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Presence of Recombinant *Potato Virus Y* Genotypes in Sweden

Asmaa Youssef

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ASMAA YOUSSEF

Supervisor: Prof. Dr. Anders Kvarnheden

Swedish University of Agricultural Sciences (SLU),
Department of Plant Biology

Examiner: Prof. Dr. Christina Dixelius

Swedish University of Agricultural Sciences (SLU),
Department of Plant Biology

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Cover picture: Symptoms of foliage necrosis on potato leaves and petal color-break of tobacco flowers. Photo: A. Youssef.

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Popular Science

Potato virus Y (PVY) is the most important and widespread virus infecting potato worldwide. PVY is a tuber-borne virus, and it is also spread mechanically and by aphid vectors of about 50 species. 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 causes a wide variety 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. PVY displays a high genetic diversity and has the ability to evolve overtime and form new strains through crosses (recombination) between different strains and through mutations. Recently, new PVY recombinant variants have emerged and their incidence is increasing throughout the world. There are numerous risks associated with the emergence of new strains/variants: overcoming resistance in potato, yield loss, reduction in tuber quality (due to potato tuber necrotic ringspot disease) …. etc.

For characterization of PVY isolates, 50 symptomatic potato samples were collected from different potato-growing regions in Sweden. All the collected samples were positive in the serological test using DAS-ELISA, except for three samples that did not react with any of the employed antibodies, but tested positive in the subsequent molecular analyses with PCR. Among 42 Swedish potato samples analysed by PCR, the tuber necrosis inducing PVY^{NTN} strain was the most common genotype with 19 samples having a single infection and 19 samples having mixed infection by PVY^{NTN} and one or several other PVY genotypes. PVY^{NTN} dominated among the analysed samples. In addition, resistance-breaking PVY^{O} isolates were detected in 12 out of 42 tested potato samples in mixed infections with other PVY genotypes. It can be concluded that the non-recombinant ordinary PVY^{O} in Sweden mainly has been replaced by recombinant PVY genotypes, such as PVY^{NTN}, as has been reported for many European countries.

A total of 36 samples were screened for their ability to induce potato tuber necrotic ringspot disease on different potato cultivars. Screening for necrotic ringspots was done on tubers developing from initial potato samples and on cv. Désirée tubers from inoculated plants. Out of 36 PVY-infected potato samples, tuber necrotic ringspots was observed for 10 samples of different potato cultivars either at harvest or after storage in dark at 23°C ± 1. These 10 potato samples were identified to contain the recombinant PVY^{NTN} strain as single or mixed infections. Furthermore, foliage necrosis was found to be associated with numerous potato samples identified to contain recombinant PVY genotypes, such as PVY^{NTN}.

Abstract

Potato virus Y (PVY) is the most important and widespread virus infecting potato (*Solanum tuberosum*) worldwide. Recently, new PVY recombinant genotypes have emerged and their incidence is increasing throughout the world. In Sweden, the non-recombinant PVY^{O} strain used to be most common, but it now seems to have been replaced by the recombinant genotypes, as has been reported for many European countries. This study intends to biologically, serologically and molecularly characterize Swedish PVY isolates from potato. To study the present genetic diversity of PVY in Swedish potato, 50 symptomatic potato samples from different parts of Sweden were selected for characterization of the infecting PVY genotypes using serology, multiplex-PCR, sequence analyses and inoculation tests. In DAS-ELISA, one sample was positive only for serotype O, 31 samples for only serotype N and 15 samples for both serotypes. None of the samples were positive for serotype C. Three samples did not react with any of the employed antibodies, but tested positive in the subsequent PCR analyses. To identify specific PVY genotypes, multiplex-PCR was used with diagnostic patterns for known genotypes. The identities of the amplification products were verified by sequencing. Among 42 Swedish samples analysed by PCR, PVY^{NTN} was the most common genotype with 19 samples having a single infection by PVY^{NTN} and 19 samples having mixed infection by PVY^{NTN} and one or several other PVY genotypes. PCR analyses also indicated frequent infections by PVY^{N}-W and other recombinants, most often in mixed infection with PVY^{NTN}. The previously dominant PVY^{O} strain was detected in 12 of the samples in mixed infections. In inoculation tests, extracts of all 16 tested samples were found to induce vein and stem necrosis on tobacco (*Nicotiana tabacum*), which is indicative of the N pathotype of PVY, including PVY^{NTN}. Leaf/tuber extracts of 12 samples initially identified to contain the PVY^{O} strain were inoculated into potato cv. Désirée (Ny_{tbr}:*nc:nz:*Nd_{tbr}) and screened for hypersensitive response (HR). Unexpectedly, no HR was observed in cv. Désirée for extract from any of these samples. These findings indicate the absence of PVY^{O} and PVY^{D} strains from the tested samples and that the PVY^{O} patterns obtained in the multiplex-PCR were generated by other related genotypes. Full-genome sequencing is required to be carried out to identify in more detail the exact PVY genotypes in selected samples. It can be concluded that PVY^{O} in Sweden mainly has been replaced by recombinant genotypes, such as PVY^{NTN} and PVY^{N}-W.

Table of Contents

List of Abbreviations

6K (1)	6 Kilo Dalton (KDa) viral protein (1)
6K (2)	6 KDa viral protein (2)
CI	Cytoplasmic inclusion
CP	Coat protein
cv.	Cultivar
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
DMSO	Dimethyl sulfoxide (C_2H_6OS)
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Extreme resistance
HC-Pro	Helper-component proteinase
HR	Hypersensitive response
In silico PCR	*In silico* Polymerase Chain Reaction
LB	Luria Broth
MEGA	Molecular Evolutionary Genetics Analysis
M-MuLV (MMLV)	Moloney Murine Leukemia Virus
NIa	First nuclear inclusion protein
NIb	Second nuclear inclusion protein
ORF	Open reading frame
P1	The first protein coded by the 5' region of a *Potyvirus* genome
P3	The third protein coded by the 5' region of a *Potyvirus* genome
P3N-PIPO	P3 N-terminal protein-pretty interesting *Potyviridae* ORF
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PTNRD	Potato tuber necrotic ringspot disease
PVY^{NTN}	PVY^{N} Tuber Necrosis
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RJs	Recombinant junctions
ROS	Reactive Oxygen Species
SASA	Science and Advice for Scottish Agriculture
Taq DNA Polymerase	*Thermus aquaticus* DNA Polymerase
TBE	Tris-Borate-EDTA
VPg	Viral protein genome-linked

1. Introduction

1.1. Potato production

Potato (*Solanum tuberosum*) is one of the major food crops in the world, including Sweden. The total cultivated area of potato in Sweden in 2013 was about 23,875 hectares distributed throughout the country, including 808 ha of organic potatoes, and with a total production of 806,000 tonnes (Jordbruksverket, 2014). Diseases and pests have a massive impact on crop yield. There are numerous pathogens infecting potato, *e.g.* viruses, oomycetes, fungi and bacteria, among which viruses are considered as being very significant. The majority of the potato pathogens are transmitted via tuber seeds and consequently there is a pressing need for producing certified pathogen-free tuber seeds in order to minimize infection levels. The Swedish needs of certified potato seed during 2012/2013 were about 17,402 tonnes (Jordbruksverket, 2014).

1.2. Potato viral diseases

There are around 40 known viruses infecting potato and among these viruses *Potato virus Y* (PVY) is one of the most significant ones, due to its worldwide spread and its significant effects on potato yield and quality (Valkonen, 2007; Karasev and Gray, 2013b; Quenouille *et al*., 2013). The spread of PVY in Sweden is expected to increase as a result of climate change favouring aphid vectors (Roos *et al*., 2011), emergence of recombinants capable of overcoming resistance genes in potato and the lack of efficient resistance in many of the common potato cultivars, *e.g*. King Edward and Asterix.

1.3. *Potato virus Y* (PVY)

1.3.1. PVY distribution and biology

PVY belongs to the genus *Potyvirus* in the family *Potyviridae*. PVY possesses a flexuous filamentous virion, approximately 730 – 740 nm in length and 11 nm in diameter. PVY has a wide range of host plants from 31 families comprising 495 species in 72 genera, including economically important solanaceous crop plants and ornamental plants: potato, tomato (*Solanum lycopersicum*), pepper (*Capsicum sp.*), tobacco (*Nicotiana tabacum*), *Physalis sp.* and *Petunia sp.* (Kerlan, 2006). The potyviral genome is a positive-sense, single-stranded RNA molecule of about 9.7 kb. The 5'-end of its genome is covalently linked to the VPg protein via a tyrosine residue and the 3'-end consists of a polyadenylated sequence. The potyviral genome is translated into a main single polyprotein chain of about 3062 amino acid residues. Subsequently, the polyprotein chain is cleaved by three viral proteases, P1 serine, HC-Pro cysteine and NIa-Pro cysteine proteinases, into ten multifunctional proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and CP). In addition, the potyviral genome contains the short ORF P3N-PIPO, which is generated by a +2 frameshift in the P3 cistron (Valkonen, 2007; Karasev and Gray, 2013a; Quenouille *et al*., 2013) (Fig. 1).

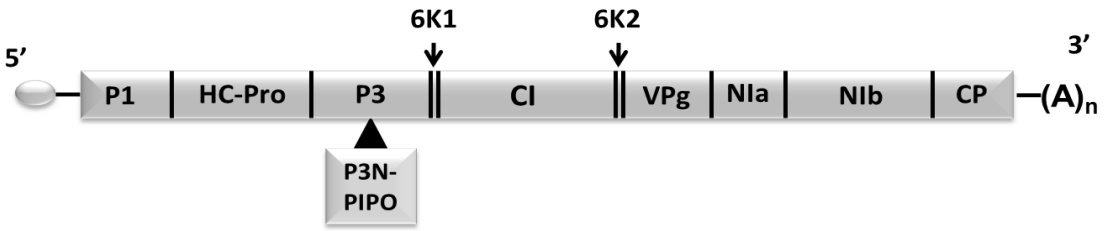

Figure 1. *Potato virus Y* (PVY) genome structure. P1: the first protein encoded by the 5' genomic region. HC-Pro (helper-component proteinase): the second encoded protein. P3: the third encoded protein. P3N-PIPO (P3 N-terminal protein-pretty interesting *Potyviridae* open reading frame (ORF)): A short ORF generated by a +2 frameshift in the P3 cistron. 6 K1 (6 Kilo Dalton (KDa) viral protein 1): the fourth encoded protein. CI (cytoplasmic inclusion): the fifth encoded protein. 6 K2 (6 KDa viral protein 2): the sixth encoded protein. VPg (viral protein genome-linked): the seventh encoded protein. NIa, the first inclusion body and the eighth encoded protein. NIb: the second inclusion body and the ninth encoded protein. CP (coat protein): the last encoded protein.

1.3.2. PVY economic significance

PVY causes a wide variety of symptoms on potato foliage ranging from symptomless or mild mosaic to defoliation and death. The level and severity of these symptoms are basically dependent upon PVY isolate/strain, degree of host-plant resistance/susceptibility, and presence of co-infection with other PVY strains and/or other viruses. In addition, PVY causes potato tuber necrotic ringspot disease, and this greatly affects tuber marketability (Karasev and Gray, 2013b). The potato tuber necrotic ringspot disease symptoms may be obvious already at harvest or develop during storage, especially when the growing and/or storage temperature is around 22 °C (Glais *et al*., 2015). Many isolates of PVY strains are known to cause the aforementioned disease, *e.g*. PVY^{NTN} (Karasev and Gray, 2013a; Quenouille *et al*., 2013). Yield losses caused by PVY infection can be up to 80%, depending on infection incidence of tuber seeds (vertical transmission), potato cultivar, PVY isolate, activity of aphid-vectors (horizontal transmission) and environmental conditions (Quenouille *et al*., 2013).

1.3.3. PVY transmission

PVY is a tuber-borne virus, and it is also spread mechanically and by aphid vectors in a non-persistent manner (Quenouille *et al*., 2013). PVY is transmitted by aphids of about 50 species. PVY acquisition and inoculation during aphid probing/feeding occur within a few minutes (Radcliffe and Ragsdale, 2002; Radcliffe and Lagnaoui, 2007). The green peach aphid, *Myzus persicae*, is the most efficient vector, whereas the efficiency of the bird-cherry oat aphid (*Rhopalosiphum padi*) in transmitting PVY is low compared with *M. persicae* (Sigvald, 1984; Verbeek *et al*., 2010; Nanayakkara *et al*., 2012). However, *R. padi* is considered to be the most significant vector species in Sweden for PVY, because it often occurs in high numbers (Sigvald, 1984; 1989).

1.3.4. PVY genetic diversity

PVY displays a high genetic diversity. There are six non-recombinant strain groups: stipple streak (PVY^{C}), the ordinary (PVY^{O}), tobacco veinal necrosis (PVY^{N}), the North American strain NA-PVY^{N}, PVY^{Z} and the recently identified strain PVY^{D}. In addition, there are recombinant strains: tuber necrosis inducing PVY^{N} (PVY^{NTN}), PVY^{N}-Wilga (PVY^{N}-W or $PVY^{N:O}$), PVY^{E}, PVY^{Z}-NTN, PVY^{NTN-NW} and PVY-NE11 (Chikh Ali *et al*., 2010b; Kerlan *et al*., 2011; Galvino-Costa *et al*., 2012; Quenouille *et al*., 2013; Kehoe and Jones, 2016). PVY has the ability to evolve overtime and form new strains through recombination between different strains and through mutations (Karasev and Gray, 2013a). For instance, there are numerous recombinants between the non-recombinant PVY^{O} and PVY^{N} strains, such as PVY^{NTN} and PVY^{N}-W, which subsequently have recombined to form the new recombinant strain PVY^{NTN-NW} (Chikh Ali *et al*., 2010a). These recombinant strains have different biological properties from their parents. There are different recombination structures of these strains; PVY^{Z}-NTN and PVY^{NTN} (A) harbour three recombination junctions (RJs) at HC-Pro/P3, VPg and the 3'-end of the CP cistron, whereas the second variant PVY^{NTN} (B) has the previously mentioned RJs and one additional RJ in P1 (Hu *et al*., 2009; Chikh Ali *et al*., 2010b; Kerlan *et al*., 2011) (Fig. 2). Historically, the non-recombinant PVY^{O} strain has been the most prevalent one in Sweden. During the recent decades, there has been a large increase in the incidence of recombinant strains, such as PVY^{NTN} and PVY^{N}-W, in Europe, *e.g*. in Poland, Germany, France, the Netherlands, Scotland and Belgium (Glais *et al*., 2005; Vlugt *et al*., 2008; Quenouille *et al*., 2013; Davie, 2014; Kamangar *et al*., 2014). Consequently, continuous and precise identification, as well as characterization of emergent strains, is tremendously important for controlling PVY spread and for subsequent breeding of virus-resistant cultivars, in order to minimize economic losses.

