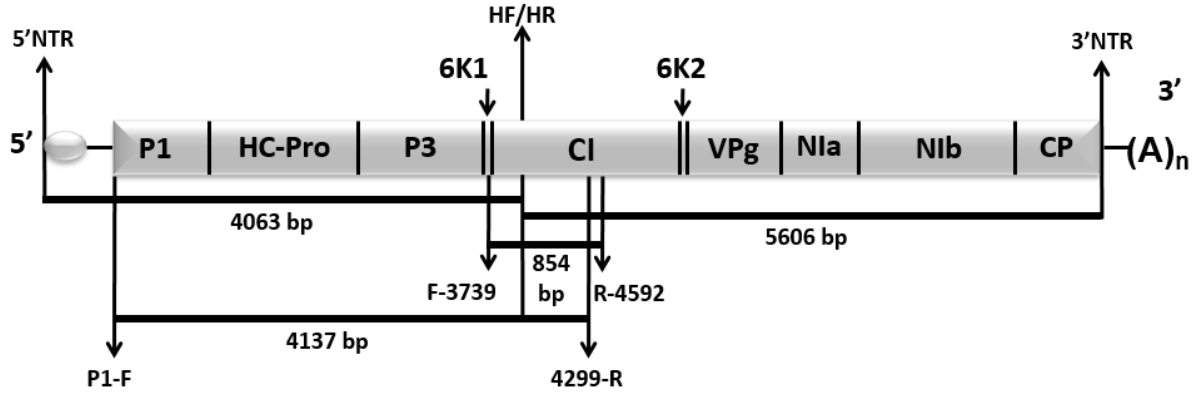

Figure 2. Genomic structure of PVY showing locations of the primers, which were used for amplifying the PVY genome, and expected product sizes (bp).

2.2.2. Cloning and sequencing of PCR fragments

GeneJET gel extraction kit (Thermo Scientific) was utilized for purifying PCR products from agarose gels. A total amount of 150 – 250 ng of purified DNA was ligated into pJET cloning vector (50 ng) using Clone JET PCR cloning kit (Thermo Scientific), and then transformed into lab-made competent cells of *Escherichia coli* strain DH5α. The transformation was run following standard procedures. Plasmid DNA was purified from overnight *E. coli* cultures using GeneJET plasmid miniprep kit (Thermo Scientific), and digested by Fast Digest BglII (Thermo Scientific). Clones with the expected insert size were selected for sequencing utilizing Sanger sequencing technology at Macrogen (Amsterdam, The Netherlands). One to three clones per fragment were sequenced using pJET forward and reverse primers. Sequencing

reads were checked for quality and sequence identity to PVY sequences available in GenBank utilizing BLASTn search engine offered by the National Center for Biotechnology Information (NCBI) {available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>}. All analyses were done after excluding primers. Walking primers were designed for obtaining sequence overlaps of at least 100 bp. Overlapping sequences sharing 99 – 100% nucleotide identity were assembled using the multiple alignment option offered by BLASTn.

The complete coding sequence of isolate O-MB was assembled from two PCR fragments covering genomic positions 210 – 4298 and 4064 – 9673. Partial genome sequences (covering genomic positions 180 – 769) for Swedish samples NTN-2, NWO-KE1 and NNW-KE2 from a previous M.Sc. thesis by Youssef (2017) were included in this study (Appx. Table A). The complete coding sequences of isolates NTN-2 and NTN-KE2 were assembled from four overlapping PCR fragments covering genomic positions 180 – 769, 386 – 4033, 3760 – 4569 and 4064 – 9673. In addition, the complete genome of isolate NTN-5 was assembled from three overlapping PCR fragments covering genomic positions 32 – 4033, 3760 – 4569 and 4064 – 9673. All PCR fragments were assembled after removing forward and reverse primers.

2.3. Nucleotide and amino acid sequence identity analyses

ORFs of the sequenced Swedish PVY isolates NTN-2, NTN-5, NTN-KE2 and O-MB were identified using the search engine ORFfinder offered by NCBI. Screening for the P3N-PIPO was done based on data provided by Cuevas *et al*. (2012). Calculations of nucleotide and amino acid sequence identities for full-length and D-RNAs were done using the search engines BLASTn, tBLASTx and BLASTx offered by NCBI. All analyses were done after removing primers. Sequences were aligned using CLUSTAL-W in MEGA programme version 7.0 (Kumar *et al*., 2016) using the default parameters. Sequences with deletions were aligned manually. All alignment sessions were imported in fasta format. Nucleotide and amino acid identities were determined using multiple sequence alignment by uploading fasta files in the search engines BLASTn and tBLASTx, respectively. Molecular classification of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

PVY polyprotein cleavage sites were determined though sequence comparisons based on information provided by Adams *et al*. (2005). The amino acid sequence of HC-Pro and P3 for six Swedish isolates (NTN-2, NTN-5, NWO-KE1-1, NTN-KE2, NWO-L-1 and O-MB) as well as isolates from different strain groups obtained from GenBank (Appx. Table B) were screened for unique amino acid differences through sequence comparisons. The six Swedish isolates and complete genome sequences of 22 PVY isolates from GenBank (Appx. Table B) were compared at amino acid positions 236 to 302 in the HC-Pro protein. This regions has been identified by Tian and Valkonen (2013) to be responsible for triggering/overcoming resistance by the Ny_{tbr} gene.

2.4. Phylogenetic analyses

The phylogenetic analyses included nucleotide sequences available in GenBank of 23 PVY isolates of different strain groups (Appx. Table B), as well as four complete coding sequences (NTN-2, NTN-5, NTN-KE2 and O-MB) and six partial genome sequences (NWO-KE1-1, NNW-KE3, NNW-KE6, NNW-KE278, NWO-L-1 and NWO-L-2) of Swedish isolates from this study. The complete nucleotide sequence of an isolate of *Pepper severe mosaic virus* (PepSMV; Ahn *et al*., 2006) was used as an outgroup (Appx. Table B). Phylogenetic analyses were run after removing primers as well as proximal 5'- and 3'-regions. Sequence alignment was done using CLUSTAL-W in MEGA programme version 7.0 (Kumar *et al*., 2016) using default parameters. Two separate phylogenetic analyses were run, with the first analysis including nucleotide sequences covering genomic positions 180 – 4033 and the second analysis including positions 4064 – 9589. Phylogenetic relationships of aligned nucleotide sequences were inferred by the Maximum-Likelihood method implemented in MEGA programme version 6.0 (Tamura *et al*., 2013) with 1000 bootstrap replicates for branch evaluation (Felsenstein, 1985; Tamura and Nei, 1993; Tamura *et al*., 2013).

2.5. Recombination analyses

The nucleotide sequences of NTN-2, NTN-5, NTN-KE2 and O-MB were analyzed for evidences of genomic recombination. Recombination analysis was run using recombination detection programme version 4.0 (RDP4) package (Martin *et al*., 2015) employing RDP, GENECONV, Chimaera, BootScan, MaxChi, SiScan, PhylPro, LARD and 3SEQ programmes. A recombination pattern was considered when detected by at least four of the previously mentioned programmes with a *P*-value of $< 1 \times 10^{-6}$.

3. Results

3.1. Nucleotide and amino acid sequence identity analyses

The complete coding sequences of the four Swedish PVY isolates NTN-2, NTN-5, NTN-KE2 and O-MB were determined. In addition, partial genome sequences of six Swedish PVY isolates and ten sequences of PVY D-RNAs, including one PVY D-RNA sequence (NW-DeI) from the Netherlands, were determined.

Sequence analysis of cloned amplification products intended to cover nucleotide positions 1 – 4063 revealed mis-priming, as sequences starting at position 386 were obtained for isolates NTN-2, NWO-KE1-1 and NTN-KE2, at position 1036 for a fragment from sample NWO-KE1, and at position 3354 for an isolate from sample NNW-KE3 (data not shown). These mis-priming problems occurred because the reverse primer HR-4063 annealed as a forward primer, instead of the forward primer 5'NTR. A full-length fragment of 5606 bp (genomic positions 4034 – 9706) using the primer pair HF/3'NTR was obtained for samples NTN-2, NTN-5, NNW-KE2, NNW-KE6, NNW-KE278, NWO-L and NWO-MB. For sample NWO-KE1, a full-length fragment was not obtained using the same primer pair, although high-quality RNA extracted at different stages of viral infection was utilized and the PCR assay was run under optimal conditions.

The overlapping PCR fragment of 854 bp generated by the primer pair F-3739/R-4592 (Fig. 2) was obtained for isolates NTN-2, NTN-5 and NTN-KE2. The overlapping regions of the 854 bp fragments with PCR fragments (genomic positions 1 – 4063 and 4034 – 9706) generated by the primer pairs 5'NTR/HR-4063 and HF/3'NTR for the previously mentioned isolates shared a nucleotide sequence identity of 99 – 100%. These findings confirmed that these fragments came from the same genotype enabling the assembly of nearly complete genomes. The overlapping regions for fragments from sample NWO-L shared a nucleotide sequence identity of 98%, and these sequences were then not assembled into a longer contig (data not shown).