1.3.5. Natural sources of resistance against PVY

Plants have evolved numerous layers of defence against invading pathogens. The plant basal defence recognizes pathogen-associated molecular patterns (PAMPs) and elicits a range of plant defence responses. In turn, viruses evolve effectors (also called virulence proteins) to suppress plant defence. These effectors are recognized by the second layer of plant defence, strain-specific R genes (Valkonen,

2015). This model of interaction is known as gene-for-gene, which was identified by Flor (1946). R genes elicit rapid and effective defence responses that can be in form of extreme resistance (ER) or hypersensitive resistance response (HR). R genes are found in wild and cultivated potato species against PVY. The dominant PVY-specific *Ry* genes confer ER to all PVY strains, whereas strain-specific R genes confer resistance to specific PVY variants, with ER being epistatic to HR. Cultivars harbouring the dominant resistance gene Nc_{tbr}, *e.g.* King Edward, develop HR against PVY^{C}, those with the Ny_{tbr} gene, *e.g.* Désirée, develop HR against PVY^{O} and PVY^{O}-O5 and those with the Nz_{tbr} gene, *e.g.* Maris Bard, develop HR against PVY^{Z} and PVY^{Z}-NTN (Singh *et al*., 2008; Karasev *et al*., 2011; Kerlan *et al*., 2011). On the other hand, both PVY^{N} and PVY^{E} strains overcome the three resistance genes (Karasev and Gray, 2013a). The PVY^{N} strain is distinct from the strains PVY^{Z} and PVY^{O} by its ability to induce veinal necrosis in tobacco. Recently, a new PVY strain, PVY^{D}, has been suggested to elicit a new resistance gene, the Nd_{tbr} gene (Kehoe and Jones, 2016). Up to date, PVY proteins that are recognized by resistance genes of potato are still unknown (Valkonen, 2015).

1.3.6. Characterization of PVY strains

PVY is classified according to its serological, molecular and biological characteristics. Precise PVY characterization is carried out using different methods:

i. Serological assays are used for detection of the CP of PVY and there are specific antibodies for the serotypes C, O and N. The PVY^{C} strain reacts positively with serotype C, PVY^{O}, PVY^{NTN-NW} and PVY^{N}-W react with serotype O, and NA-PVY^{N}, PVY^{E}, PVY^{N}, PVY-NE11, PVY^{NTN}, PVY^{Z} and PVY^{Z}-NTN react with serotype N (Chikh Ali *et al*., 2007; Kerlan *et al*., 2011; Karasev and Gray, 2013a; Karasev and Gray, 2013b). Meanwhile, the reactivity of the PVY^{D} strain with PVY serotypes has not been reported yet. Enzyme-linked immunosorbent assay (ELISA) is the most common serological assay as being a rapid, cheap and efficient method for large scale monitoring of PVY (Karasev and Gray, 2013a).

ii. Molecular characterization is a fast and reliable method for differentiating PVY strains (Karasev *et al*., 2011; Chikh Ali *et al*., 2013). Molecular identification utilizing RJs as a tool for differentiating different strains through RT-PCR is an efficient method (Glais *et al*., 2005; Lorenzen *et al*., 2006a; Chikh Ali *et al*., 2010b; Karasev and Gray, 2013a). For more precise characterization of PVY isolates and for identification of new variants, identification methods based on genome sequencing may be necessary (Lorenzen *et al*., 2006b; Karasev *et al*., 2011; Gao *et al.*, 2014).

iii. Biological characterization with a set of potato cultivars harbouring different resistance genes is commonly used as a biological indicator for differentiating PVY strains and tobacco is used for identifying the pathotypes O and N. In addition, other solanaceous indicator plants are used, such as *Physalis sp*. and *Datura sp*. as well as *Chenopodium sp.* (Chenopodiaceae) (Singh *et al*., 2008).

Although ELISA may be used for PVY identification, it fails to differentiate the recombinant PVY^{NTN} from the non-recombinant PVY^{N} strain, or the recombinants PVY^{N}-W and PVY^{NTN-NW} from the non-recombinant PVY^{O} strain (Glais *et al*., 2005; Karasev *et al*., 2011; Chikh Ali *et al*., 2013). In addition, PCR-based identification methods could lead to inaccurate or wrong characterization (Chikh Ali *et al*., 2010b), especially under mixed infections of different variants. Therefore, PVY characterization should be done on the basis of serological, molecular and biological criteria. The aim of this study was to characterize PVY strains infecting potato in Sweden based on serological, molecular and biological criteria.

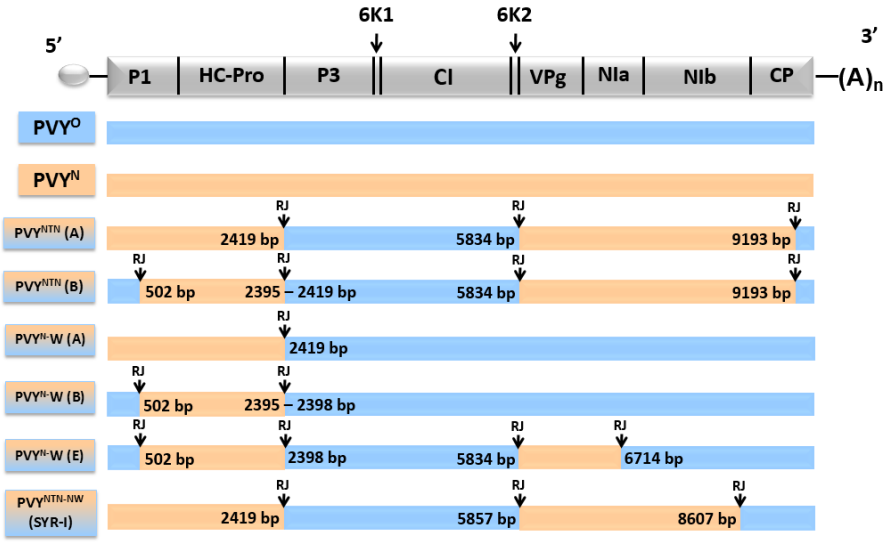

Figure 2. Genome structure for common strain/variants of *Potato virus Y* (PVY). Light blue colour represents PVY^{O} genotype and light orange colour represents PVY^{N} genotype. Positions of recombinant junctions (RJs) are indicated (Hu *et al*., 2009).

2. Materials and Methods

2.1. PVY sample sources

For characterization of PVY isolates, 45 symptomatic potato foliage samples, a black aphid (*Aphis fabae*) sample and five tuber samples from PVY-infected mother plants were collected from 13 locations in Sweden. Systemic foliage symptoms of the collected potato samples were scored according to visual observation (Table 1).

In 2015, five foliage samples of cv. King Edward (253, 266, 276, 278 and 287) were supplied from Stubbetorp Potatis through Uppsala Ecology Center. Six foliage and two tuber samples of cv. Solist as well as six foliage samples of cv. Sava were collected from a field in Ekhaga through Uppsala Ecology Center. In the same year, one aphid (*A. fabae*) and seven foliage samples (NTN-A (1), NNW-B, NWO-C, NTN-D, NTN-E, NWO-F3 and NTN-G) of unknown potato cultivars and one foliage sample of cv. Maritema (NWO-Mt) were collected. A potato plantlet growing outside Uppsala Ecology Center, Ultuna was found to be infested by three black aphids (*A. fabae*). The potato plant and the three *A. fabae* aphids were tested separately and given the identification code NTN-A (1 and 2, respectively). Sample NTN-G was grown in a field in Uppsala and collected from Uppsala Ecology Center, whereas the exact origin of sample NWO-Mt is unknown. Moreover, 15 foliage samples of numerous potato cultivars, *e.g*. Folva and King Edward, were collected by Ms Åsa Rölin from different locations in Sweden: Bodaholm, Brålanda, Enköping, Gagnef, Götlunda, Mora, Norrtälje, Säffle, Stora Skedvi and Uddevalla. Furthermore, five PVY-infected tubers of cv. Désirée were detected among imported tuber seeds from Bakker Holland, the Netherlands (Table 1).

In 2013, four symptomatic potato plants (King Edward 1, 2, 3 and 4) had been observed in the greenhouse at the Biocenter, Ultuna. By propagation through tubers, these virus isolates had been maintained in the greenhouse. Similarly, plants multiplied from a necrotic potato tuber collected on Öland in 2008 had been maintained in the greenhouse (NTN-0, NTN-2 and NTN-5).

Collected foliage samples exhibited various symptoms ranging from mosaic to leaf necrosis and death, whereas tuber samples showed no symptoms, except for samples NTN-0, NTN-2 and NTN-5 as tubers exhibited potato tuber necrotic ringspot disease. PVY infection was confirmed by ELISA as well as multiplex- and uniplex-PCR assays. To maintain PVY isolates, potato material was propagated by growing cuttings or through tubers under greenhouse conditions of 23 °C ± 2 and 16 h photoperiod.

Table 1. List of collected PVY-infected potato samples

Variety/Sample	Sample ID	Foliage Symptoms	Year of collection	Origin
Folva (1)	NTN-F1	Mo	2015	Norrtälje
Folva (2)	NTN-F2	Mo, CS	2015	Gagnef
King Utsäde (2)	NTN-KU	Mo, CS	2015	Norrtälje
Asterix (3)	NNW-A	Mo, CS	2015	Svallby, Enköping
Magnum Bonum (4)	NWO-MB	Mo	2015	Norrtälje
King Edward (5)	NNW-KE5	Ch, Mo	2015	Säffle
King Edward (6)	NNW- KE6	Mo, LN	2015	Säffle
King Edward (7)	NTN-KE7	Mo, CS, SN, SP	2015	Uddevalla
King Edward (8)	NNW-KE8	Mo, SSN	2015	Götlunda, Närke
King Edward (9)	NWO-KE9	Mo, CS	2015	Gagnef
King Edward (10)	E-KE10	Mo	2015	Brålanda, Västra Götaland
Solist (9A)	NNW-S9	Mo, LN	2015	Mora
Vivi (10)	NWO-V	Mo, SSN	2015	Stora Skedvi
Blue de la marche (11)	NWO-Bdlm	Mo, CS	2015	Säffle
Mandel (12)	NTN-Mn	Mo, LR, NL	2015	Säffle
Lurö (13)	NWO-L	Mo, NL	2015	Säffle
King Edward (253)	NTN-KE253	TNYL, Mo	2015	Stubbetorp, Motala
King Edward (266)	NNW-KE266	TNYL, Mo	2015	Stubbetorp, Motala
King Edward (276)	NTN-KE276	TNYL	2015	Stubbetorp, Motala
King Edward (278)	NNW-KE278	TNYL	2015	Stubbetorp, Motala
King Edward (287)	NTN-KE287	TNYL, ChYL, Mo	2015	Stubbetorp, Motala
Maritema	NWO-Mt	SeM	2015	Unknown
Solist (A)	NNW-SA	N.A.	2015	Ekhaga
Solist (B)	NTN-SB	N.A.	2015	Ekhaga
Solist (170)	NTN-S170	ChYL, Mo	2015	Ekhaga
Solist (171)	NTN-S171	ChYL	2015	Ekhaga
Solist (204)	N-S204	ChYL, Mo	2015	Ekhaga
Unknown (G)	NTN-G	M	2015	Uppsala
Sava (3)	N-S3	M, NL	2015	Ekhaga
Sava (4)	N-S4	M, NL	2015	Ekhaga
Sava (5)	N-S5	M, NL	2015	Ekhaga
Sava (6)	N-S6	M, NL	2015	Ekhaga
Sava (7)	N-S7	M, NL	2015	Ekhaga
Sava (10)	N-S10	M, NL	2015	Ekhaga
Solist (1)	N-S1	M, NL	2015	Ekhaga
Solist (2)	N-S2	M, NL	2015	Ekhaga
Solist (9B)	N-S9	M, NL	2015	Ekhaga
Désirée (I)	NW-DeI	Mo, SeSt	2015	The Netherlands
Désirée (II)	NWO-DeII	N.A.	2015	The Netherlands
Désirée (III)	N-DeIII	SeM, Mo	2015	The Netherlands
Désirée (IV)	O-DeIV	N.A.	2015	The Netherlands
Désirée (V)	O-DeV	M, Ch	2015	The Netherlands

Ch = chlorosis, ChYL = chlorosis in young leaves, CS = cup shape, LN = leaf necrosis, LR = leaf-roll, M = mosaic, Mo = mottling, N.A. = non-available, NL = necrotic lesions, SeM = severe mosaic, SeSt = severe stunting, SN = stem necrosis, SP = stem split, SSN = slight stem necrosis, TNYL = tip necrosis in young leaves.

Continued Table 1. List of collected PVY-infected potato samples

Variety/Sample		Sample ID	Foliage Symptoms	Year of collection	Origin
Unknown (A)		NTN-A (1)	M	2015	Ultuna, Uppsala
Aphis fabae		NTN-A (2)	N.A.	2015	Ultuna, Uppsala
Unknown (B)		NNW-B	Mo	2015	Ultuna, Uppsala
Unknown (C)		NWO-C	Ch	2015	Ultuna, Uppsala
Unknown (D)		NTN-D	Ch	2015	Ultuna, Uppsala
Unknown (E)		NTN-E	M	2015	Ultuna, Uppsala
Unknown (F)		NWO-F3	M	2015	Ultuna, Uppsala
King Edward (1)		NWO-KE1	Ch, Mo	2013	Ultuna, Uppsala
King Edward (2)		NNW-KE2	SM, Mo	2013	Ultuna, Uppsala
King Edward (3)		NNW-KE3	SM, Mo	2013	Ultuna, Uppsala
King Edward (4)		NWO-KE4	Ch	2013	Ultuna, Uppsala
Originate from the same tuber	Unknown (0)	NTN-0	Mo, LD, PTNRD	2008	Öland, Kalmar
	Unknown (2)	NTN-2	Mo, LD, PTNRD	2008	Öland, Kalmar
	Unknown (5)	NTN-5	Mo, LD, PTNRD	2008	Öland, Kalmar

Ch = chlorosis, ChYL = chlorosis in young leaves, LD = leaf death, M = mosaic, Mo = mottling, N.A. = non-available, NL = necrotic lesions, SeM = severe mosaic, SeSt = severe stunting, PTNRD = potato tuber necrotic ringspot disease.

2.2. Serological characterization of PVY samples

The collected samples were initially tested by double-antibody sandwich ELISA (DAS-ELISA) for the presence of PVY. The monoclonal $PVY^{O/C}$, PVY^{C} and PVY^{N} antibodies from SASA (Science and Advice for Scottish Agriculture) were employed for differentiating PVY strains. All antibodies were diluted (1:1000) and DAS-ELISA was run according to the manufacturer's instructions. Readings of plates were taken at 405 nm (OD_{405}) using a BIO-RAD Benchmark microplate reader at different time points after 10 – 135 min of incubation in dark at room temperature. Virus-free plant samples of potato, tobacco and quinoa (*Chenopodium quinoa*) were used as negative controls and a PVY-infected potato sample obtained from the Ecology Center in Uppsala was used as a positive control. Mean optical density values of tested samples, which were at least twice the mean value of the negative control were considered to be positive.

2.3. RNA extraction and multiplex-PCR

2.3.1. RNA extraction and Reverse Transcription

Young leaves from virus-free and infected plants, in addition to aphids, were utilized for RNA extraction using Spectrum plant total RNA kit (Sigma-Aldrich). Total RNA was extracted according to the manufacturer's instructions. The quality of the extracted RNA was checked by running 1 µl of the RNA extract on a 1.2% agarose gel, and the RNA concentration was measured using a NanoDrop. Reverse Transcription (RT) was carried out using 4 µl of the extracted RNA and M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's instructions and utilizing oligo $(dT)_{18}$ primer (Thermo Scientific) at a final concentration of 10 µM. The RT reaction was incubated at 37°C for 60 min, followed by 70°C for 10 min for terminating the reaction.

2.3.2. Multiplex- and uniplex-PCR conditions

The primary molecular classification was carried out on the basis of uniplex- and multiplex-PCR results (Glais *et al.*, 2005; Chikh Ali *et al*., 2010b; 2013) (Table 2, Fig. 3). Six primer pairs were utilized for

multiplex-PCR (Chikh Ali *et al*., 2010b) and one pair was separately employed for uniplex-PCR. Uniplex-PCR assay was run for detecting the presence of PVY^{N}-W using the primer pair W2253/W6343 (Glais *et al.*, 2005). A sample was considered to contain PVY^{N}-W when generating a band of 4114 bp in uniplex-PCR and/or two bands of 835 + 441 bp in multiplex-PCR assays. Four different DNA polymerases were used (see below). All primers were obtained from TAG Copenhagen A/S and used at a final concentration of 0.2 µM, while dNTP mix (Thermo Scientific) was used at a final concentration of 0.2 mM. *Taq* DNA polymerase was primarily used for multiplex-PCR assays, with PCR mix of 20 µl containing a final concentration of 0.02 U/µl *Taq* DNA polymerase (Thermo Scientific), 1.25 mM $MgCl_2$, 1x buffer, sterile MQ and 2 µl cDNA. DNA polymerases with high proofreading efficiency, Platinum *Pfx* DNA Polymerase (Invitrogen), Expand High Fidelity PCR System or Phusion High-Fidelity DNA Polymerase (Thermo Scientific) were also employed for multiplex- and uniplex-PCR assays, especially for amplifying PCR fragments, which were subsequently cloned and sequenced. The 20 µl PCR mix for Platinum *Pfx* DNA Polymerase contained a final concentration of 0.02 U/µl Platinum *Pfx* DNA Polymerase, 1 mM $MgSO_4$, 1x buffer, 0.45x enhancer, sterile MQ and 2 µl cDNA. The 20 µl PCR mix using Expand High Fidelity PCR System contained a final concentration of 0.035 U/µl Expand High Fidelity PCR System, 1.25 mM $MgCl_2$, 1x buffer, sterile MQ and 2 µl cDNA. The 20 µl PCR mix using Phusion High-Fidelity DNA Polymerase contained a final concentration of 0.02 U/µl Phusion High-Fidelity DNA Polymerase, 1 mM $MgCl_2$, 0.5x HF buffer, 0.5x GC buffer, 3% DMSO, sterile MQ and 2 µl cDNA. Multiplex- and uniplex-PCR conditions for Expand High Fidelity PCR System and Phusion High-Fidelity DNA Polymerase are shown in Table S1. PCR conditions for *Taq* DNA polymerase and Platinum *Pfx* DNA Polymerase were set as follows: initial denaturation of 2 min at 94 °C, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 2 min and a final extension of 10 min at 72°C. The amplification products were analysed by agarose gel electrophoresis.

Table 2. Multiplex- and uniplex-PCR product sizes (bp) and molecular classification of PVY according to Glais *et al*. (2005), Chikh Ali *et al*. (2010b; 2013)

PCR product size (bp)	Molecular classification	PCR product size (bp)	Molecular classification
1307 + 633 + 398	PVY^{N}	633	PVY-NE11
4114 + 853 + 633 + 441	PVY^{N}-W (A) = $PVY^{N:O}$	853 + 532	PVY^{O}
4114 + 853 + 441	PVY^{N}-W (B)	1076 + 633 + 441	PVY^{NTN-NW} (SYR-I)
4114 + 853 + 441 + 278	PVY^{N}-W (C)	1076 + 441	PVY^{NTN-NW} (SYR-II)
1307 + 633 + 441	PVY^{NTN} (A)	1076 + 441 + 278	PVY^{NTN-NW} (SYR-III)
1307 + 441	PVY^{NTN} (B)	633 + 441	PVY^{E}
1307	NA-PVY^{N}		

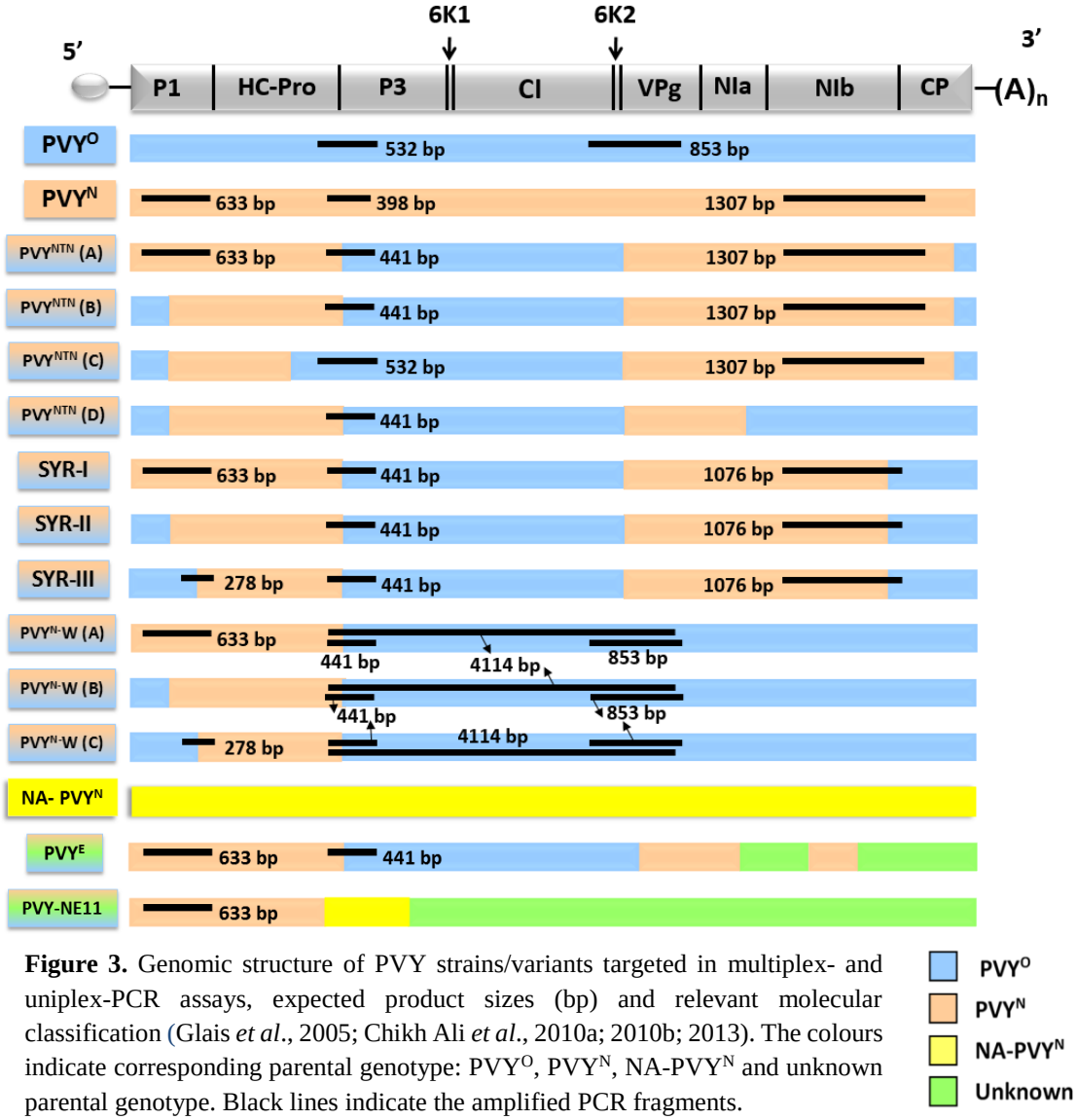

Figure 3. Genomic structure of PVY strains/variants targeted in multiplex- and uniplex-PCR assays, expected product sizes (bp) and relevant molecular classification (Glais *et al*., 2005; Chikh Ali *et al*., 2010a; 2010b; 2013). The colours indicate corresponding parental genotype: PVY^{O}, PVY^{N}, NA-PVY^{N} and unknown parental genotype. Black lines indicate the amplified PCR fragments.

2.4. Cloning of PCR fragments and sequence analysis

Multiplex-PCR fragments generated by high fidelity polymerases were cloned for sequencing. GeneJET PCR purification kit (Thermo Scientific) was utilized for purifying PCR products, and the DNA concentration was measured using a NanoDrop. In addition, 5 µl of the purified DNA was run on an agarose gel (1.2%) to check purity and concentration. The purified PCR product was ligated into pJET cloning vector using Clone JET PCR cloning kit (Thermo Scientific), and then transformed into commercial (Life Technology) or lab-made competent cells of *Escherichia coli* strain DH5α according to the manufacturer's instructions. Plasmid DNA was purified from overnight cultures of selected *E. coli* colonies using GeneJET plasmid miniprep kit (Thermo Scientific) and digested by Fast Digest BglII (Thermo Scientific). The digests were analysed by agarose gel electrophoresis. Clones with expected insert size were selected for sequencing utilizing Sanger sequencing technology at Macrogen (Amsterdam, The Netherlands). One to three clones for each sample and PCR product were sequenced. CLUSTAL-W in MEGA program version 6.0 (Tamura *et al*., 2013) was employed for multiple sequence alignment using default parameters. The obtained sequences were aligned after removing the forward and reverse primers. Sequence identity was determined using the nucleotide BLAST search engine offered by the National Center for Biotechnology Information (NCBI) {available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>}. Molecular classification of GenBank matched isolates was done based on information provided by Hu *et al*. (2009), Kerlan *et al*. (2011) and Schubert *et al*. (2015).

2.5. Biological assay and hypersensitive response (HR) test

Biological characterization of PVY isolates was done using the indicator plants tobacco (*Nicotiana tabacum*) cv. Samsun, quinoa and potato cv. Désirée. The potato cv. Désirée (*Ny_{tbr}:nc:nz:Nd_{tbr}*) was used because it has the corresponding resistance gene *Ny_{tbr}* against the ordinary PVY^{O} strain and the hypothetical resistance gene *Nd_{tbr}* against the PVY^{D} strain, and does not have the resistance genes *Nc_{tbr}* and *Nz_{tbr}* (Kehoe and Jones, 2016). Tobacco was utilized to screen for tobacco veinal necrosis and Désirée to screen for HR, which is elicited by the non-recombinant PVY^{O} strain. The PVY pathotype was determined on the basis of the capability to induce veinal necrosis on tobacco (Singh *et al*., 2008). Désirée tuber seeds were bought from Bakker Holland. Before virus inoculation, Désirée tuber seeds were tested by ELISA for PVY infection. Seeds of tobacco and quinoa were sown in plastic pots filled with S-jord substrate (Hasselforsgarden, Sweden). Ten days post-sowing, plantlets were transplanted individually into plastic pots (VEFI Europa) (each 7.9 cm × 7.8 cm × 6.2 cm; length × width × height). All plants were grown under greenhouse conditions of 23°C ± 2 and 16 h photoperiod. For inoculation, plant sap was extracted from 1 g of fresh PVY-infected leaves by grinding in 20 ml phosphate buffer (0.1 M). Two fully-expanded middle leaves of three to four plants of each species were dusted with carborundum and mechanically inoculated with plant extract of each sample. As mock treatment, three to four plants of each species were dusted with carborundum and inoculated with phosphate buffer. Virus-free Désirée plants were mechanically inoculated at eight/ten-leaf stage, tobacco at three/four-leaf stage and quinoa at the four/five-leaf stage. Foliar symptoms were monitored starting from seven days post-inoculation (dpi) and were daily recorded for 30 days for tobacco and quinoa, and for 45 days for Désirée. Infections of inoculated tobacco, quinoa, and potato cv. Désirée were validated by DAS-ELISA at 14 dpi for tobacco, 21 dpi for quinoa, and 30 dpi for Désirée. In DAS-ELISA, systemic leaves of tobacco and potato cv. Désirée were tested, and inoculated and systemic leaves of quinoa. Harvested potato tubers from virus-free and PVY-infected plants were stored in dark at 23°C ± 1 and scanned for potato tuber necrotic ringspot disease symptoms after four and eight weeks.