The determined sequence of NTN-2 is of 9547 nt, NTN-5 is of 9637 nt, NTN-KE2 is of 9548 nt and O-MB is of 9516 nt. The large ORF of NTN-2, NTN-5 and O-MB encodes a putative polyprotein of 3061 aa. Meanwhile, sequence of NTN-KE2 contains a nucleotide substitution at position 2277 leading to the creation of a stop codon (UAG) and the split of the large ORF of 3061 aa into two ORFs of 2299 aa and 695 aa. NTN-KE2 The length of the ORF P3N-PIPO varied among PVY isolates. The ORF P3N-PIPO was identified for the Swedish isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2, NWO-L-1 and O-MB to start at nucleotide position 2919 with the $G_{1\text{-}2}$ $A_{3\text{-}7}$ motif (GGAAAAAAA) and terminating with the stop codon (UAA) at position 3150. The ORF P3N-PIPO of the Swedish isolates encodes a putative protein of 76 aa, which is similar to several isolates from the strains PVY^{C}, PVY^{O}, PVY^{E}, PVY^{Z}-NTN, PVY^{NTN}, PVY^{N}-W and $PVY^{NTN\text{-}NW}$. Isolates NTN-2 and NTN-5 shared 100% amino acid identity of the translated P3N-PIPO ORF and the two isolates shared 99% amino acid identity with NWO-KE1-1, NTN-KE2 and NWO-L-1, and 96% with O-MB (data not shown).

Isolate NTN-2 shared nucleotide identity of 99% with NTN-5, NWO-KE1-1 (nucleotide positions 180 – 4033), NTN-KE2, NNW-KE278 (nucleotide positions 4064 – 9673) and NWO-L-1 (nucleotide positions 210 – 4298) as well as 91% with NNW-KE6, 90% with NWO-L-2 and 89% with O-MB. For nucleotide positions 4064 – 9673, isolate O-MB shared nucleotide and amino acid identities of 99% with NNW-KE6 and NWO-L-2, while the complete sequence of O-MB shared nucleotide and amino acid identities of 99% with PVY^{O} isolates, *e.g.* ID1_5_62A, CO2140 and PVY^{O}-Oz from the USA, and FL from Canada, as well as with the PVY^{Z} isolate CRM2 from the UK (Tables 1 and 2). When comparing publicly available sequences, it was found that the nucleotide sequence identity among isolates of the PVY^{O} strain ranged from 97 to 99%, which is the same identity as when comparing sequences of the PVY^{O} and PVY^{Z} strains (data not shown). The Swedish isolates NTN-2, NTN-5 and NTN-KE2 shared highest nucleotide and amino acid identities at 99% with PVY^{NTN} (A) isolates, such as 3D from Serbia, NTN from Slovenia, IUNG-4 from Poland, PB312 from the USA, IUNG-8 from Germany and La Union from Colombia (Tables 1 and 2). Isolates NWO-KE1-1 (nucleotide positions 180 – 4033), NNW-KE278 (nucleotide positions 4064 – 9673) and NWO-L-1 (nucleotide positions 210 – 4298) shared nucleotide and amino acid identities of 99 – 100% with isolates of the PVY^{NTN} and PVY^{Z}-NTN strains. Isolate NNW-KE3 shared nucleotide identity of 99% and amino acid identity of 100% with the PVY^{N}-W strain. Similarly, isolate NNW-KE6 shared nucleotide and amino acid identities of 99% with the PVY^{N}-W strain. Isolate NWO-L-2 shared nucleotide and amino acid identities of 99% with the PVY^{O}, PVY^{Z} and PVY^{N}-W strains (Tables 1 and 2).

Cleavage sites of the polyprotein were identified through sequence comparisons for the Swedish isolates, along with other isolates from GenBank. The motif MIQF was found to be the putative cleavage site for P1/HC-Pro for the Swedish isolate O-MB, in addition to isolates of PVY^{C}, PVY^{D}, PVY^{NTN-NW}, PVY^{O}, PVY^{O}-O5 and PVY^{Z}. The motif MIQF was found to be the putative cleavage site for P1/HC-Pro for the Swedish PVY^{NTN} isolates NTN-2, NTN-5. The motif MVQF was found to be the putative cleavage site for P1/HC-Pro for the Swedish isolates NWO-KE1-1, NTN-KE2 and NWO-L-1, and isolates from the PVY^{C}, NA-PVY^{N}, PVY^{N}, PVY^{NTN}, PVY^{NTN-NW}, PVY^{N}-W and PVY^{Z}-NTN strains. The motif YRVG was found to be the putative cleavage site for HC-Pro/P3 for all PVY strain groups, with some exceptions (data not shown).

A total of 22 complete genome sequences of PVY isolates from different strain groups, in addition to six Swedish PVY isolates, were screened for the amino acid residues 236 to 302 in the HC-Pro protein that Tian and Valkonen (2013) identified as being responsible for triggering/overcoming resistance by the *Ny_{tbr}* gene. The Swedish isolate O-MB, together with one PVY^{C} isolate and isolates of PVY^{O}, PVY^{O}-O5 and PVY^{Z} were found to have the eight amino acid residues (I_{236}, K_{238}, S_{247}, V_{252}, Q_{262}, R_{296}, K_{270} and I_{301}) located in the HC-Pro protein and previously identified as being responsible for triggering the *Ny_{tbr}* gene in potato (Tian and Valkonen, 2013). Whereas, the Swedish PVY^{N} isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2 and NWO-L-1, together with isolates from PVY^{E}, PVY^{N}, PVY^{NTN}, PVY^{N}-W, PVY^{NTN-NW} and PVY^{Z}-NTN possess the eight amino acid residues (N_{236}, L_{238}, A_{247}, I_{252}, R_{262}, K_{296}, R_{270} and V_{301}) identified to be required for overcoming *Ny_{tbr}* resistance (Fig. 3).

The P1, HC-Pro and P3 proteins were found to be highly variable among PVY isolates from different strain groups, including the Swedish PVY isolates, and no unique amino acid differences between PVY^{Z} and PVY^{O} were detected. HC-Pro protein of isolate NTN-2 differed by having leucine at position 314 and that of NTN-KE2 by having glutamine at position 411 from all analyzed PVY isolates of different strain groups. For the same protein, NWO-L-1 was found to differ by having serine at position 459 from the strains PVY^{E}, PVY^{N}, PVY^{NTN}, PVY^{N}-W, PVY^{NTN-NW} and PVY^{Z}-NTN, and O-MB differed by having valine at position 90 from all isolates belonging to the PVY^{O} and PVY^{Z} strains. For the P3 protein, isolates NTN-2 and NTN-5 were found to differ at positions 175, 249, 255 and 272, and isolate NWO-L-1 differed at positions 268 and 316 from other Swedish PVY^{NTN} (A) isolates with a few exceptions. Isolate O-MB differed from numerous PVY^{O} isolates by having arginine at position 155 and glutamine at position 276 in the P3 protein. Furthermore, the foliage-necrosis-inducing isolate NTN-2 differed from the non-inducing isolate NTN-5 in two amino acid residues located in the HC-Pro and VPg proteins (data not shown).

A total of ten different patterns of PVY D-RNAs from five potato samples were determined. The sequenced PVY D-RNAs were associated with samples NTN-5, NW-DeI, NWO-KE1, NNW-KE2 and NNW-KE266. The NWO-KE1-2 D-RNA shared 99% nucleotide identity with numerous PVY^{NTN} (A) isolates, *e.g.* 3D, La Union, IUNG-4, PB312 and IUNG-13, as well as the Japanese PVY^{Z}-NTN isolate Eu-12Jp. The D-RNAs NTN-5-2, NWO-KE1-2, NWO-KE1-4, NWO-KE1-6, NWO-KE1-7, NNW-KE2 and NNW-KE266 shared a nucleotide identity of 99 – 100% with numerous isolates of the PVY^{NTN} and PVY^{Z}-NTN strains. The D-RNA of the Dutch isolate NW-DeI shared a nucleotide identity of 99% with the PVY^{N}-W strain and the D-RNA of the Swedish isolate NWO-KE1-3 shared 99% identity with isolates from the PVY^{NTN}, PVY^{N}-W and PVY^{NTN-NW} strain groups. The D-RNA of NWO-KE1-5 shared a nucleotide identity of 97 – 100% with isolates from numerous PVY strain groups (Table 3).

An in-frame single-deletion of 1582 nucleotide residues spanning the NIb to CP genomic region was identified for the D-RNA NNW-KE2 (pattern 1) and an in-frame single-deletion of 2994 nucleotide residues spanning the 6K2 to CP genomic region was identified for D-RNA NWO-KE1-4 (pattern 4). In-frame single-deletions of 2756 – 3953 nucleotide residues spanning the CI to NIb genomic region were detected for the D-RNAs NWO-KE1-5 (pattern 2), NWO-KE1-2 (pattern 3), NW-DeI (pattern 5) and NWO-KE1-3 (pattern 6). Moreover, in-frame single-deletions of 4126 – 5149 nucleotide residues spanning the CI to CP genomic region were detected for the D-RNAs NNW-KE266 (pattern 7), NTN-5-2 (pattern 8), NWO-KE1-7 (pattern 9) and NWO-KE1-6 (pattern 10) (Table 4, Fig. 4). D-RNAs patterns 1, 4, 7, 8, 9 and 10 have a deletion the 5'- proximal part of the CP cistron and D-RNA pattern 4 has a deletion in the 3'-proximal part of the 6K2 cistron (Fig. 4).

The sequenced PVY D-RNAs were found to have 5 to 7 nucleotide residues long direct repeats (DR) flanking deletion junction sites, except for D-RNA NWO-KE1-2 Additionally, NWO-KE1-2 was found to have a duplication of 383 nt located within the CI cistron (nucleotide positions 4193 – 4575) (Table 4, Fig. 4). The D-RNA NWO-KE1-2 has a single-deletion of 2907 nucleotide residues spanning the CI to NIb genomic region.