3. Results

3.1. Serological characterization

The presence of PVY infection in the potato samples was tested by DAS-ELISA. Out of 50 Swedish samples, one sample was positive for only serotype O, 31 of the samples were positive for only serotype N and 15 samples reacted positively for both serotypes (Table S2, Fig. 4.A). Four Swedish samples (NWO-C, NTN-D, NTN-E and NWO-F3) did not react with any of the employed antibodies (Table S2), but these samples were subsequently found to be positive for PVY infection using multiplex-PCR assay. Inoculation with extract of sample NWO-F3 resulted in PVY-infected potato plants of cv. Désirée, which were found to be positive for serotypes N and O. This positive DAS-ELISA test was obtained for sample NWO-F3 when systemic leaves from inoculated Désirée plants at 45 dpi were tested. Out of 21 Dutch Désirée tuber seed samples tested by DAS-ELISA, four samples were found to be PVY-infected with three samples (NW-DeI, O-DeIV and O-DeV) being positive for serotype O and one sample (N-DeIII) positive for serotype N. One Dutch sample (NWO-DeII) was PVY-free based on the DAS-ELISA tests, but was subsequently found to be PVY-infected using multiplex-PCR assay. None of the tested samples from Sweden or the Netherlands were positive for serotype C (Table S2).

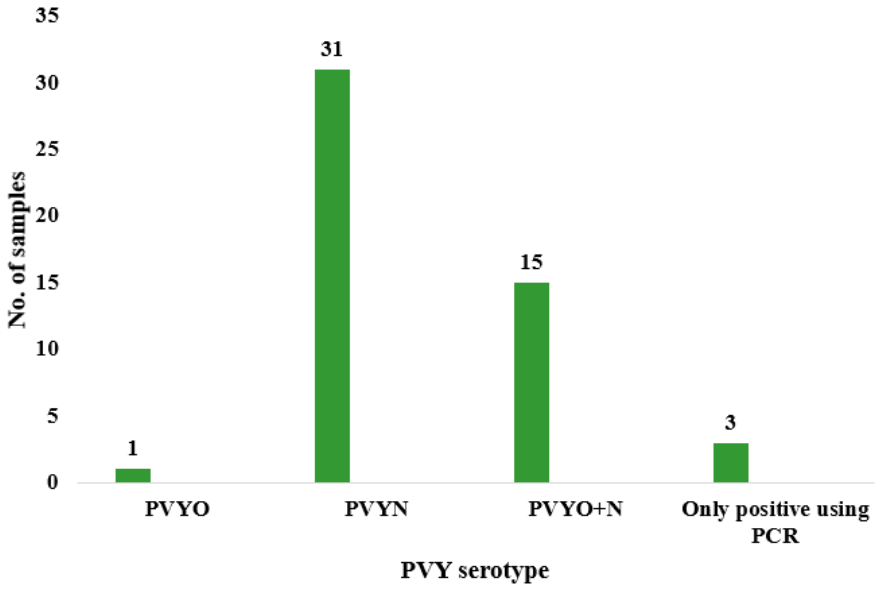

Figure 4.A. Percentage of PVY serotypes in potato samples collected from different regions in Sweden.

3.2. Molecular characterization and sequence analysis

For identifying PVY genotypes, 42 Swedish and two Dutch samples were tested by multiplex- and uniplex-PCR assays. Out of 42 Swedish samples, 19 were characterized to have PVY^{NTN} (A) and one sample was identified to contain PVY^{E}, as a single infection. One sample, NWO-F3, was identified to contain a mixed infection of PVY^{E} and PVY^{O}. One sample (N-S204) was identified to contain a mixed infection of NA-PVY^{N} and PVY-NE11 or the variant PVY^{NTN} (A) with a missing band of 441 bp in the multiplex-PCR assay. For two samples, NWO-L and NWO-MB, the PVY genotypes could not be properly identified, because a band of 441 bp was missing in the multiplex-PCR assay. However, NWO-L and NWO-MB seem to contain the PVY^{O} strain in mixed infections with PVY^{NTN} (A) and PVY^{NTN-NW} (SYR-I/SYR-II). In addition, 19 samples contained mixed infections of different PVY genotypes, *e.g*. PVY^{NTN} and PVY^{O} (Table 3, Fig. S1). Nineteen of the samples with mixed infection were identified to contain the variant PVY^{NTN} (A), 12 samples contained PVY^{O}, 11 samples contained PVY^{N}-W and eight samples contained PVY^{NTN-NW}. The variant PVY^{NTN} (A) was the most common genotype, as being found in 38 samples in single and mixed infections with other genotypes (Fig. 4.B). The Dutch sample NWO-DeII was identified to contain mixed infections of PVY^{O}, PVY^{NTN} (A) and PVY^{N}-W (A) or PVY^{N}-W (B) (Table 3, Fig. S1). The presence of mixed infections of PVY genotypes resulted in having numerous probabilities when analysing the RT-PCR results.

In the uniplex- and multiplex-PCR assays, additional unexpected bands were sometimes obtained. Two additional bands of 3079 and 6376 bp were generated for NNW-KE3, and an additional band of about 324 bp was amplified for many samples, *e.g*. NWO-KE1, in multiplex-PCR assay (Fig. S2). The primer pair W2253/W6343, which is specific for PVY^{N}-W (Wilga), amplified four to six bands (375 – 2500 bp) in uniplex-PCR assay for samples NNW-KE8, NWO-KE9 and E-KE10, but not the expected 4114 bp band (data not shown). The employed DNA polymerases differed in their sensitivity for detecting PVY genotypes present in the samples. Out of the four employed polymerases, Phusion High-Fidelity DNA Polymerase generated most bands, particularly for samples with mixed infections of different PVY genotypes (Fig. 4.A, Fig. S3).

Sequences were determined for cloned PCR fragments of samples NTN-0, NTN-2, NTN-5, NW-DeI, NWO-KE1, NNW-KE2, NNW-KE3, NWN-KE266, NNW-KE278 and NTN-S170. Sequence analyses of the majority of clones verified the expected genomic location and band length, with a few exceptions (Table S3). For instance, sequence analysis of at least two clones per sample of the 633 bp band, which is diagnostic for PVY^{N} and other recombinants such as PVY^{NTN} (A) and PVY^{N}-W (A), revealed the expected genomic location and band length. Meanwhile, sequence analysis of the unexpected bands of

563 bp and 809 bp generated for NNW-KE2 and NNW-KE3, respectively, revealed that the forward primer n7577 had annealed to its corresponding genomic position, while the reverse primer could not be detected. Thus, the 563 bp and 809 bp bands have probably been generated instead of a 1307 bp fragment, due to a deletion or mis-priming. In a similar manner, sequence analysis of the 354 bp fragment from NNW-KE3 demonstrated that the forward primer n156 had annealed to the correct genomic position, but it did not extend to the length of 633 bp, because the reverse primer did not anneal to the correct genomic position. In a multiplex-PCR assay, there were many possible primer combinations that sometimes resulted in amplification of bands with larger sizes than expected. For example, the 3079 bp band obtained for NNW-KE3 was generated as a result of amplification with the primer pair S5585m/YO3-8648 and the 6376 bp band as a result of amplification with the primer pair n2258/YO3-8648. Sequence analysis showed that the fragment of 324 bp amplified from sample NWO-KE1 using the primer pair S5585m/YO3-8648 contained a large deletion of a 2756 bp located within CI/NIb.

All the sequenced replicate clones of the same fragment size were found to belong to the same PVY genotypes, even for samples that contained mixed infections of different PVY genotypes according to multiplex-PCR results. Sequenced clones for the same RT-PCR fragment (both when comparing clones for the same sample and between samples) shared 98 – 100% nucleotide sequence identity. The 633 bp fragment for samples NTN-0, NTN-2, NTN-5, NWO-KE1, NNW-KE2 and NNW-KE3 shared 99 – 100% identity with PVY^{NTN}, PVY^{Z}-NTN, PVY^{N}-W (A) and PVY^{E}. The 532 bp fragment for sample NWO-KE1 was 99% identical to isolates belonging to the strains PVY^{O} and PVY^{Z} and 98% identical to the variant PVY^{O}-O5, *e.g.* ME173 (GenBank no. FJ643479.1). The 853 bp fragment for samples NWO-KE1, NNW-KE3 and NW-DeI shared 99% identity with PVY^{O}, PVY^{N}-W/$PVY^{N:O}$, PVY^{O}-O5 and PVY^{Z}. The 1307 bp fragment for samples NNW-KE266, NNW-KE278 and NTN-S170 shared 99% identity with isolates belonging to PVY^{NTN} and PVY^{Z}-NTN (Table S3).

Table 3. Classification of PVY genotypes in two Dutch and 42 Swedish potato samples using multiplex- and uniplex-PCR assays[a]

Sample ID	Band size[b]	Molecular classification
NTN-0	441 + 633 + 1307	PVY^{NTN} (A)
NTN-2	441 + 633 + 1307	PVY^{NTN} (A)
NTN-5	441 + 633 + 1307	PVY^{NTN} (A)
NNW-A	441 + 633 + 1307 + 4114	PVY^{NTN} (A) and PVY^{N}-W
NTN-A (1)[c]	441 + 633 + 1307	PVY^{NTN} (A)
NTN-A (2)[d]	441 + 633 + 1307	PVY^{NTN} (A)
NNW-B	441 + 633 + 853 + 1307	PVY^{NTN} (A) and PVY^{N}-W (A or B)
NWO-Bdlm	441 + 532 + 633 + 853 + 1076 + 1307	PVY^{O}, PVY^{NTN} (A) and SYR-I/SYR-II
NWO-C	441 + 532 + 633 + 853 + 1307	PVY^{O} and PVY^{NTN} (A)
NTN-D	441 + 633 + 1307	PVY^{NTN} (A)
NW-DeI	441 + 633 + 853 + 1076 + 4114	PVY^{N}-W (A or B) and SYR-I/SYR-II
NWO-DeII	441 + 532 + 633 + 853 + 1307 + 4114	PVY^{O}, PVY^{NTN} (A) and PVY^{N}-W (A or B)
NTN-E	441 + 633 + 1307	PVY^{NTN} (A)
NTN-F1	441 + 633 + 1307	PVY^{NTN} (A)
NTN-F2	441 + 633 + 1307	PVY^{NTN} (A)
NWO-F3	441 + 532 + 633 + 853	PVY^{O} and PVY^{E}
NTN-G	441 + 633 + 1307	PVY^{NTN} (A)

[a]Multiplex- and uniplex-PCR assays were run according to Glais *et al.* (2005), Chikh Ali *et al*. (2010b; 2013), [b]Band size in base pair, [c]RT-PCR results for potato foliage sample. [d]RT-PCR results for a black aphid sample.

Cont. Table 3. Classification of PVY genotypes in two Dutch and 42 Swedish potato samples using multiplex- and uniplex-PCR assays[a]

Sample ID	Band size[b]	Molecular classification
NWO-KE1	441 + 532 + 633 + 853 + 1307 + 4114	PVY^{O}, PVY^{NTN} (A) and PVY^{N}-W (A or B)
NNW-KE2	441 + 633 + 853 + 1307 + 4114	PVY^{NTN} (A) and PVY^{N}-W (A or B)
NNW-KE3	441 + 633 + 853 + 1076 + 4114	PVY^{N}-W (A or B) and SYR-I/SYR-II
NWO-KE4	441 + 532 + 633 + 853 + 1307	PVY^{O} and PVY^{NTN} (A)
NNW-KE5	441 + 633 + 853 + 1307	PVY^{NTN} (A) and PVY^{N}-W (A or B)
NNW-KE6	441 + 633 + 853 + 1076 + 1307 + 4114	PVY^{NTN} (A), PVY^{N}-W (A or B) and SYR-I/SYR-II
NTN-KE7	441 + 633 + 1307	PVY^{NTN} (A)
NNW-KE8	441 + 633 + 853 + 1076 + 1307	PVY^{NTN} (A), PVY^{N}-W (A or B) and SYR-I/SYR-II
NWO-KE9	441 + 532 + 633 + 853 + 1076 + 1307	PVY^{O}, PVY^{NTN} (A) and SYR-I/SYR-II
E-KE10	441 + 633	PVY^{E}
NTN-KE253	441 + 633 + 1307	PVY^{NTN} (A)
NNW-KE266	441 + 532 + 633 + 853 + 1307	PVY^{O} and PVY^{NTN} (A)
NTN-KE276	441 + 633 + 1307	PVY^{NTN} (A)
NNW-KE278	441 + 532 + 633 + 853 + 1307 + 4114	PVY^{O}, PVY^{NTN} (A) and PVY^{N}-W (A or B)
NTN-KE287	441 + 633 + 1307	PVY^{NTN} (A)
NTN-KU	441 + 633 + 1307	PVY^{NTN} (A)
NWO-L	532 + 633 + 853 + 1076 + 1307	PVY^{O}, PVY^{NTN} (A) and SYR-I/SYR-II
NWO-MB	532 + 633 + 853 + 1076 + 1307	PVY^{O}, PVY^{NTN} (A) and SYR-I/SYR-II
NTN-Mn	441 + 633 + 1307	PVY^{NTN} (A)
NWO-Mt	441 + 532 + 633 + 853 + 1076 + 1307	PVY^{O}, PVY^{NTN} (A) and SYR-I/SYR-II
NNW-SA	441 + 633 + 853 + 1307 + 4114	PVY^{NTN} (A) and PVY^{N}-W (A or B)
NTN-SB	441 + 633 + 1307	PVY^{NTN} (A)
NNW-S9	441 + 633 + 853 + 1307	PVY^{NTN} (A) and PVY^{N}-W (A or B)
NTN-S170	441 + 633 + 1307	PVY^{NTN} (A)
NTN-S171	441 + 633 + 1307	PVY^{NTN} (A)
N-S204	633 + 1307	NA-PVY^{N} and PVY-NE11
NWO-V	441 + 532 + 633 + 853 + 1307	PVY^{O} and PVY^{NTN} (A)

[a]Multiplex- and uniplex-PCR assays were run according to Glais *et al.* (2005), Chikh Ali *et al*. (2010b; 2013), [b]Band size in base pair.

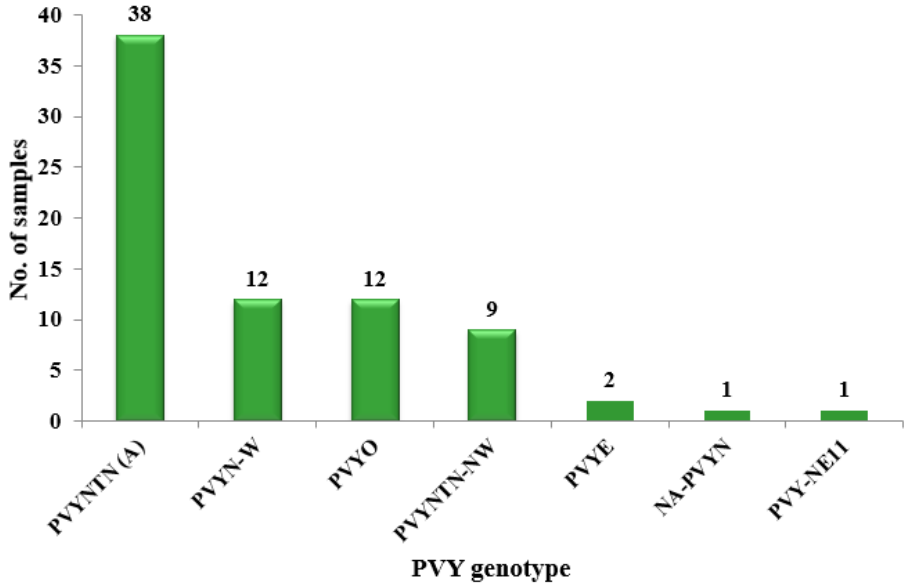

Figure 4.B. Distribution of PVY genotypes in potato samples collected from different regions in Sweden.

3.3. Biological characterization

Out of 16 tested samples, inoculation with extracts of samples NTN-2, NW-DeI, NWO-KE1, NNW-KE3, NWO-KE4, NTN-KE253, NTN-KE287, NTN-SB, NTN-S171 and N-S204 induced chlorotic local lesions on inoculated leaves of quinoa plants at 10 – 14 dpi. Meanwhile, inoculation with extract of sample NTN-G induced mosaic on inoculated leaves of quinoa plants (Table 4, Fig. S4). Using DAS-ELISA, PVY infection could be verified in inoculated leaves of quinoa for samples NW-DeI, NWO-KE1 and NWO-KE4, while for systemic leaves, none of the quinoa plants tested positive (Table 5). Positive reactions were only obtained with the monoclonal antibodies $PVY^{O/C}$ (Table 5), even if the original potato samples were positive for both N and O serotypes.

After inoculation with extract from 16 PVY-positive samples, symptoms emerged at seven dpi in tobacco. It was found that all of the tested samples contained PVY of the N pathotype, as inoculation with extracts of all samples induced veinal and stem necrosis on tobacco, but at different levels (Table 4, Fig. S5). Positive DAS-ELISA results were obtained at 30 dpi for tobacco plants inoculated with extracts of all tested samples, except for those inoculated with extract of sample N-S204 (Table 5). Death of the four oldest leaves was observed at 17 dpi in tobacco plants inoculated with extracts of samples NTN-0, NTN-2, NTN-5, NWO-KE1, NNW-KE3 and NTN-KE276. Few days later (at 20 – 25 dpi), some recovery was observed in tobacco plants inoculated with extracts of all tested samples, except for sample NTN-KE276, as vein and stem necrosis was restricted to the middle leaves and did not develop on the uppermost leaves. Extract of sample NNW-KE266 induced a special necrotic pattern on tobacco stems that looked like a snake-leather-like pattern (Fig. S5). Tobacco plants inoculated with extract of samples NTN-0 and NTN-2 showed severe stunting and leaf death. Extract of sample NTN-KE276 induced the most severe symptoms on tobacco that resulted in plant death for all inoculated plants at different stages of infection starting from 14 dpi. Extract of sample N-S204 induced very mild foliage symptoms on tobacco up to 30 dpi that developed to irregular alternation between light-green and dark-green islands at about 130 dpi. These mild symptoms were accompanied by negative DAS-ELISA results at 30 dpi (Table 5), but positive for multiplex-PCR assay (data not shown). At 90 dpi, tobacco leaves inoculated with extract of sample N-S204 reacted positively with SASA monoclonal PVY^{N} antibodies employing DAS-ELISA. Interestingly, petal colour-break of flowers was observed at 90 dpi for tobacco plants inoculated with extract of sample N-S204 (Table 4, Fig. S6).

After inoculation with extract from 26 PVY-positive samples, including those identified to contain the PVY^{O} strain, symptoms emerged at 14 – 17 dpi in potato cv. Désirée. Uniquely, mosaic symptom was observed at three dpi in Désirée plants inoculated with extract of sample NWO-Bdlm. Unexpectedly,

there was no HR observed on Désirée leaves in response to any of the tested extracts of samples that had been classified as containing the non-recombinant PVY^{O} strain (Fig. 5). Symptoms from thrips infestation were observed prior and post inoculation that sometimes interfered with the monitoring of virus-induced symptoms, especially for Désirée plants inoculated with extract of sample NWO-F3 (Fig. 5). Inoculation with extracts of samples NTN-0, NW-DeI, NNW-KE2, NNW-KE3, NTN-KE276, NTN-KE287, NTN-SB and NTN-S171, which had been found not to contain the non-recombinant PVY^{O} strain, resulted in veinal and/or leaf necrosis on inoculated leaves of potato plants cv. Désirée (Fig. S7). Veinal and/or leaf necrosis was visible on systemic leaves of potato plants cv. Désirée at 45 dpi in response to inoculation with extracts of samples NWO-KE1, NWO-KE9, NWO-MB and NWO-V containing the non-recombinant PVY^{O} strain (Tables 4 and S4, Fig. 6). On the other hand, veinal and/or leaf necrosis was visible on almost all systemic leaves of potato plants cv. Désirée at 30 dpi and 45 dpi in response to inoculation with extracts of samples NTN-0, NTN-2, NW-DeI, NNW-KE2, NNW-KE3, NTN-KE276, NTN-KE287 and NTN-S171 not containing the non-recombinant PVY^{O} strain (Table 4, Fig. 7). Systemic veinal and/or leaf necrosis was also observed for potato plants of several cultivars infected with different PVY genotypes as a result of tuber-borne infection or aphid transmission. For example, systemic necrosis was recorded for potato plants of cv. King Edward (NNW-KE6, NTN-KE7 and NNW-KE8), cv. Lurö (NWO-L), cv. Mandel (NTN-Mn), cv. Solist (NNW-S9) and cv. Vivi (NWO-V). A special pattern of necrosis that was restricted to tips of the uppermost leaves was recorded for some potato plants of cv. King Edward raised from tuber-borne infection (NTN-KE253, NNW-KE266, NTN-KE276, NNW-KE278 and NTN-KE287) (data not shown). Generally, it was found that systemic necrosis was associated with samples containing recombinant genotypes, *e.g.* PVY^{NTN} (A). In addition, leaf death, especially for the lower leaves, was associated with inoculation of extracts from samples NTN-0, NTN-5, NWO-Bdlm, NWO-F3, NTN-G, NWO-KE1, NNW-KE2, NWO-KE4, NWO-KE9, NNW-KE266, NTN-KE287, NWO-L, NWO-MB, NTN-SB, NTN-S170 and NWO-V (Tables 4 and S4). Among 12 samples identified to contain the PVY^{O} strain, sample extracts from systemic leaves of Désirée plants inoculated with NWO-F3, NWO-KE1, NWO-KE4, NWO-MB, NWO-V, NWO-Bdlm, NWO-KE9 and NWO-L reacted positively with SASA monoclonal $PVY^{O/C}$ antibodies employing DAS-ELISA, which is consistent with the initial DAS-ELISA and with multiplex-PCR assay results. Sample extract from systemic leaves of Désirée plants inoculated with NWO-C did not react with any of the employed monoclonal antibodies, which is consistent with the initial DAS-ELISA results. On the other hand, sample extracts from systemic leaves of Désirée plants inoculated with NNW-KE266 and NWO-Mt did not react with any of the employed monoclonal antibodies, which is inconsistent with the initial DAS-ELISA results (Table 5).

Potato plants of cv. Désirée raised from infected tubers of the Dutch sample NW-DeI did not give any tubers, although there were tubers harvested from the mechanically inoculated Désirée plants with extract of the same sample. On the other hand, Désirée plants raised from tuber-borne infection with NW-DeI slightly recovered about 45 days from sowing, as the systemic chlorosis disappeared and was replaced by mottling, but these plants grew slower than the mechanically inoculated plants. These findings indicate that the tuber-borne infection of sample NW-DeI had more significant impact on yield than secondary infection.

A total of 36 samples were screened for their ability to induce potato tuber necrotic ringspot disease on different potato cultivars. The screening was done on tubers developing from initial samples and on cv. Désirée tubers from inoculated plants. Out of 16 screened potato samples from tuber-borne infections, necrotic ringspots were observed on freshly harvested tubers of NTN-5 (unknown cultivar), NWO-C (cv. Désirée) and NTN-F1 (cv. Folva). Necrotic ringspots were observed after eight weeks of storage for NTN-0 (unknown cultivar) and NTN-2 (unknown cultivar), NNW-A (cv. Asterix), NWO-Mt (cv. Désirée), NNW-S9 (cv. Solist), NTN-S170 (cvs. Désirée and Solist) and NTN-S171 (cv. Solist) (Table S5). No necrotic ringspots were observed on fresh or stored tubers of the Dutch samples NW-DeI and N-DeIII (cv. Désirée). Among 25 samples analysed, necrotic ringspots were was observed on freshly harvested tubers from plants of cv. Désirée inoculated with extract of sample NWO-C, whereas the disease was observed on tubers after eight weeks of storage for samples NTN-S170, NTN-S171 and NWO-Mt (Table S5, Fig. S8).

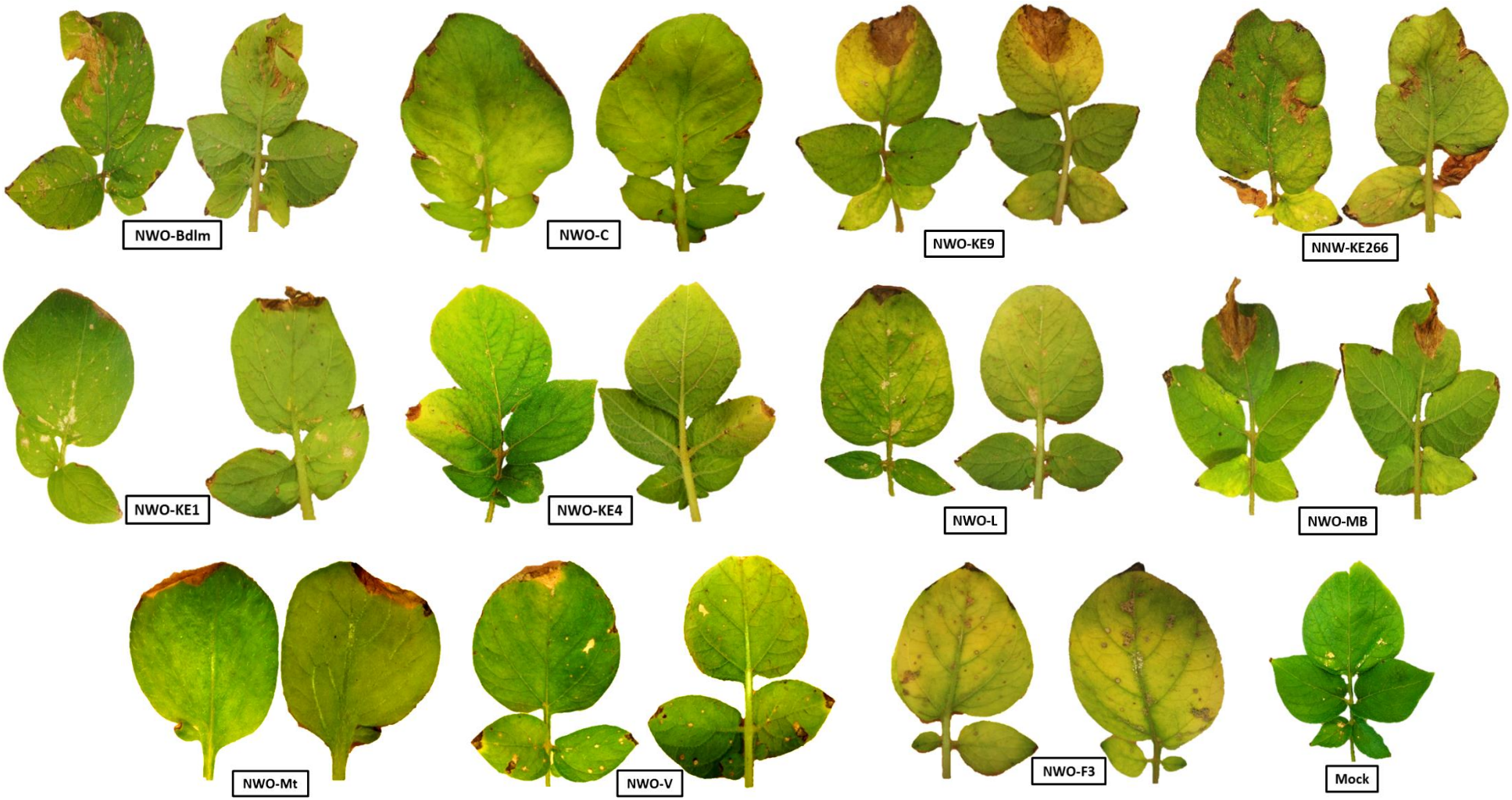

Figure 5. Screening potato cv. Désirée for hypersensitive response after mechanical inoculation with extract from eleven potato samples identified as having a mixed infection of PVY genotypes, including the non-recombinant PVY^{O}. A mock-inoculated plant was included as a negative control.