Table 1. Results of GenBank searches with BLASTn for sequences of Swedish PVY isolates

Sample ID	Isolate ID	Sequence length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NTN-2	NTN-2	9489	180 – 9673	3D	PVY^{NTN}	100	99	KJ946936.2
				IUNG-4	PVY^{NTN}	100	99	JF927752.1
				PB312	PVY^{NTN}	100	99	EF026075.1
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
				NTN	PVY^{NTN}	100	99	KM396648.1
NTN-5	NTN-5	9637	32 – 9673	IUNG-8	PVY^{NTN}	100	99	JF927756.1
				IUNG-4	PVY^{NTN}	100	99	JF927752.1
				NTN	PVY^{NTN}	100	99	KM396648.1
				IUNG-13	PVY^{NTN}	100	99	JF927761.1
				La Union	PVY^{NTN}	100	99	KR149260.1
NNW-KE2	NTN-KE2	9490	180 – 9673	IUNG-4	PVY^{NTN}	100	99	JF927752.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				IUNG-13	PVY^{NTN}	100	99	JF927761.1
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
				NTN	PVY^{NTN}	100	99	KM396648.1
NWO-MB	O-MB	9460	210 – 9673	CO2140	PVY^{O}	100	99	HQ912914.1
				SCRI-O	PVY^{O}	100	99	AJ585196.1
				CRM2	PVY^{Z}	100	99	KP691322.1
				ID130	PVY^{O}	100	99	HQ912888.1
				ID1_5_62A	PVY^{O}	100	99	HQ912890.1
NWO-KE1	NWO-KE1-1	3854	180 – 4033	3D	PVY^{NTN}	100	99	JF927752.1
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
				IUNG-4	PVY^{NTN}	100	99	JF927752.1
				PB312	PVY^{NTN}	100	99	EF026075.1
				NTN	PVY^{NTN}	100	99	KM396648.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Cont. Table 1. Results of GenBank searches with BLASTn for sequences of Swedish PVY isolates

Sample ID	Isolate ID	Sequence length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NNW-KE3	NNW-KE3	710	3355 – 4033	261-4	PVY^{N}-W	100	99	AM113988.1
				1107	PVY^{NTN-NW}	100	99	KC296439.1
				1106	PVY^{NTN-NW}	100	99	KC296438.1
				IUNG-14	PVY^{N}-W	100	99	JF927762.1
				IUNG-5	PVY^{N}-W	100	99	JF927753.1
NNW-KE6	NNW-KE6	5594	4064 – 9662	PN10A	PVY^{N}-W	100	99	DQ008213.1
				IUNG-6	PVY^{N}-W	100	99	JF927754.1
				LR	PVY^{N}-W	100	99	HQ912896.1
				N1	PVY^{N}-W	100	99	HQ912863.1
				N3	PVY^{N}-W	100	99	HQ912868.1
NNW-KE278	NNW-KE278	5606	4064 – 9673	IUNG-15	PVY^{NTN}	100	99	JF927763.1
				11627-12	PVY^{NTN}	100	99	KC634007.1
				11227-2	PVY^{NTN}	100	99	KC634004.1
				ID155	PVY^{NTN}	100	99	HQ912869.1
				Eu-12Jp	PVY^{Z}-NTN	100	99	AB702945.1
NWO-L	NWO-L-1	4089	210 – 4298	11439	PVY^{NTN}	100	99	KC634005.1
				11627-10	PVY^{NTN}	100	99	KC634006.1
				11629-9	PVY^{NTN}	100	99	KC634008.1
				1101	PVY^{NTN}	100	99	KC296434.1
				Linda	PVY^{NTN}	100	99	AJ890345.1
	NWO-L-2	5606	4064 – 9673	CRM2	PVY^{Z}	99	99	KP691322.1
				ID1_5_62A	PVY^{O}	99	99	HQ912890.1
				ID14_2_14a,	PVY^{N}-W	99	99	HQ912870.1
				A95	PVY^{N}-W	99	99	HQ912866.1
				ICIA	PVY^{O}	99	99	HQ912864.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Table 2. Results of GenBank searches with BLASTx for sequences of Swedish PVY isolates

Sample ID	Isolate ID	Sequence length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% query cover	% identity	GenBank Accession no. (protein data)
NTN-2	NTN-2	9489	180 – 9373	NTN	PVY^{NTN}	100	99	AIY63190.1
				3D	PVY^{NTN}	100	99	AJT60331.2
				PB312	PVY^{NTN}	100	99	ABK13680.1
				IUNG-4	PVY^{NTN}	100	99	AFJ05128.1
				IUNG-8	PVY^{NTN}	100	99	AFJ05132.1
NTN-5	NTN-5	9637	180 – 9373	IUNG-4	PVY^{NTN}	100	99	AFJ05128.1
				IUNG-8	PVY^{NTN}	100	99	AFJ05132.1
				NTN	PVY^{NTN}	100	99	AIY63190.1
				IUNG-13	PVY^{NTN}	100	99	JF927761.1
				3D	PVY^{NTN}	100	99	AJT60331.2
NNW-KE2	NTN-KE2	9490	180 – 9373	3D	PVY^{NTN}	100	99	AJT60331.2
				IUNG-4	PVY^{NTN}	100	99	AFJ05128.1
				PB312	PVY^{NTN}	100	99	ABK13680.1
				IUNG-8	PVY^{NTN}	100	99	AFJ05132.1
				La Union	PVY^{NTN}	100	99	AKG94974.1
NWO-MB	O-MB	9460	211 – 9373	ID1_5_62A	PVY^{O}	100	99	AEI52933.1
				CO2140	PVY^{O}	100	99	AEI52957.1
				FL	PVY^{O}	100	99	ADO14470.1
				PVY-Oz	PVY^{O}	100	99	ABK13679.1
				CRM2	PVY^{Z}	100	99	ALH24905.1
NWO-KE1	NWO-KE1-1	3854	180 – 4033	IUNG-4	PVY^{NTN}	99	99	AFJ05128.1
				3D	PVY^{NTN}	99	99	AJT60331.2
				NTN	PVY^{NTN}	99	99	AIY63190.1
				PB312	PVY^{NTN}	99	99	ABK13680.1
				La Union	PVY^{NTN}	99	99	AKG94974.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Cont. Table 2. Results of GenBank searches with BLASTx for sequences of Swedish PVY isolates

Sample ID	Isolate ID	Sequence length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% query cover	% identity	GenBank Accession no. (protein data)
NNW-KE3	NNW-KE3	710	3355 – 4033	IUNG-14	PVY^{N}-W	99	100	AFJ05138.1
				SASA-207	PVY^{N}-W	99	100	CAE50910.1
				ME162	PVY^{N}-W	99	100	AEI52915.1
				IUNG-12	PVY^{N}-W	99	100	AFJ05136.1
				ID431	PVY^{N}-W	99	100	AEI52905.1
NNW-KE6	NNW-KE6	5594	4064 – 9373	N3	PVY^{N}-W	99	99	AEI52911.1
				PN10A	PVY^{N}-W	99	99	AAY25497.1
				N1	PVY^{N}-W	99	99	AEI52906.1
				ID14_2_14a	PVY^{N}-W	99	99	AEI52913.1
				ID431	PVY^{N}-W	99	99	AEI52905.1
NNW-KE278	NNW-KE278	5606	4064 – 9373	11289-1	PVY^{NTN}	99	100	AGL81300.1
				ID155	PVY^{NTN}	99	100	AEI52912.1
				L26	PVY^{Z}-NTN	99	100	ACO35930.1
				N4	PVY^{NTN}	99	100	ACO35929.1
				NTNK1	PVY^{NTN}	99	99	BAN16650.1
NWO-L	NWO-L-1	4089	211 – 4298	1105	PVY^{NTN}	99	99	AGH27744.1
				I-16	PVY^{NTN}	99	99	AMW92179.1
				Eu-12Jp	PVY^{Z}-NTN	99	99	BAN58738.1
				11627-10	PVY^{NTN}	99	99	AGM51291.1
				11227-2	PVY^{NTN}	99	99	AGM51289.1
	NWO-L-2	5606	4064 – 9373	ID243	PVY^{O}	99	99	AEI52938.1
				ID281	PVY^{O}	99	99	AEI52936.1
				FL	PVY^{O}	99	99	ADO14470.1
				CRM2	PVY^{Z}	99	99	ALH24905.1
				N3	PVY^{N}-W	99	99	AEI52911.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Table 3. Results of GenBank searches with BLASTn for sequences of PVY D-RNAs

Sample ID	D-RNAs	Fragment length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NTN-5	NTN-5	582	4064 – 4291	F89II	PVY^{NTN}	100	100	KX184819.1
				F17	PVY^{NTN}	100	100	KX184817.1
				Eu-12Jp	PVY^{Z}-NTN	100	100	AB702945.1
				NTNTK1	PVY^{NTN}	100	100	AB711146.1
				IUNG-15	PVY^{NTN}	100	100	JF927763.1
			9317 – 9673	F89II	PVY^{NTN}	100	99	KX184819.1
				F17	PVY^{NTN}	100	99	KX184817.1
				GBVC_PVY_26	PVY^{N}-W	100	99	JQ969039.2
				11627-12	PVY^{NTN}	100	99	KC634007.1
				M3	PVY^{Z}-NTN	100	99	KF850513.1
NWO-KE1	NWO-KE1-2	3053	4064 – 5218	IUNG-13	PVY^{NTN}	100	99	JF927761.1
				I-17	PVY^{NTN}	100	99	KT599908.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				NTN	PVY^{NTN}	100	99	KM396648.1
				Eu-12Jp	PVY^{Z}-NTN	100	99	AB702945.1
			4193 – 4575	IUNG-13	PVY^{NTN}	100	100	JF927761.1
				I-17	PVY^{NTN}	100	100	KT599908.1
				3D	PVY^{NTN}	100	100	KJ946936.2
				NTN	PVY^{NTN}	100	100	KM396648.1
				Eu-12Jp	PVY^{Z}-NTN	100	100	AB702945.1
			8127 – 9642	3D	PVY^{NTN}	100	100	KJ946936.2
				NTN	PVY^{NTN}	100	100	KM396648.1
				IUNG-13	PVY^{NTN}	100	99	JF927761.1
				I-17	PVY^{NTN}	100	99	KT599908.1
				Eu-12Jp	PVY^{Z}-NTN	100	99	AB702945.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Cont. Table 3. Results of GenBank searches with BLASTn for sequences of PVY D-RNAs