Table 4. Foliage symptoms recorded for quinoa, tobacco cv. Samsun, and potato cv. Désirée after inoculation with extract of 16 different potato samples

Plant species		NTN-0	NTN-2	NTN-5	NW-DeI	NTN-G	NWO-KE1	NNW-KE2	NNW-KE3	NWO-KE4	NTN-KE253	NNW-KE266	NTN-KE276	NTN-KE287	NTN-SB	NTN-S171	N-S204
Quinoa		Ch	ChL	Ch	ChL	MM, M	ChL	Ch	ChL	ChL	ChL	Ch	Ch	ChL	ChL, St	ChL	ChL
Tobacco cv. Samsun		Mo, Ch, VN, PN, SN, SeSt, DLL	Mo, Ch, SeVN, SePN, SeSN, SeSt, DLL	Mo, SeVN, SePN, SeSN, DLL	Mo, VN, SPN, SN, St	Mo, SeVN, SePN, SN	Ch, VN, PN, SN, St, DLL	Mo, Ch, SeVN, SePN, SeSN, St	Mo, Ch, SeVN, SePN, SeSN, St, DLL	Mo, SeVN, SePN, SeSN	M, VC, VN, PN, SN	Mo, SeVN, SePN, SeSN	Mo, SeSt, SeVN, SePN, SeSN, DLL	Mo, SeVN, SePN, SeSN	Mo, SeVN, SepN, SeSN	Mo, VN, PN, SN, LD, St	MM, SVN[b]
Potato cv. Désirée	15 dpi	NS	NS	NS	M	MM, Ch, LD	MM	NS	MM	MM	-[a]	MM	MM	MM	M, LD	MM, Mo	MM
	20 dpi	M	Ch	M, SYCh, LD	Mo, ChL, SYCh, SeSt	M, SYCh, LD	M, SYCh, LD	M	MM	SYCh	-	MM	M, SYCh	MM, LD	MM, ChL, LD	CS, Mo	MM, M
	30 dpi	M, SYCh, LN, VN, LD	SYCh	Mo, SYCh, LD	SYCh, VN, LN	Mo, SYCh, LD	SYCh, LD, VN, LN	Mo, SYCh, SeVN, SeLN, PN, LD	Ch, Mo, VN, LN	SYCh	-	MM, M, DLL	Mo, SYCh, VN	Mo, Ch, VN, LD	Mo, ChYL, SYCh, LD	SeM, Mo, SYSCh	MM, SeM
	45 dpi	Mo, ChYL, LN, VN, LD	Mo, SYCh, ChYL, VN, LN	SeM, SYCh, LD	SYCh, VN, LN	MM, M	SYCh, LD, VN, LN	Mo, SeVN, SeLN, PN, LD	SeM, Mo, VN, LN	SYCh, ChYL, LD	-	M, SeM, DLL	M, Mo, SYCh, ChYL, VN	M, ChYL, SVN	ChL, ChYL	Mo, ChL, SYSCh, ChYL, VN	M, ChLL

[a]Non-available, [b]Veinal necrosis developed approximately 40 dpi, Ch = chlorosis, ChLL = chlorosis of lower leaves, ChL = chlorotic lesion, ChYL = chlorosis in young leaves, CS = cup shape, DLL = death of lower leaves, dpi = days post inoculation, LD = leaf death, LN = leaf necrosis, M = mosaic, MM = mild mosaic, Mo = Mottling, NS = no symptoms, PN = petiole necrosis, SeM = severe mosaic, SePN = severe petiole necrosis, SeSN = severe stem necrosis, SeSt = severe stunting, SeVN = severe veinal necrosis, SN = stem necrosis, SPN = slight petiole necrosis, St = stunting, SVN = slight vein necrosis, SYCh = Systemic chlorosis, VC = vein clearing, VN = veinal necrosis.

Table 5. ELISA results for systemic leaves of potato plants cv. Désirée inoculated with extracts of 25 PVY samples, as well as inoculated and systemic leaves of quinoa and systemic tobacco leaves of plants inoculated with extracts of 16 PVY samples

Sample ID	Cultivar/Species							
	Potato cv. Désirée		Quinoa				Tobacco	
	Systemic Leaves		Inoc. Leaves		Systemic Leaves		Systemic Leaves	
	$PVY^{O/C}$	PVY^{N}	$PVY^{O/C}$	PVY^{N}	$PVY^{O/C}$	PVY^{N}	$PVY^{O/C}$	PVY^{N}
NTN-0	-[a]	+[b]	-	-	-	-	-	+
NTN-2	-	+	-	-	-	-	-	+
NTN-5	-	+	-	-	-	-	-	+
NW-DeI	+	-	+	-	-	-	+[d]	-
NWO-KE1	+	+	+	-	-	-	+	+
NNW-KE2	-	+	-	-	-	-	-	+
NNW-KE3	+	-	-	-	-	-	+[d]	-
NWO-KE4	+	+	+	-	-	-	+	+
NTN-KE253	N.A.[c]	N.A.	-	-	-	-	-	+
NNW-KE266	-	-	-	-	-	-	-	+
NTN-KE276	-	+	-	-	-	-	-	+
NTN-KE287	-	+	-	-	-	-	-	+
NTN-G	-	+	-	-	-	-	-	+
NTN-SB	-	+	-	-	-	-	-	+
NTN-S171	-	+	-	-	-	-	-	+
N-S204	-	+	-	-	-	-	-	-
NWO-Bdlm	+	-						
NWO-C	-	-						
NWO-F3	+	+						
NWO-KE9	+	-						
NWO-L	+	-			N.A.			
NWO-MB	+	+						
NWO-Mt	-	-						
NTN-S170	-	+						
NWO-V	+	+						

[a](-) = negative, [b](+) = positive, [c]N.A. = non-available, [d]Serotype O, but pathotype N.

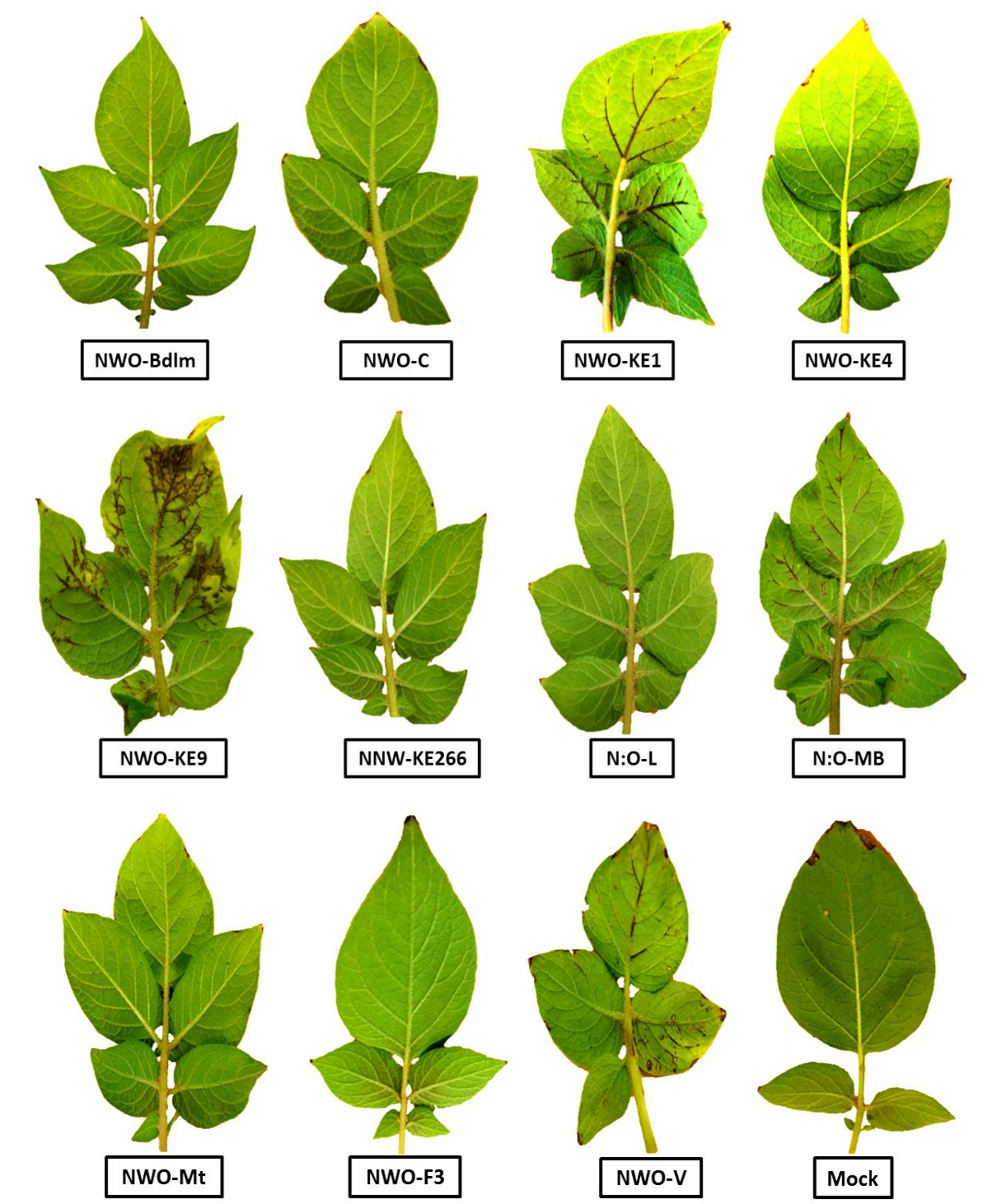

Figure 6. Foliage symptoms on the abaxial leaf surface of systemic leaves of potato cv. Désirée at 45 dpi induced by inoculation with extract from eleven potato samples identified as having a mixed infection of PVY genotypes, including the non-recombinant PVY^{O} strain. A mock-inoculated plant was included as a negative control.

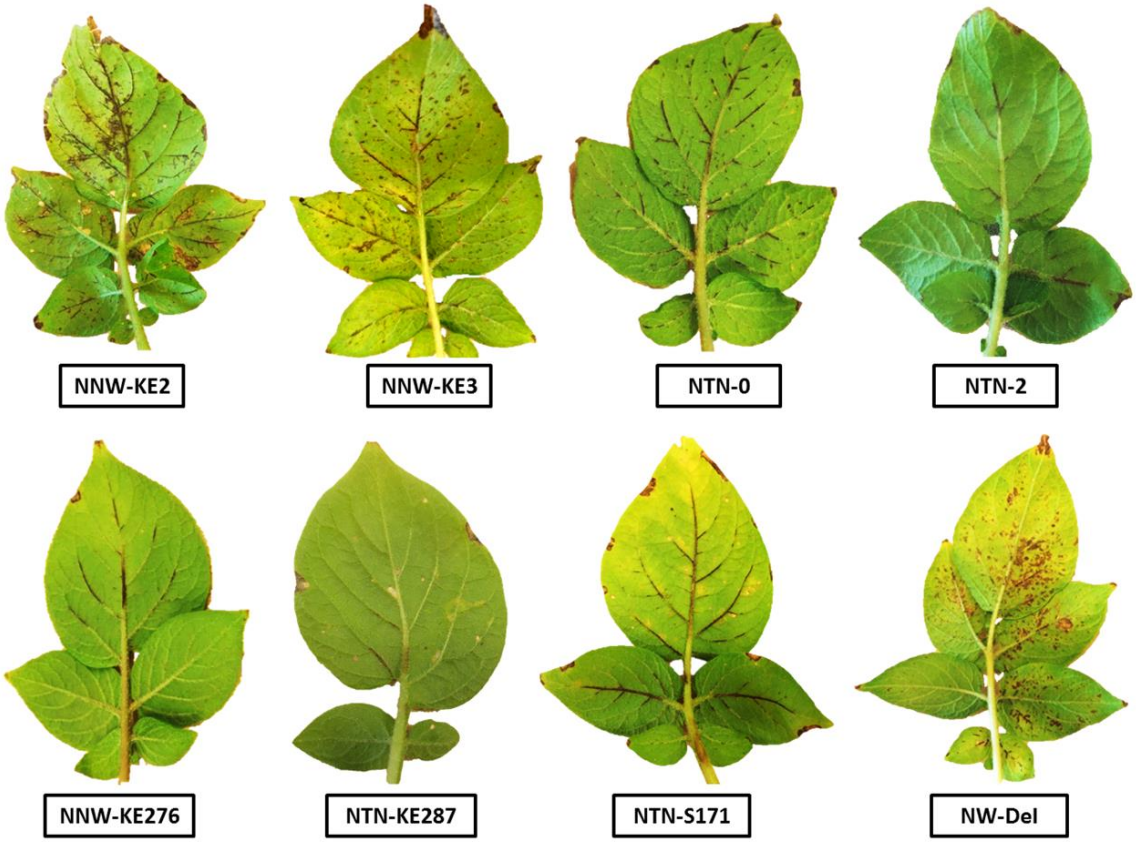

Figure 7. Systemic necrosis symptoms on the abaxial leaf surface of systemic leaves of potato cv. Désirée at 45 dpi induced by inoculation with extracts of eight PVY-infected potato samples found not to contain the non-recombinant PVY^{O} strain.

4. Discussion

Out of 50 ELISA-tested samples from different regions in Sweden, 31 of the samples were positive for only serotype N, one sample was positive for only serotype O, 15 samples reacted positively for both serotypes and three samples were negative. One of the samples, which did not react with any of the employed monoclonal antibodies, was identified using RT-PCR assays to contain a mixed infection of PVY^{NTN} (A) and PVY^{O}, and the other two samples were found to contain a single infection of PVY^{NTN} (A). In a similar manner, a few PVY^{E} and PVY^{NTN} isolates have been found not to react with SASA monoclonal PVY^{N} antibodies (Galvino-Costa *et al*., 2012). Sample NTN-Mn, which was classified as PVY^{NTN} (A) based on RT-PCR assays, gave positive results with both monoclonal PVY^{N} and $PVY^{O/C}$ antibodies. Similarly, numerous PVY^{N-}W, PVY^{N}/PVY^{NTN} and PVY^{O} isolates have been shown to react with both monoclonal $PVY^{O/C}$ and PVY^{N} antibodies (Yin *et al*., 2012). Out of 21 tested Dutch Désirée tuber seed samples, three samples were positive for only serotype O, one sample was positive for only serotype N and one sample did not react with any of the employed antibodies, but was subsequently found to have a mixed infection of PVY^{NTN} (A), PVY^{O} and PVY^{N}-W. Therefore, relying on ELISA as a single detection method could increase the incidence of false negative results. None of the Swedish or Dutch potato samples was positive for the C serotype, which is consistent with a previous study reporting the absence of the PVY^{C} strain from Europe (Quenouille *et al*., 2013). Nevertheless, it is still possible that there are PVY^{C} isolates, which react with the specific monoclonal antibodies for PVY^{N} and not with those for PVY^{C} (Blanco-Urgoiti *et al*., 1998). Hence, follow-up screening for presence of the PVY^{C} strain is required.

Based on the RT-PCR tests, it was found that PVY^{NTN} (A) dominated among the analysed samples, followed by PVY^{N}-W and PVY^{O}. Out of 42 PCR-tested Swedish potato samples, the PVY^{O} and/or the PVY^{Z} strains were detected in 12 samples in mixed infections with PVY recombinants. The variant PVY^{NTN} (A) was detected in 38 samples in single or mixed infections. In a similar manner, there has been an overwhelming increase in the incidence of recombinant strains reported in many European countries (Quenouille *et al*., 2013), for example, the PVY^{NTN} strain dominates the PVY population in Scotland and Belgium (Davie, 2014; Kamangar *et al*., 2014). A few decades ago, the non-recombinant ordinary PVY^{O} strain was the most common PVY strain in Sweden (Sigvald, 1985). In the U.S., the predominance of the PVY^{O} strain population has decreased (Karasev and Gray, 2013b), and it seems to be the same case in Sweden. There have been no previous reports regarding the molecular classification of PVY strains in Sweden, except for five PVY-infected potato samples, which were analysed by Beuch (2013). These five samples were identified to contain the PVY^{N}, PVY^{NTN} and PVY^{N}-W strains. In the present study PVY^{N} was not detected in any of the tested potato samples collected from 13 locations in Sweden. The shift from the non-recombinant PVY^{O} strain to recombinant genotypes, *e.g*. PVY^{NTN}, could be explained by the higher efficiency of the PVY^{NTN} strain to be transmitted by aphid vectors, as well as its capacity to maintain higher virus titres in plant cells, compared with either the PVY^{O} or the PVY^{N}-W strain (Srinivasan *et al*., 2012; Syller and Grupa, 2014). The rate of PVY^{O} spread is also hindered when cultivars harbouring the resistance gene Ny_{tbr} are grown (Carroll *et al*., 2016).

Molecular classification of PVY according to Glais *et al*. (2005) and Chikh Ali *et al*. (2010b; 2013) resulted in various problems: 1) Two samples could not be properly characterized. 2) Numerous probabilities arose from the presence of mixed infections with different PVY genotypes. 3) Mis-priming or genome deletions that lead to amplifying bands of unexpected size. For example, fragments of 3079 bp and 6376 bp were generated for NNW-KE3 in multiplex-PCR as a result of amplification with the primer pairs S5585m/YO3-8648 and n2258/YO3-8648, respectively. 4) Primers were unable to separate genotypes with high sequence identities in the targeted regions. For instance, sequence analysis of the expected 853 bp fragments generated for NWO-KE1, NNW-KE3 and NW-DeI showed a nucleotide identity of 99% to PVY^{Z}, PVY^{O}, PVY^{O}-O5 and PVY^{N}-W/$PVY^{N:O}$. Sequence analysis of the expected 441 and 633 bp fragments showed an identity of 99 – 100% to PVY^{NTN}, PVY^{Z}-NTN, PVY^{N}-W, PVY^{NTN-NW} and PVY^{E}. Therefore, additional assays are required to separate these strains.

Sequence analyses of the majority of clones for the 441, 532, 633, 853 and 1307 bp bands verified the expected genomic location and band length, with a few exceptions. From the available data, it can be

concluded that molecular classification schemes of PVY genotypes according to Glais *et al*. (2005) and Chikh Ali *et al*. (2010b; 2013) are efficient for preliminary PVY genotyping, but additional tests may be required. Accordingly, reliable PVY classification for representative samples is suggested to be done based on biological indexing, complete-genome sequencing considering number and locations of RJs, as well as serotyping.

In previous studies, isolates of the non-recombinant strains PVY^{C}, PVY^{O} and PVY^{N} induced chlorotic local lesions on quinoa, while no symptoms were observed in response to neither PVY^{N}-W nor PVY^{NTN} (Le Romancer *et al*., 1994; Lorenzen *et al*., 2006b). In consistence with these findings, none of sample extracts, which were identified to contain a single infection of the PVY^{NTN} strain, were able to infect quinoa with PVY. PVY present in the samples NWO-KE1, NWO-KE4 and NW-DeI could be transmitted to quinoa and local infections were verified by positive reaction to SASA monoclonal $PVY^{O/C}$ antibodies. Furthermore, inoculation with extract of samples NTN-0, NNW-KE3, NTN-KE253, NTN-KE287, NTN-SB and N-S204 induced chlorotic lesions, but no PVY infection was detected. These findings could indicate that the PVY level was below the detection level for DAS-ELISA and/or may indicate the presence of mixed infection (s) with another virus, *e.g*. *Potato virus X* (PVX) (Lorenzen *et al*., 2006b).

The overwhelming majority of the recombinant PVY strains induce tobacco veinal necrosis (Karasev and Gray, 2013b). Likewise, it was found in this study that extract of all tested PVY samples induced veinal, petiole and stem necrosis on tobacco indicating the presence of the N pathotype. Uniquely, extract of the sample N-S204, which was found to contain a mixed infection of NA-PVY^{N} and PVY-NE11 or a single infection with the variant PVY^{NTN} (A) with the 441 bp missing in the multiplex-PCR assay, induced petal colour-break of tobacco flowers that was observed at 90 dpi, aside from inducing very mild foliage symptoms. On the contrary, extract of sample NTN-KE276 induced severe symptoms on tobacco that resulted in plant death for all inoculated plants. Interestingly, extract of sample NNW-KE266 induced a special necrosis pattern on tobacco stems that looked like a snake-leather-like pattern. Recovery was observed in tobacco plants at about 20 – 25 dpi in response to inoculation of extracts of all tested samples, except for those inoculated with extract of sample NTN-KE276, as vein and stem necrosis was restricted to the middle leaves and did not develop on the uppermost leaves. In addition, the mosaic was milder on the uppermost leaves compared with the middle leaves. The recovery phenomenon in tobacco is associated with reduced PVY titre in the uppermost leaves. The phenomenon is host-specific and independent of salicylic acid (Nie and Molen, 2015), as it results from the induction of autophagy after PVY infection (Choi *et al*., 2016).

Karasev *et al*. (2011) reported that all tested isolates belonging to PVY^{O} and PVY^{O}-O5 induced HR in cv. Désirée. Leaves of cv. Désirée inoculated with the PVY^{O} strain developed HR at 10 to 11 dpi, which usually was accompanied by defoliation of the necrotic leaves (Kerlan *et al*., 2011). In this study, no HR was observed on inoculated cv. Désirée leaves up to 40 dpi for any extract of samples that had been classified as containing the non-recombinant PVY^{O} strain. In addition, none of the inoculated leaves defoliated, although extracts of a few samples induced systemic foliage necrosis. Instead, inoculation with extracts of eight samples, which were characterized as not containing the non-recombinant PVY^{O} strain, induced veinal and/or leaf necrosis on inoculated leaves of potato plants cv. Désirée. The non-recombinant PVY^{D} strain has been shown to induce necrotic stem streaking and death of cv. Désirée plants (Kehoe and Jones, 2016), but these symptoms were not observed for any of the tested samples. These findings indicate the absence of the ordinary PVY^{O} and PVY^{D} strains, and may indicate the presence of the non-recombinant PVY^{Z} strain or resistance-breaking isolates of the PVY^{O} strain, especially because the ability of overcoming the Ny_{tbr} or the Nz_{tbr} gene probably has not been tested before in Sweden. However, the incidence of the PVY^{Z} strain is very rare. Consequently, biological indexing using potato cultivars possessing the Nz_{tbr} gene and full-genome sequencing are required to test this hypothesis.

It was found that induction of local and/or systemic foliage necrosis in cv. Désirée is not associated with a specific PVY serotype. In the light of the findings by Karasev *et al*. (2011), a single amino acid substitution from arginine to glutamine at position 98 in the CP leads to a shift from serotype O to

serotype N. In previous studies, local and systemic foliage necrosis, as well as leaf drop was found to be associated with isolates belonging to the recombinant PVY^{NTN} strain, regardless of their serotyping (Le Romancer *et al*., 1994; Kerlan *et al*., 2011; Kogovšek *et al*., 2016). In this study, local and/or systemic foliage necrosis was found to be associated with numerous potato samples identified to contain recombinant PVY genotypes, *e.g*. PVY^{NTN} (A) and PVY^{NTN-NW}, regardless of their ability to induce potato tuber necrotic ringspot disease. Hence, it is assumed that the genetic determinants responsible for induction of foliage necrosis in potato are not linked to those responsible for induction of potato tuber necrotic ringspot disease.

Samples NW-DeI and NNW-KE3, which contained mixed infections of PVY^{N-W} and PVY^{NTN-NW} (SYR-I/SYR-II), induced severe symptoms in cv. Désirée ranging from chlorosis to systemic veinal and leaf necrosis. The PVY^{N-W} strain induces mild mosaic in most potato cultivars (Glais *et al*., 2005; Yin *et al*., 2012), while PVY^{NTN-NW} (SYR-I) induced severe leaf deformation, mosaic and stunting in plants of cv. Yukon Gold (Hu *et al*., 2011). Consequently, the presence of PVY^{NTN-NW} could be responsible for the severity of symptoms or it is because of mixed infections with the two strains. A PVY isolate may induce systemic necrosis in one potato cultivar, but not in another. In this thesis, systemic necrosis was observed for potato plants of cvs. King Edward (ny_{tbr}:*Nc*:nz_{tbr}:Nd_{tbr}) and Désirée. Systemic necrosis has also been observed for other potato cultivars, *e.g*. Maris Bard (Ny_{tbr}:Nc_{tbr}:Nz_{tbr}) and Yukon Gold (Ny_{tbr}:*nc*:Nz_{tbr}:Nd_{tbr}) (Kerlan *et al*., 2011). Induction of potato foliage necrosis is associated with aggressive strains, *e.g*. PVY^{NTN}, due to the increased accumulation of ROS-associated metabolites in leaves of susceptible cultivars, which does not occur in the resistant ones (Kogovšek *et al*., 2016). To our best knowledge, PVY genetic determinants linked to this phenomenon are still unknown. These findings may indicate the presence of resistance genes or susceptibility factors that are responsible for development of systemic necrosis in potato.

Potato tuber necrotic ringspot disease (PTNRD) symptoms may be obvious already at harvest or develop during storage, especially when the growing and/or storage temperature falls within 22 °C ± 2 (Glais *et al*., 2015). Out of 36 PVY-infected potato samples, potato tuber necrotic ringspot disease was observed for 10 samples of different potato cultivars either at harvest or after storage in dark at 23°C ± 1. The RT-PCR analyses had shown that these samples contained the PVY^{NTN} strain as single (six samples, including NTN-0, NTN-2 and NTN-5) or mixed infections (four samples). Infection with PTNRD-inducing PVY isolates through mechanical inoculation or tuber-borne transmission, induced necrotic ringspots on tubers of some potato cultivars, but not on others. Furthermore, not all samples containing the PVY^{NTN} strain induced potato tuber necrotic ringspot disease. A substitution at amino acid 419 in HC-Pro from glutamic acid (E) to aspartic acid (D) inhibits the expression of tuber necrotic ringspots (Glais *et al*., 2015). Therefore, it can be concluded that potato tuber necrotic ringspot disease expression is independent of the virus source (vertical or horizontal transmission), but dependent on PVY isolate, potato genotype and their interactions.

From the results of serological, molecular and biological analyses, it can be concluded that the non-recombinant PVY^{O} strain has been replaced by the PVY^{NTN} strain, along with other PVY recombinants. PVY evolves overtime and forms new strains through recombination between different strains and through mutations (Karasev and Gray, 2013a). Rapid PVY evolution is credited to its considerable genetic diversity. 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) …. etc. The PVY population in Sweden seems to have a high genetic diversity based on the available biological, serological, molecular and sequence data. This genetic diversity may lead to increased PVY spread, due to the lack of resistance against recombinant and virulent strains, *e.g*. PVY^{NTN}, especially when it is combined with appropriate environmental conditions for spread by aphid vectors. Furthermore, the spread of non-virulent isolates, *e.g*. N-S204, may also increase to serious levels as such isolates maintain very low virus titres and induce symptomless infection or very mild symptom. Such isolates may escape from detection, spread, evolve and cause sudden outbreaks.

5. Conclusion

In Sweden, the PVY^{O} strain has mainly been replaced by the PVY^{NTN} strain, along with other recombinant genotypes, as reported in many European countries, and the variant PVY^{NTN} (A) now seems to be the common PVY genotype. The characterized Swedish PVY^{O} isolates were able to overcome the Ny_{tbr} in potato cv. Désirée, resistance-breaking. From the available data, the PVY population in Sweden seems to have a high genetic diversity. It is necessary to improve the current diagnostic tools for rapid and accurate identification of PVY isolates, especially the new recombinants, and to have a frequently updated international classification of PVY variants and strains.

6. Further Prospective

Complete-genome sequencing of a few resistance-breaking PVY^{O} isolates and other PVY isolates with novel biological properties is required for screening of genetic determinants responsible for these biological properties and for screening of the presence of new PVY recombinants. It is important to screen for PVY isolates associated with potato tuber necrotic ringspot disease and for the presence of the PVY^{C} and PVY^{E} strains in Sweden. Therefore, large-scale collection and inspection of symptomatic potato plants and suspected secondary hosts from the majority of the potato-growing areas in Sweden is required.

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9. Supplementary Materials

Table S1. Multiplex- and uniplex-PCR conditions using different DNA polymerases

Cycle	No. of cycles	Expand High Fidelity PCR System	Phusion High-Fidelity DNA Polymerase
Initial denaturation	1x	94°C/2 min	98°C/30 s
Denaturation	11x	94°C/15 s	98°C/10 s
Annealing		60°C/30 s	62°C/30 s
Extension		72°C/2 min	72°C/2 min
Denaturation	11x	94°C/15 s	98°C/10 s
Annealing		62°C/30 s	63°C/30 s
Extension		72°C/2 min	72°C/2 min
Denaturation	11x	94°C/15 s	98°C/10 s
Annealing		64°C/30 s	64°C/30 s
Extension		72°C/2 min	72°C/2 min
Final extension	1x	72°C/10 min	72°C/10 min

Table S2. Serotyping of 55 PVY samples based on DAS-ELISA results using three monoclonal antibodies

Sample ID	Serotyping[a]	Sample ID	Serotyping[a]	Sample ID	Serotyping[a]
NTN-0	N	NWO-KE1	O + N	NWO-Mt	O + N
NTN-2	N	NNW-KE2	N	N-S1	N
NTN-5	N	NNW-KE3	O	N-S2	N
NTN-A (1)[b]	N	NWO-KE4	O + N	N-S3	N
NNW-A	O + N	NNW-KE5	N	N-S4	N
NNW-B	N	NNW-KE6	O + N	N-S5	N
NWO-Bdlm	O + N	NTN-KE7	N	N-S6	N
NWO-C	None	NNW-KE8	O + N	N-S7	N
NTN-D	None	NWO-KE9	O + N	NNW-S9	O + N

[a] O = serotype PVY^O, N = serotype PVY^N, O + N = serotypes PVY^O and PVY^N, None = no serotype detected using SASA monoclonal antibodies.