Sample ID	D-RNAs	Fragment length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NWO-KE1	NWO-KE1-3	1627	4090 – 4495	IUNG-14	PVY^{N}-W	100	99	JF927762.1
				IUNG-12	PVY^{N}-W	100	99	JF927760.1
				MAF-VOY	PVY^{N}-W	100	99	JQ924286.1
				F89II	PVY^{NTN}	100	99	KX184819.1
				F17	PVY^{NTN}	100	99	KX184817.1
			8450 – 9673	IUNG-14	PVY^{N}-W	100	99	JF927762.1
				SYR-II-DrH	PVY^{NTN-NW}	100	99	AB461453.1
				IUNG-7	PVY^{N}-W	100	99	JF927755.1
				IUNG-3	PVY^{N}-W	100	99	JF927751.1
				MAF-VOY	PVY^{N}-W	100	99	JQ924286.1
	NWO-KE1-4	2612	4064 – 5730	IUNG-13	PVY^{NTN}	100	99	JF927761.1
				NTN	PVY^{NTN}	100	99	KM396648.1
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				VNP413	PVY^{NTN}	100	99	HG810950.1
			8726 – 9673	IUNG-13	PVY^{NTN}	100	100	JF927761.1
				NTN	PVY^{NTN}	100	100	KM396648.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
				VNP413	PVY^{NTN}	100	99	HG810950.1
	NWO-KE1-5	280	5967 – 6021	SGS-AG	PVY^{N}-W	98	98	JQ924288.1
				MAF-VOY	PVY^{N}-W	98	98	JQ924286.1
				FL	PVY^{O}	98	98	HM367075.1
				La Union	PVY^{NTN}	98	97	KR149260.1
				SYR-III-S2	PVY^{NTN-NW}	98	97	KP793715.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Cont. Table 3. Results of GenBank searches with BLASTn for sequences of PVY D-RNAs

Sample ID	D-RNAs	Fragment length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NWO-KE1	NWO-KE1-5	280	8779 – 9047	CRM2	PVY^{Z}	100	100	KP691322.1
				WA-13	PVY^{O}	100	100	HM590407.1
				SCRI-O	PVY^{O}	100	100	AJ585196.1
				IUNG-14	PVY^{N}-W	100	99	JF927762.1
				IUNG-10	PVY^{N}-W	100	99	JF927758.1
	NWO-KE1-6	460	4065 – 4260	I-6	PVY^{NTN}	100	100	KT599906.1
				NTN	PVY^{NTN}	100	100	KM396648.1
				GBVC_PVY_3	PVY^{NTN}	100	100	JQ969035.2
				GBVC_PVY_37	PVY^{NTN}	100	100	JQ969033.2
				Eu-12Jp	PVY^{Z}-NTN	100	100	AB702945.1
			9411 – 9673	I-6	PVY^{NTN}	100	99	KT599906.1
				NTN	PVY^{NTN}	100	99	KM396648.1
				IUNG-13	PVY^{NTN}	100	99	JF927761.1
				LR	PVY^{N}-W	100	99	HQ912896.1
				Yarumal_varB	PVY^{NTN}	100	100	KX184819.1
	NWO-KE1-7	565	4064 – 4611	F89II	PVY^{NTN}	100	99	KX184819.1
				I-17	PVY^{NTN}	100	99	KT599908.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				GBVC_PVY_37	PVY^{NTN}	100	99	JQ969033.2
				Eu-12Jp	PVY^{Z}-NTN	100	99	AB702945.1
			9656 – 9673	Yarumal_varB	PVY^{NTN}	100	100	KX184819.1
				Yarumal	PVY^{NTN}	100	100	KX184819.1
				F89II	PVY^{NTN}	100	100	KX184819.1
				F65	PVY^{NTN}	100	100	KX184819.1
				F17	PVY^{NTN}	100	100	KX184817.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

Cont. Table 3. Results of GenBank searches with BLASTn for sequences of PVY D-RNAs

Sample ID	D-RNAs	Fragment length[a]	Genomic coverage[b]	Matched isolates	Strain group[c]	% Query cover	% identity	GenBank Accession no.
NNW-KE2	NNW-KE2	4024	4064 – 7666	IUNG-4	PVY^{NTN}	100	99	JF927752.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				VNP413	PVY^{NTN}	100	99	HG810950.1
				IUNG-15	PVY^{NTN}	100	99	JF927763.1
				IUNG-8	PVY^{NTN}	100	99	JF927756.1
			9250 – 9673	IUNG-4	PVY^{NTN}	100	100	JF927752.1
				IUNG-8	PVY^{NTN}	100	100	JF927756.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				IUNG-15	PVY^{NTN}	100	99	JF927763.1
				11627-12	PVY^{NTN}	100	99	KC634007.1
NNW-KE266	NNW-KE266	1946	4150 – 5831	VNP413	PVY^{NTN}	100	99	HG810950.1
				3D	PVY^{NTN}	100	99	KJ946936.2
				9703-4	PVY^{NTN}	100	99	KC296441.1
				IUNG-4	PVY^{NTN}	100	99	JF927752.1
				HN1	PVY^{NTN}	100	99	HQ631374.1
			9345 – 9609	3D	PVY^{NTN}	100	100	KJ946936.2
				IUNG-4	PVY^{NTN}	100	100	JF927752.1
				VNP413	PVY^{NTN}	100	99	HG810950.1
				I-17	PVY^{NTN}	100	99	KT599908.1
				1101	PVY^{NTN-NW}	100	99	KC296434.1
NW-DeI (Dutch)	NW-DeI	2198	4064 – 5118	IUNG-2	PVY^{N}-W	100	99	JF927750.1
				GBVC_PVY_34	PVY^{N}-W	100	99	JQ969041.2
				IUNG-5	PVY^{N}-W	100	99	JF927753.1
				09-3a	PVY^{N}-W	100	99	JF795485.1
				SASA207	PVY^{N}-W	100	99	AJ584851.1
			8446 – 9589	IUNG-2	PVY^{N}-W	100	99	JF927750.1
				GBVC_PVY_34	PVY^{N}-W	100	99	JQ969041.2
				IUNG-5	PVY^{N}-W	100	99	JF927753.1
				09-3a	PVY^{N}-W	100	99	JF795485.1
				SASA207	PVY^{N}-W	100	99	AJ584851.1

[a]Length excluding forward and reverse primers. [b]Genomic position is based on our alignment. [c]Strain grouping of GenBank matched isolates were done based on information provided by Lorenzen *et al*. (2008), Hu *et al*. (2009), Karasev *et al*. (2011), Kerlan *et al*. (2011), Galvino-Costa *et al*. (2012), Kehoe and Jones (2016) and our findings.

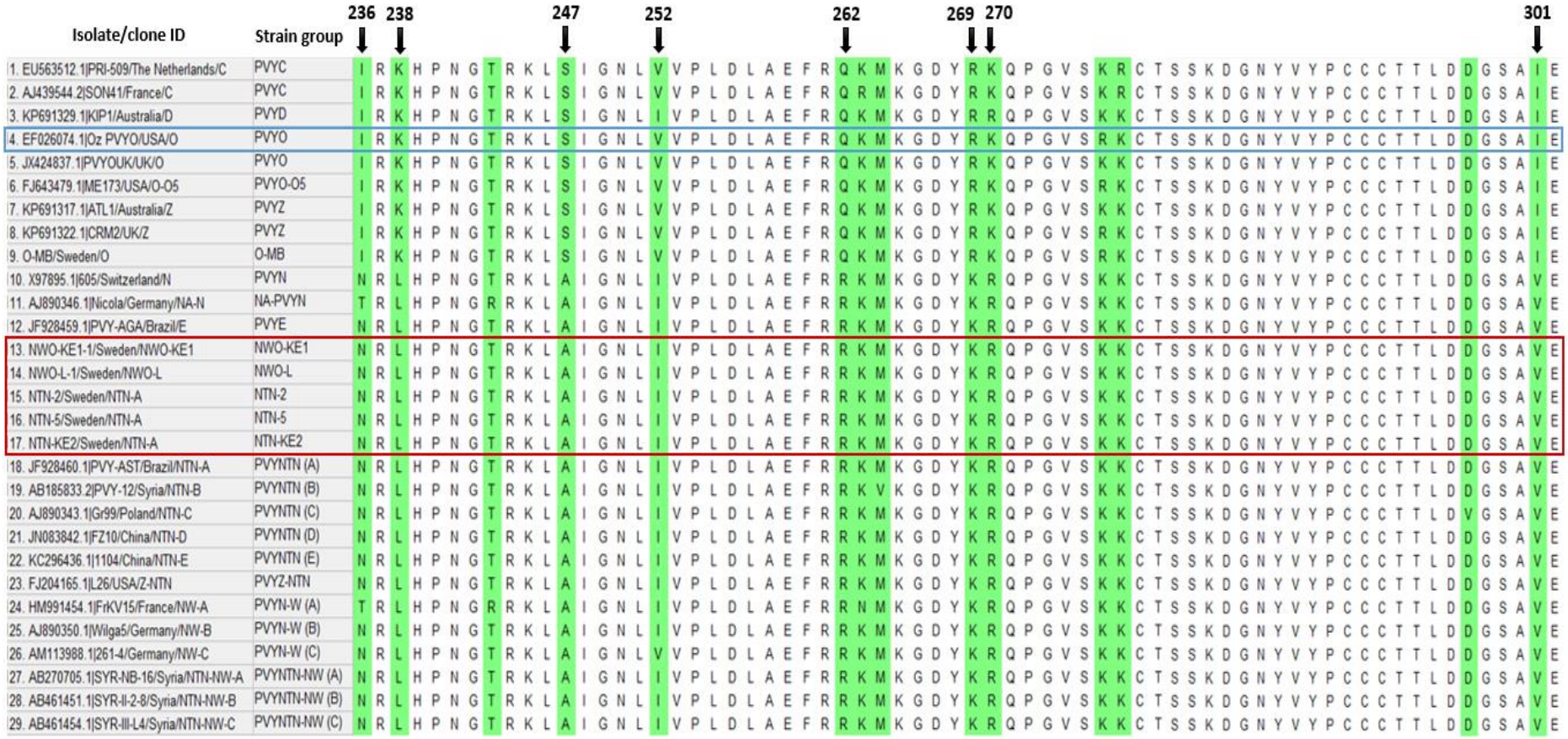

Figure 3. Amino acid sequence alignment of HC-Pro residues 236 to 302 of PVY isolates from all strain groups, including the Swedish isolates O-MB, NTN-2, NTN-5, NWO-KE1-1, NTN-KE2 and NWO-L-1 sequenced in this study. The Swedish isolate O-MB was found to have the amino acid residues N_{236}, L_{238}, A_{247}, I_{252}, R_{262}, K_{296}, R_{270} and V_{301} previously determined to be responsible for overcoming resistance by the *Ny$_{tbr}$* gene (Tian and Valkonen, 2013). The Swedish isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2 and NWO-L-1 were found to have the amino acid residues I_{236}, K_{238}, S_{247}, V_{252}, Q_{262}, R_{296}, K_{270} and I_{301} determined to be responsible for triggering the *Ny$_{tbr}$* gene (Tian and Valkonen, 2013). Isolate O-MB is marked in blue and the Swedish PVY^{NTN} isolates are marked in red.

Table 4. Sequenced PVY D-RNA molecules

D-RNA Pattern	D-RNA	Deleted region[a]	Deletion size[b]	Sequence motif[c]	Defective cistrons	Strain group
D-RNA (1)	NNW-KE2	7667 – 9249	1582	CATTGAA	NIb and CP	PVY^{NTN}
D-RNA (2)	NWO-KE1-5	5638 – 8394	2756	ACAGCA	CI to NIb	NG[d]
D-RNA (3)	NWO-KE1-2	5218 – 8125	2907	Genome duplication[e]	CI to NIb	PVY^{NTN}
D-RNA (4)	NWO-KE1-4	5731 – 8725	2994	TCCAAAA	6K2 to CP	PVY^{NTN}
D-RNA (5)	NW-DeI	5119 – 8444	3325	AAGCA	CI to NIb	PVY^{N}-W, PVY^{O}
D-RNA (6)	NWO-KE1-3	4496 – 8449	3953	ATGGCA	CI to NIb	NG
D-RNA (7)	NNW-KE266	5218 – 9344	4126	TGCATAC	CI to CP	NG
D-RNA (8)	NTN-5-2	4292 – 9316	5024	AGAGAGG	CI to CP	PVY^{NTN}, PVY^{Z}-NTN
D-RNA (9)	NWO-KE1-7	4612 – 9656	5044	GTGGT	CI to CP	NG
D-RNA (10)	NWO-KE1-6	4260 – 9409	5149	AAGTATT	CI to CP	NG

[a]Deleted nucleotide region based on our alignment. [b]Deletion size in base pair (bp). [c]Nucleotide sequence motifs found at one end for the sequenced PVY D-RNAs and repeated at both ends of the corresponding deletion junction sites in wild-type genomes. [d]Numerous strain groups including PVY^{NTN}, PVY^{Z}-NTN, PVY^{NTN-NW}, PVY^{N}-W, PVY^{E}, PVY^{O} and PVY^{Z}. [e]Duplication of 383 nt located within the CI cistron at nucleotide positions 4193 – 4575.

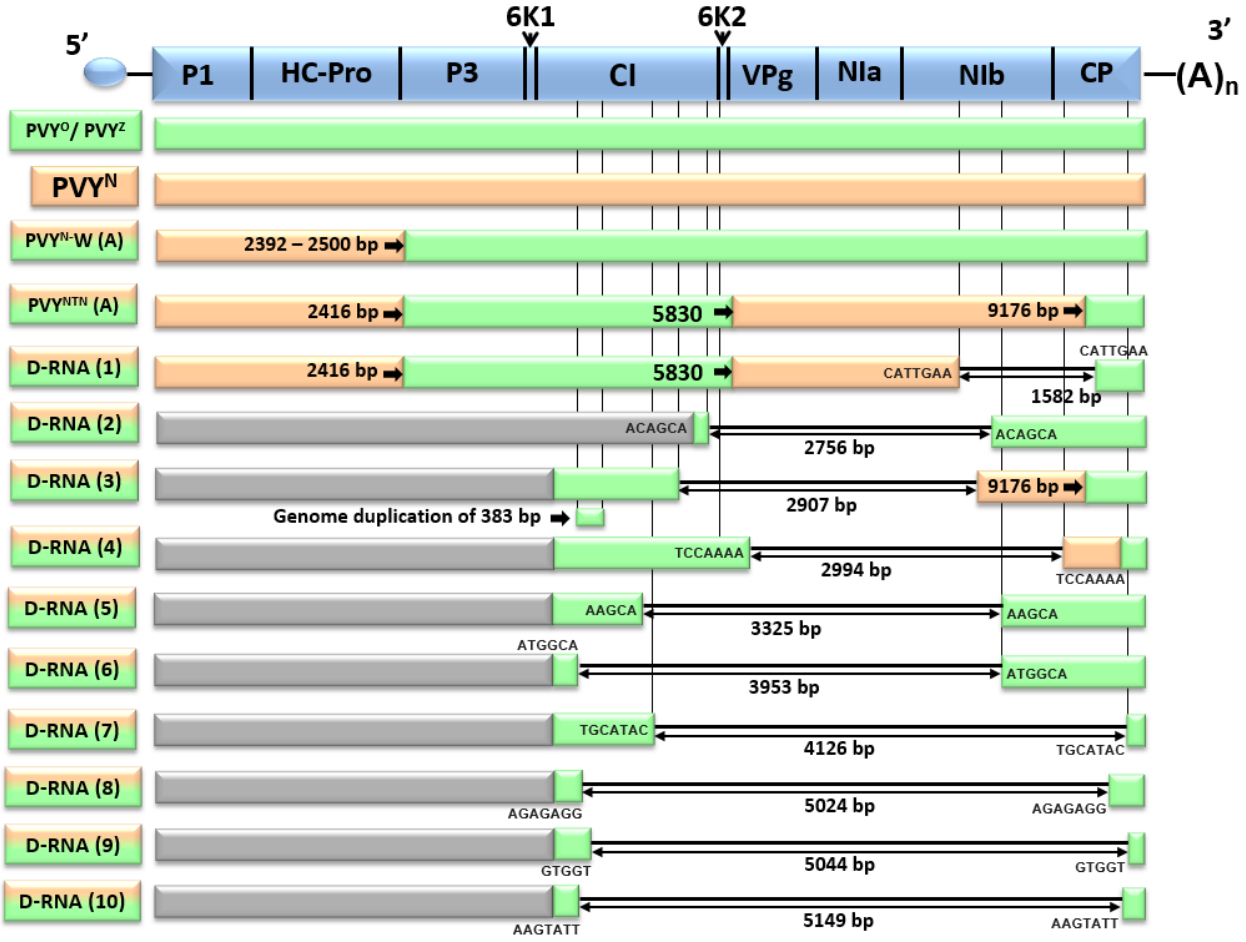

Figure 4. Genomic structure of PVY^{O}, PVY^{N}, PVY^{N}-W (A) and PVY^{NTN} (A), together with the ten identified PVY D-RNA patterns. Light green colour represents the PVY^{O} or PVY^{Z} strains, orange colour represents the PVY^{N} strain and grey colour represents a PVY genotype of unknown strain group. Positions of recombinant junctions (RJs) between PVY^{O} and PVY^{N} are marked. Positions and sizes (bp) of deleted genomic regions as well as nucleotide sequence motifs flanking deletion junction sites are indicated.

3.2. Phylogenetic analyses

In phylogenetic analyses of sequences covering genomic positions 180 – 4033, the Swedish isolate O-MB clustered with PVY^{Z} isolate CRM2 and PVY^{O} isolate SCRI-O from the UK, and were in phylogenetic group $PVY^{O/Z}$ together with isolates of PVY^{O}-O5, PVY^{C} and PVY^{D} (Fig. 5). The Swedish isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2 and NWO-L-1 clustered with isolates of PVY^{NTN} (A) and PVY^{Z}-NTN, and were in phylogenetic group PVY^{NTN}. Furthermore, NNW-KE3 showed a close relationship with PVY^{N}-W (A) isolate FrKV15 from France in phylogenetic group PVY^{NTN} (Fig. 5).

In phylogenetic analyses for sequences covering genomic positions 4064 – 9589, isolate O-MB again clustered with PVY^{O} isolate SCRI-O. NWO-L-2 clustered with PVY^{Z} isolate CRM2 and NNW-KE6 clustered with isolates of the PVY^{N}-W strain (Fig. 6). Isolate O-MB as well as NNW-KE6 and NWO-L-2, and isolates of the PVY^{O}, PVY^{Z} and PVY^{N}-W strains were in phylogenetic group $PVY^{O/Z}$. The Swedish isolates NTN-2, NTN-5 and NTN-KE2 clustered with isolates of the variant PVY^{NTN} (A), and were in phylogenetic group PVY^{NTN}. NNW-KE278 clustered with isolates of the PVY^{Z}-NTN strain and different variants of PVY^{NTN} in phylogenetic group PVY^{NTN} (Fig. 6).

The results of the two phylogenetic analyses (covering genomic positions 180 – 4033 and 4064 – 9589) confirmed the presence of the PVY^{NTN} strain in Sweden. Isolate O-MB, and isolates of the PVY^{O}, PVY^{Z} and PVY^{N}-W strains were in phylogenetic group $PVY^{O/Z}$ (Fig. 6). NWO-L-2 clustered with PVY^{Z} isolate CRM2 and NNW-KE6 clustered with isolates of the PVY^{N}-W strain in the phylogenetic group $PVY^{O/Z}$. Isolates NTN-2 and NTN-5 as well as isolates NTN-KE2 and NWO-KE1-1 were found to be closely related based on the phylogenetic relationships (Fig. 6).

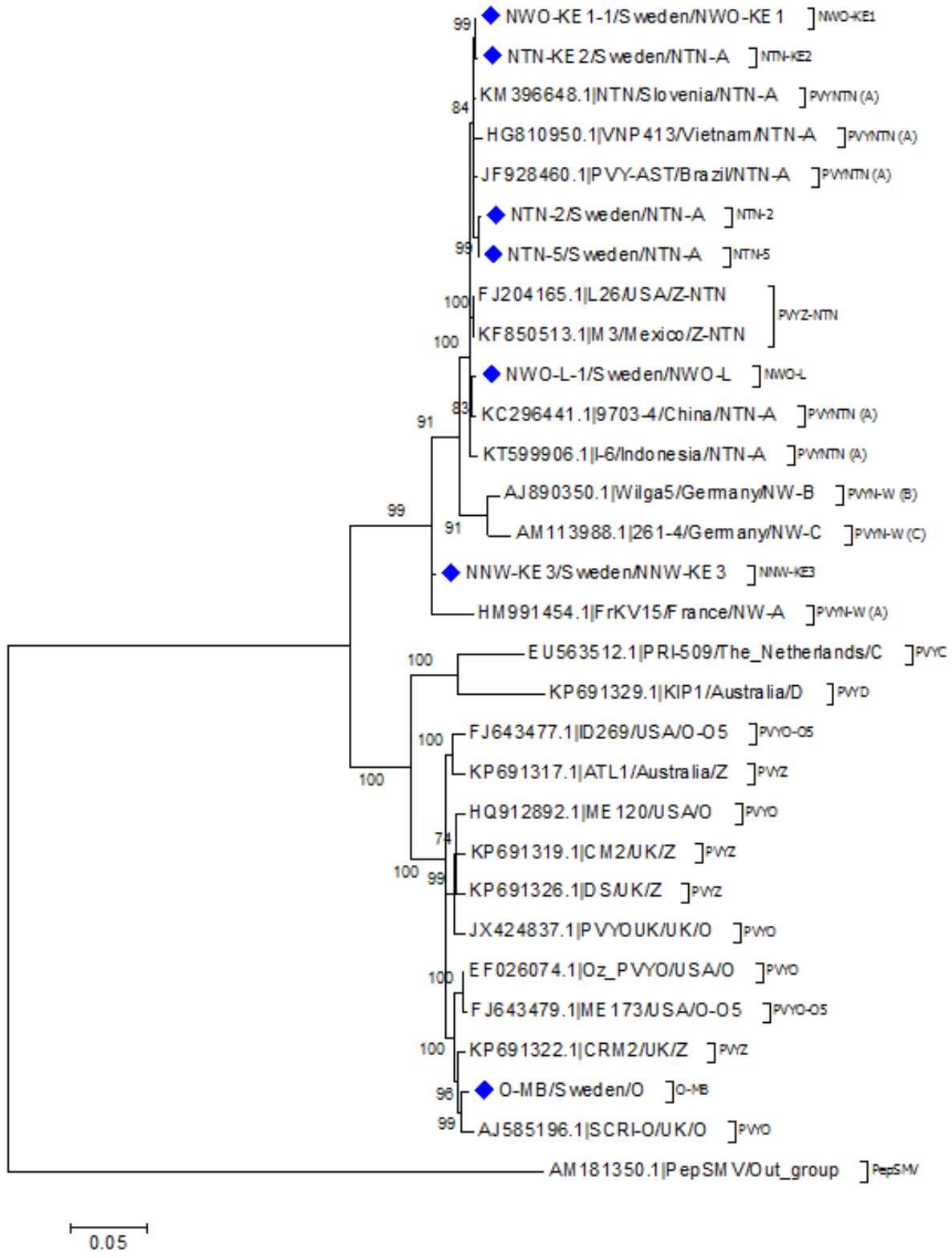

Figure 5. Phylogenetic analysis by Maximum-Likelihood method of nucleotide sequences covering genome positions 180 – 4033 of 22 PVY isolates from GenBank, together with the Swedish isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2, NNW-KE3, NWO-L-1 and O-MB. The percentages of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. Branches with low bootstrap value (less than 70%) have been collapsed. Branch length is drawn to scale with the bar indicating 0.05 nt substitutions per site. The nucleotide sequence of an isolate of PepSMV (Ahn *et al*. 2006) was used as an outgroup. Swedish PVY isolates are indicated.

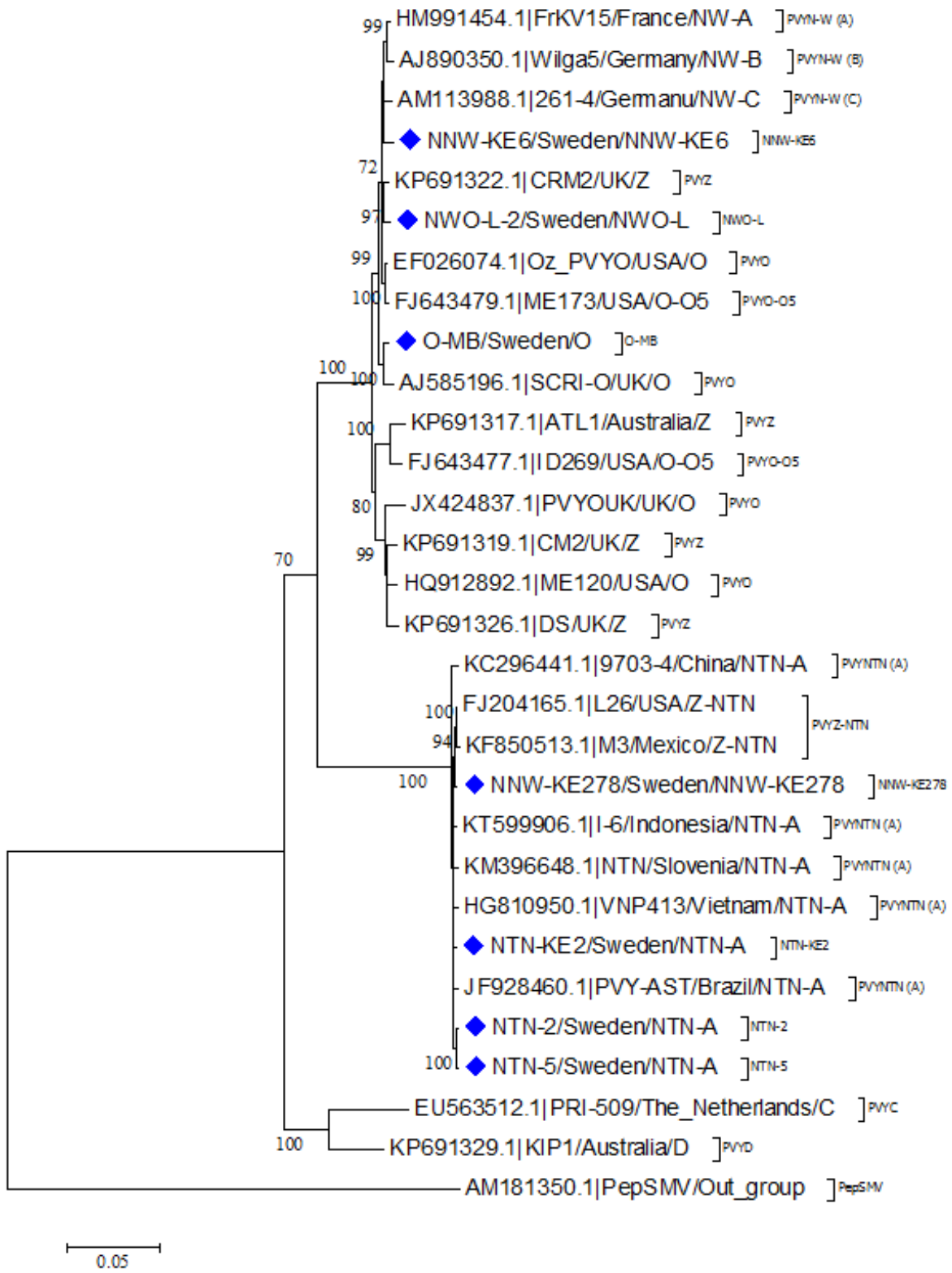

Figure 6. Phylogenetic analysis by Maximum-Likelihood method of nucleotide sequences covering genome positions 4064 – 9673 of 22 PVY isolates from GenBank, together with the Swedish isolates NTN-2, NTN-5, NTN-KE2, NNW-KE6, NNW-KE278, NWO-L-2 and O-MB. The percentages of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. Branches with low bootstrap value (less than 70%) have been collapsed. Branch length is drawn to scale with the bar indicating 0.05 nt substitutions per site. The nucleotide sequence of an isolate of PepSMV (Ahn *et al*. 2006) was used as an outgroup. Swedish PVY isolates are indicated.

3.3. Recombination analysis

No recombination breakpoints were detected for the Swedish isolate O-MB, which is similar to isolates from PVY^{O}, PVY^{O}-O5 and PVY^{Z}. Recombination analysis of NTN-2, NTN-5 and NTN-KE2 revealed that these isolates are putative recombinants with three RJs at nucleotide positions 2416 located within HC-Pro/P3, at 5830 in VPg and at 9176 in the CP. This is similar to isolates of the variant PVY^{NTN} (A) and of the PVY^{Z}-NTN strain (Table 5; Appx. Table B).

Table 5. Putative recombination breakpoint sites of Swedish PVY genomes detected by recombination detection programmes

Isolate ID	Strain Group	Recombination site and corresponding cistron		*P*-value[b]	Length (nt)	Possible parents
		Recombination site (s)[a]	**Corresponding cistron (s)**			
NTN-2	PVY^{NTN}	2416, 5830, 9176	HC-Pro/P3, VPg and CP	5.5×10^{-12}	9547	PVY^{O}, PVY^{Z} and PVY^{N}
NTN-5	PVY^{NTN}	2416, 5830, 9176	HC-Pro/P3, VPg and CP	1.45×10^{-12}	9637	PVY^{O}, PVY^{Z} and PVY^{N}
NTN-KE2	PVY^{NTN}	2416, 5830, 9176	HC-Pro/P3, VPg and CP	7.3×10^{-12}	9548	PVY^{Z} and PVY^{N}
O-MB	PVY^{Oc}	None-recombinant	-	-	9516	PVY^{Z}

[a]Nucleotide positions of recombination breakpoints. [b]Greatest *P*-value among recombinants identified by the recombination-detecting programmes RDP, GENECONV, BootScan, MAXChi, Chimaera, SiScan and PhylPro implemented in RDP4. [c]Isolate O-MB is primarily classified as a resistance-breaking PVY^{O} isolate, and not as PVY^{Z}, as it has not been tested before for its ability to trigger the *Nz* gene.

4. Discussion

In this thesis, the complete coding sequence of one resistance-breaking PVY^{O} isolate (O-MB) and three PVY^{NTN} isolates (NTN-2, NTN-5 and NTN-KE2) from Sweden were determined. In addition, partial genome sequences of six Swedish PVY isolates and ten sequences of PVY D-RNAs, including one Dutch PVY D-RNA, were determined. To the best of our knowledge, this is the first report of PVY D-RNAs. The nearly complete genome sequences of NTN-2, NTN-5 and NTN-KE2 were found to vary in length (9547 – 9637 nt). The difference in length of the Swedish PVY^{NTN} isolates occurred as a result of mis-priming, as the reverse primer HR-4063 annealed (at genomic position 386) as a forward primer, instead of primer 5'NTR (genomic position 1). A full-length fragment of 5606 bp (genomic positions 4034 – 9706) using the primer pair HF/3'NTR was obtained for samples NTN-2, NTN-5, NNW-KE2, NNW-KE6, NNW-KE278, NWO-L and NWO-MB. A fragment of 5606 bp was not amplified for sample NWO-KE1, although high-quality RNA extracted at different stages of viral infection was utilized and the PCR assay was run under optimal conditions. Instead several PCR fragments of different molecular weights were amplified with the largest size being of 3053 bp. These results may suggest that there was no amplification of the full-length fragment, because the D-RNAs were preferentially amplified in the PCR.

The preliminary classification of Swedish isolates as belonging to PVY^{NTN} using serology, multiplex-RT-PCR and inoculation tests (Youssef, 2017) was confirmed in this study by sequence analyses. The complete coding sequences of NTN-2, NTN-5 and NTN-KE2 isolates, and partial genome sequences of NWO-KE1-1, NNW-KE278 and NWO-L-1 shared an amino acid identity of 99% to isolates of the PVY^{NTN} strain. The complete coding sequence of O-MB and partial genome sequence of NWO-L-2 shared amino acid identity of 99% with the PVY^{O} and PVY^{Z} strains, and two partial genome sequences shared 99% with the PVY^{N}-W strain. The Swedish isolates O-MB, NNW-KE6 and NWO-L-2, together with isolates belonging to the PVY^{O} and PVY^{Z} strains were in phylogenetic group $PVY^{O/Z}$.

Despite the fact that the PVY^{O} and PVY^{Z} strains have shown different biological properties after inoculation on potato cultivars carrying the genes *Ny*$_{tbr}$ and *Nz*$_{tbr}$ (Kehoe and Jones, 2016), they clustered in the same phylogenetic group, $PVY^{O/Z}$. In a similar manner, for phylogenetic analyses of sequences covering genomic positions 180 – 4033, isolates of the PVY^{C} and PVY^{D} strains were in phylogenetic group $PVY^{O/Z}$. These findings revealed a disagreement between biological and phylogenetic grouping of PVY strains/variants. Isolates NTN-2, NTN-5 and NTN-KE2 as well as isolates NWO-KE1-1, NNW-KE278 and NWO-L-1 were in phylogenetic group PVY^{NTN}. It was found that NTN-2 and NTN-5 as well as NTN-KE2 and NWO-KE1-1 are closely related based on the phylogenetic relationships. Isolates NTN-2 and NTN-5 originated from the same tuber source, whereas NWO-KE1-1 and NTN-KE2 originated from the same seed lot (Youssef, 2017).

Recombination analyses revealed that the Swedish PVY^{NTN} isolates NTN-2, NTN-5 and NTN-KE2 have typical features of the variant PVY^{NTN} (A) and the PVY^{Z}-NTN strain with three RJs located within the HC-Pro/P3, VPg and CP cistrons, which are the same RJs as

numerous PVY^{NTN} (A) isolates from Europe (Kamangar *et al*., 2014). These findings confirmed the presence of PVY recombinant genotypes, such as PVY^{NTN}, in Sweden, as has been reported in several countries from Europe, *e.g.* the Netherlands, Scotland and Belgium (van der Vlugt *et al.*, 2008; Davie, 2014; Kamangar *et al*., 2014). In addition, isolate O-MB was found to be a non-recombinant, which is similar to isolates from PVY^{O}, PVY^{O}-O5 and PVY^{Z} (Karasev *et al*., 2011; Ogawa *et al*., 2012; Kehoe and Jones, 2016). It was assumed that novel symptoms observed in PVY-infected potato plants were associated with the presence of new PVY recombinants in Sweden (Youssef, 2017), but no new recombination patterns were detected in this thesis. Instead, numerous PVY D-RNAs were found in different potato samples.

The large ORF of NTN-2, NTN-5 and O-MB encodes a putative polyprotein of 3061 aa, which is similar to PVY isolates from all strain groups. The presence of a nonsense mutation at position 2277 generated a stop codon (UAG) that led to the split of the large ORF of NTN-KE2 into two ORFs encoding two putative proteins of 2299 aa and 695 aa. Presence of nonsense mutations may lead to the generation of non-functional viruses, which require a helper virus for its replication. In order to verify these findings, more clones from sample NNW-KE2 are needed to be sequenced and checked for the presence of this nucleotide substitution. The length of the ORF P3N-PIPO has been found to vary among PVY isolates (Cuevas *et al*., 2012). The ORF P3N-PIPO for the Swedish isolates (NTN-2, NTN-5, NWO-KE1-1, NTN-KE2, NWO-L-1 and O-MB) and several PVY isolates from different strain groups was identified to encode a putative protein of 76 aa.

Based on serological tests, multiplex RT-PCR and sequencing of PCR fragments, sample NWO-MB was previously found to have a mixed infection of PVY^{O}, PVY^{NTN} and PVY^{NTN-NW} (Youssef, 2017). Inoculation of potato plants of cv. Désirée suggested that the PVY^{O} variant in this sample was breaking the resistance of the Ny_{tbr} gene, because no visible HR was triggered and the inoculated plants became systemically infected (Youssef, 2017). Sequencing of the complete PVY^{O} coding region of this isolate revealed that the putative HC-Pro amino acid sequence contained the eight amino acid residues identified as being responsible for recognition by the Ny_{tbr} gene (Tian and Valkonen, 2013). Isolates of the PVY^{C} and PVY^{Z} strains have also been shown to overcome resistance based on the Ny_{tbr} gene (Dullemans *et al*., 2011; Kehoe and Jones, 2016), even if they have these eight amino acid residues in the HC-Pro protein. On the other hand, the Swedish isolates NTN-2, NTN-5, NWO-KE1-1, NTN-KE2 and NWO-L-1 were found to possess the eight amino acid residues required for overcoming resistance based on the Ny_{tbr} gene. Therefore, it is assumed that resistance to PVY by the Ny_{tbr} gene may be determined by numerous genetic determinants within and outside the HC-Pro cistron (Moury *et al*., 2011). Sequence comparisons revealed that O-MB differs from all studied isolates belonging to the PVY^{O} and PVY^{Z} strains by having a valine residue at position 90 in the HC-Pro protein, and from some isolates of the PVY^{O} and PVY^{Z} strains by having arginine at position 155 and glutamine at position 276 in the P3 protein. These amino acid residues may play a role in overcoming resistance by the Ny_{tbr} gene. I hypothesized that the non-recombinant PVY^{Z} strain and resistance-breaking PVY^{O} isolates have evolved from the ordinary PVY^{O} strain by mutations. In a similar manner, a tuber-necrosis-inducing isolate of

the NA-PVY^{N} strain was found to have evolved from a non-inducing NA-PVY^{N} isolate by mutations (Nie and Singh, 2003). It is also possible that the presence of PVY^{O} isolates in mixed infection with PVY recombinants, including D-RNAs, may lead to overcoming resistance by the *Ny$_{tbr}$* gene. This is especially because the resistance-breaking PVY^{O} variant was always found in mixed infections with various PVY recombinants. This hypothesis has to be proven by further inoculation studies. Furthermore, sequence comparisons revealed that the foliage-necrosis-inducing isolate NTN-2 differs from the non-inducing isolate NTN-5 by two amino acid residues located in the HC-Pro and VPg proteins. Therefore, it is suggested that these amino acid residues could be responsible for the induction of foliage necrosis in potato.

Recombination plays a crucial role in PVY evolution (Karasev and Gray, 2013). It is likely that D-RNA formation and genomic RNA-RNA recombination of RNA viruses both occur as a result of template switching or replicase jumping during regular replication (Pathak and Nagy, 2009) and it is assumed that similar processes occur for PVY. Biochemical assays have suggested that strong hairpin structures, AU-rich sequence (s) or breakpoints in the donor RNA promote template switching of the viral replicase to the acceptor RNA. The replicase utilizes short sequence (2 to 5 nt) complementarity between the donor and the acceptor templates as a primer for resumption of the replication on the acceptor RNA (Pathak and Nagy, 2009). For example, deletion in RNA-2 of BBMV is induced by duplication of a complementary sequence close to the base of the hairpin formed by duplication in the reverse order (Hull, 2002). Interestingly, the sequenced PVY D-RNAs were found to have nucleotide sequence motifs of 5 – 7 nt flanking deletion junction sites, except for D-RNA NWO-KE1-2. Instead, evidence of duplication was detected for NWO-KE1-2. These motifs were found to be repeated at both ends of the corresponding junction sites in wild-type genomes, which may induce deletion within the corresponding junction sites. Furthermore, *cis*-acting elements may also play a role in guiding template-switching events (Pathak and Nagy, 2009).

In this thesis, a total of ten different patterns of PVY D-RNAs from five potato samples were identified. The sequenced D-RNAs were found to have in-frame single-deletions ranging from 1582 – 5149 nucleotide residues in length spanning the regions NIb to CP, CI to NIb, 6K2 to CP and CI to CP. Single-deletions D-RNAs and DI-RNAs have been reported for numerous plant viruses from different families, such as AMV, BBMV and CMV from family *Bromoviridae* and CTV from family *Closteroviridae*.

CMV D-RNAs of RNA3 were found neither to be associated with virus accumulation nor symptoms intensity, whereas those associated with BBMV induced severe symptoms in some host plants (Hull, 2002). In this thesis, two group of PVY D-RNAs were found. The first group of PVY D-RNAs (patterns 1, 2, 3, 4, 5, 6, 9 and 10) were found to be associated with severe foliage symptoms in potato cv. Désirée. The second group of PVY D-RNAs (patterns 7 and 8) were found to be associated with mild foliage symptoms in potato cv. Désirée (Youssef, 2017). Hence, infectious clones of these D-RNAs are required to investigate the possible role (s) of D-RNAs in PVY pathogenicity/virulence, adaption and/or evolution. It is also worth investigating whether the PVY D-RNAs are encapsidated within the CP of the functional virus or not.

5. Conclusions

The variant PVY^{NTN} (A) was found in Sweden and seems to be common. Recombination analyses revealed no new recombination patterns. It is suggested that there are other genetic determinants responsible for triggering the *Ny_{tbr}* gene in potato other than the amino acid residues I_{236}, K_{238}, S_{247}, V_{252}, Q_{262}, R_{296}, K_{270} and I_{301} located in the HC-Pro protein. The amino acid residues valine at position 90 in the HC-Pro protein as well as arginine at position 155 and glutamine at position 276 in the P3 protein, together with other genetic determinants, may play a role in overcoming resistance by the *Ny_{tbr}* gene. It is also suggested that the non-recombinant PVY^{Z} strain and the resistance-breaking PVY^{O} isolates have evolved by mutation from the ordinary PVY^{O} strain. D-RNA molecules of PVY are common and could play an important role in virus pathogenicity/virulence, adaption and/or evolution.

6. Further Prospective

Studying the role of PVY D-RNAs in virus pathogenicity/virulence is necessary for a better understanding of PVY epidemiology and evolution. Identification of the genetic determinants in PVY that are responsible for overcoming resistance in cultivated potato cultivars is important for breeding. Developing new PCR-based diagnostic methods is required for rapid and efficient characterization of the Swedish PVY isolates.

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9. Appendices

Table A. Previously determined sequences for Swedish PVY isolates

Sample ID	Clone ID	Genomic coverage[a]	Genomic location	Host	Reference
NTN-2	NTN-2-22	180 – 769	P1	*S. tuberosum* cv. Unknown	Youssef (2017)
NWO-KE1	KE1-4	180 – 769	P1	*S. tuberosum* cv. King Edward	Youssef (2017)
NNW-KE2	KE2-21	180 – 769	P1	*S. tuberosum* cv. King Edward	Youssef (2017)

[a]Genomic coverage is based on our alignment after removing forward and reverse primers

Table B. Published potyvirus sequences included in the analyses

Strain	Isolate ID	Host	Country	GenBank accession no.	Reference
PVY^{Z}	ATL1	*Solanum tuberosum* cv. Atlantic	Australia	KP691317.1	Kehoe and Jones (2016)
	CM2	*S. tuberosum* cv. Cara	UK	KP691319.1	Kehoe and Jones (2016)
	CRM2	*S. tuberosum* cv. Pentland Crown	UK	KP691322.1	Kehoe and Jones (2016)
	DS	*S. tuberosum* cv. Desiree	UK	KP691326.1	Kehoe and Jones (2016)
PVY^{O}	ME120	*S. tuberosum*	USA	HQ912892.1	Karasev *et al*. (2011)
	PVY^{O}UK	*S. tuberosum*	UK	JX424837.1	Tian and Valkonen (2013)
	PVY^{O}-Oz	Unknown	USA	EF026074.1	Kerlan *et al*. (2011)
	SCRI-O	*S. tuberosum*	UK	AJ585196.1	Schubert *et al*. (2007)
PVY^{O}-O5	ID269	*S. tuberosum*	USA	FJ643477.1	Karasev *et al*. (2011)
	ME173	*S. tuberosum*	USA	FJ643479.1	Karasev *et al*. (2010)
PVY^{C}	Chile3	*C. baccatum* cv. Crystal	Chile	FJ214726.1	Moury (2010)
	PRI-509	*S. tuberosum* cv. Zeeuwse Blauwe	The Netherlands	EU563512.1	Dullemans *et al*. (2011)
PVY^{D}	KIP1	*S. tuberosum* cv. Kipfler	Australia	KP691329.1	Kehoe and Jones (2016)
PVY^{N}	605	*S. tuberosum*	Switzerland	X97895.1	Jakab *et al*. (1997)
NA-PVY^{N}	Nicola	*N. tabacum*	Germany	AJ890346.1	Schubert *et al*. (2007)
PVY^{E}	PVY-AGA	*S. tuberosum* cv. Agata	Brazil	JF928459.1	Galvino-Costa *et al*. (2012)
PVY-NE11	NE-11	Unknown	USA	DQ180180.1	Lorenzen *et al*. (2008)
PVY^{Z}-NTN	L26	*S. tuberosum*	USA	FJ204165.1	Hu *et al.* (2009)
	M3	*S. tuberosum* cv. Fianna	Mexico	KF850513.1	Quintero-Ferrer *et al*. (2014)
PVY^{NTN} (A)	9703-4	*N. tabacum*	China	KC296441.1	Schubert *et al.* (2015)
	I-6	*S. tuberosum* cv. Granola (Super John)	Indonesia	KT599906.1	Chikh Ali *et al*. (2016)
	NTN	*S. tuberosum* cv. Pentland Squire	Slovenia	KM396648.1	Kutnjak *et al*. (2015)

Cont. Table B. Published potyvirus sequences included in the analyses

Strain group	Isolate ID	Host	Country	GenBank accession no.	Reference
PVY^{NTN} (A)	PVY-AST	*S. tuberosum* cv. Asterix	Brazil	JF928460.1	Galvino-Costa *et al*. (2012)
	VNP413	*S. tuberosum*	Vietnam	HG810950.1	Schubert *et al*. (2015)
PVY^{N}-W (A)	FrKV15	*S. tuberosum*	France	HM991454.1	Kamangar *et al*. (2014)
PVY^{N}-W (B)	Wilga5	*S. tuberosum*	Germany	AJ890350.1	Schubert *et al*. (2007)
PVY^{N}-W (C)	261-4	*S. tuberosum*	Germany	AM113988.1	Schubert *et al*. (2007)
PVY^{NTN-NW} (A)	SYR-NB-16	*S. tuberosum* L.	Syria	AB270705.1	Chikh Ali *et al.* (2007)
PVY^{NTN-NW} (B)	SYR-II-2-8	Unknown	Syria	AB461451.1	Chikh Ali *et al.* (2010)
PVY^{NTN-NW} (C)	SYR-III-L4	Unknown	Syria	AB461454.1	Schubert *et al*. (2015)
PepSMV	PepSMV	*C. annuum*	South Korea	AM181350.1	Ahn *et al*. (2006)

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