Cont. Table S2. Serotyping of 55 PVY samples based on DAS-ELISA results using three monoclonal antibodies

Sample ID	Serotyping[a]	Sample ID	Serotyping[a]	Sample ID	Serotyping[a]
NW-DeI	O	E-KE10	N	N-S9	N
NWO-DeII	None	NTN-KE253	N	N-S10	N
N-DeIII	N	NNW-KE266	N	NTN-S170	N
O-DeIV	O	NTN-KE276	N	NTN-S171	N
O-DeV	O	NNW-KE278	N	N-S204	N
NTN-E	None	NTN-KE287	N	NNW-SA	O + N
NTN-F1	N	NTN-KU	N	NTN-SB	N
NTN-F2	N	NWO-L	O + N	NWO-V	O + N
NWO-F3	O + N	NWO-MB	O + N		
NTN-G	N	NTN-Mn	O + N		

[a] O = serotype PVY^O, N = serotype PVY^N, O + N = serotypes PVY^O and PVY^N, None = no serotype detected using SASA m onoclonal antibodies.

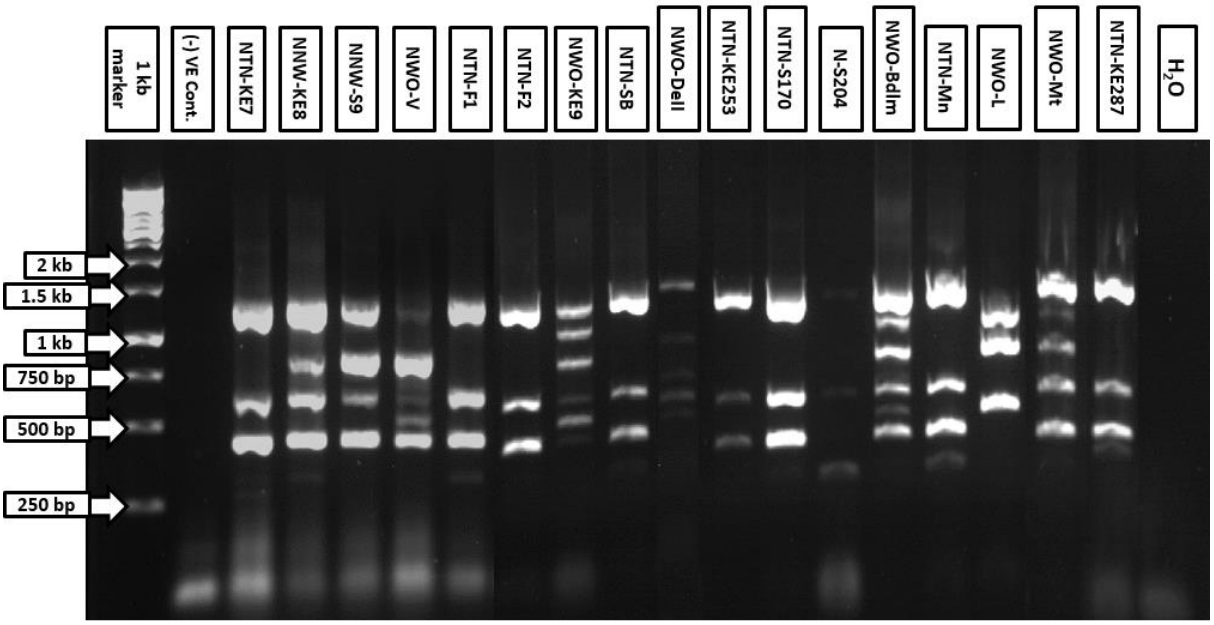

Figure S1.A. Gel electrophoresis of multiplex RT-PCR assay for PVY genotyping of different potato samples using Phusion High-Fidelity DNA Polymerase. (-) ve control = PVY-free sample.

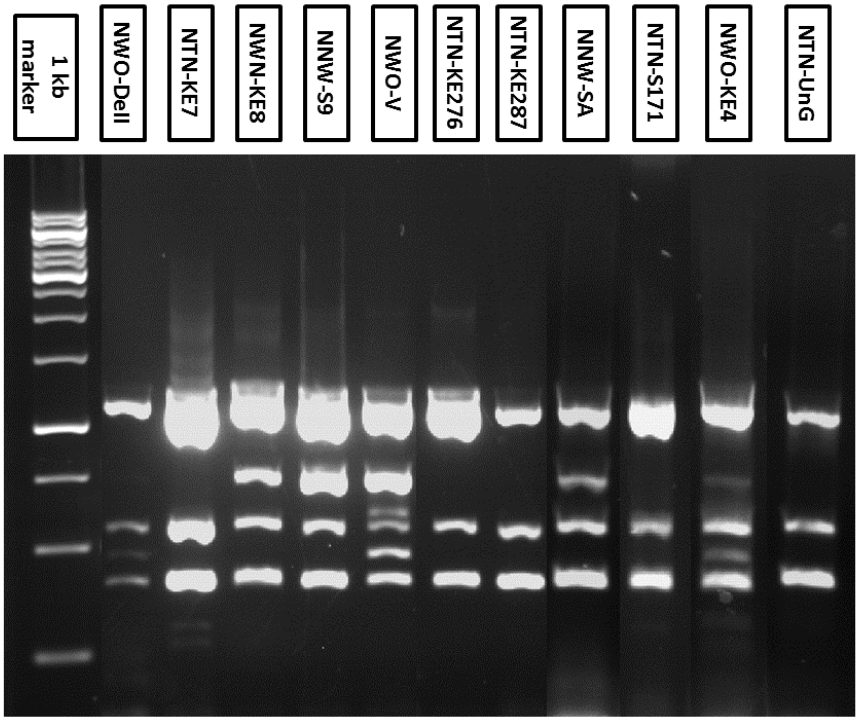

Figure S1.B. Gel electrophoresis of multiplex RT-PCR assay for PVY genotyping of different potato samples using Phusion High-Fidelity DNA Polymerase.

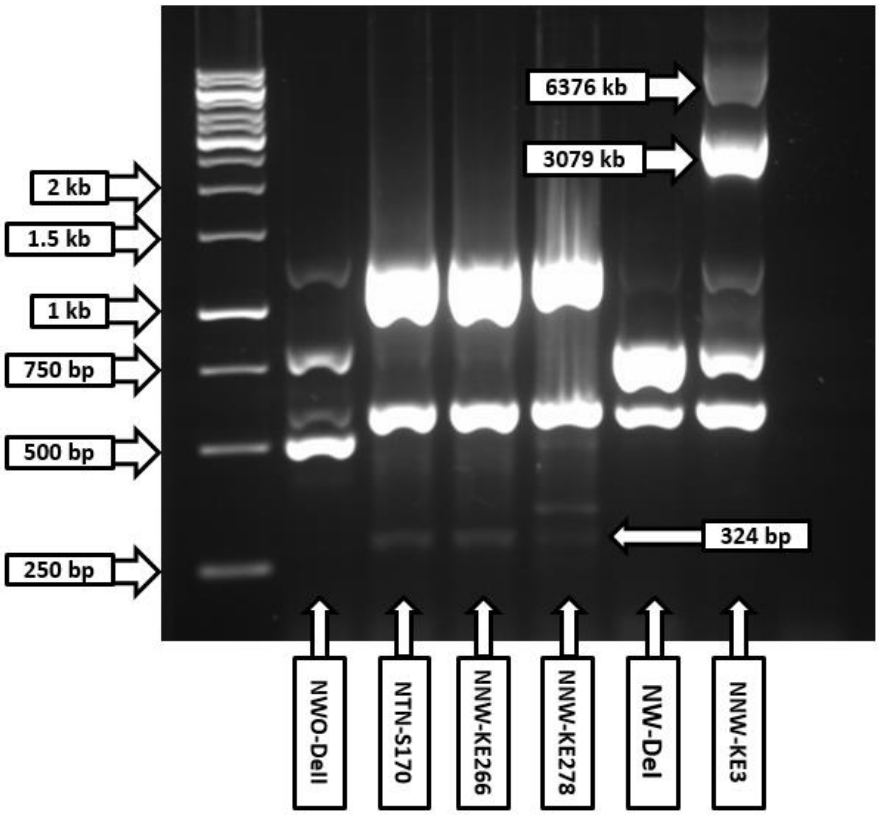

Figure S2. Gel electrophoresis of multiplex-PCR assay for PVY genotyping in different potato samples utilizing Phusion High-Fidelity DNA Polymerase showing unexpected bands of approximately 324, 370, 3079 and 6376 bp.

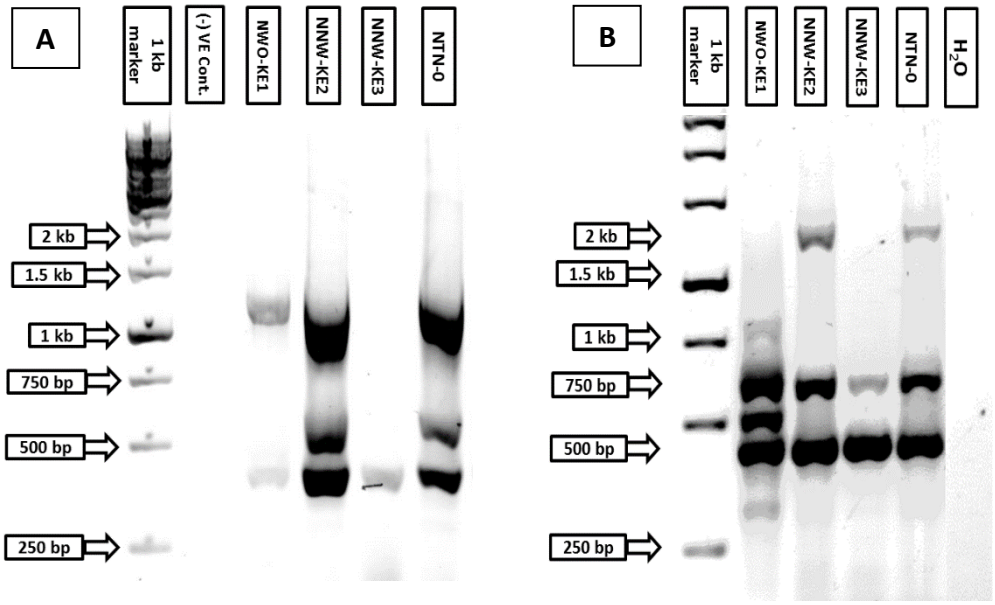

Figure S3. Gel electrophoresis of multiplex-PCR assay for PVY genotyping in samples NWO-KE1, NNW-KE2, NNW-KE3, and NTN-0 utilizing (A) *Taq* DNA polymerase (Thermo Scientific). (B) Platinum Pfx DNA Polymerase (Invitrogen).

Table S3. Results of GenBank searches with BLASTn for sequences of cloned PCR fragments

Sample ID	Genomic Coverage	Strain group	% Query cover	% identity	GenBank Accession no.
NTN-0	160 – 792 (633 bp)	PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN}	100	99	JF928460.1
		$PVY^{N:O}$	100	99	HQ912871.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{E}	100	99	JF928459.1
NTN-2	160 – 792 (633 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		$PVY^{N:O}$	100	99	HQ912872.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{E}	100	99	JF928459.1
NTN-5	160 – 792 (633 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		$PVY^{N:O}$	100	99	HQ912871.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{Z}-NTN	100	99	FJ204165.1
NW-DeI	5578– 6430 (853 bp)	PVY^{Z}	100	99	KP691322.1
		PVY^{N}-W	100	99	JQ924286.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{O}-O5	100	99	FJ643479.1
		$PVY^{N:O}$	100	99	HQ912872.1
NWO-KE1	160 – 792 (633 bp)	PVY^{Z}-NTN	100	100	KF850513.1
		PVY^{NTN}	100	100	HQ912869.1
		PVY^{Z}-NTN	100	100	FJ204165.1
		PVY^{NTN}	100	99	AB711146.1
		PVY^{NTN}	100	99	AB711145.1
	2169 – 2700 (532 bp)	PVY^{O}	100	99	HQ912889.1
		PVY^{O}	100	99	HQ912865.1
		PVY^{Z}	100	99	KP691322.1
		PVY^{O}	100	99	HQ912888.1
		PVY^{O}-O5	100	98	FJ643479.1
	2260 – 2700 (441 bp)	PVY^{NTN}	100	100	KM396648.1
		PVY^{NTN}	100	100	JQ969033.2
		PVY^{Z}-NTN	100	100	KF850513.1
		PVY^{Z}-NTN	100	100	FJ204165.1
		PVY^{NTN-NW}	100	100	AB461453.1

Cont. Table S3. Results of GenBank searches with BLASTn for sequences of cloned PCR fragments

Sample ID	Genomic Coverage	Strain group	% Query cover	% identity	GenBank Accession no.
NWO-KE1	5578– 6430 (853 bp)	PVY^{Z}	100	99	KP691322.1
		PVY^{N-W}	100	99	JQ924286.1
		PVY^{O}	100	99	HQ912889.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{O}	100	99	HQ912865.1
	8359 – 8634 (240 bp)	PVY^{Z}	100	100	KP691322.1
		PVY^{O}	100	100	HM590407.1
		PVY^{O}	100	100	AJ585196.1
		PVY^{O}	100	99	HQ912865.1
		PVY^{N-W}	100	99	JQ924286.1
NNW-KE2	160 – 792 (633 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN-NW}	100	99	AB461455.1
		PVY^{Z}-NTN	100	99	FJ204165.1
		$PVY^{N:O}$	100	99	AY745492.1
	2260 – 2700 (441 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN}	100	99	JQ969033.2
		PVY^{NTN-NW}	100	99	AB461453.1
		PVY^{NTN-NW}	100	99	AB461451.1
	7582 – 8145 (563 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN-NW}	100	99	AB461451.1
		PVY^{NTN-NW}	100	99	AB461453.1
		PVY^{Z}-NTN	100	99	FJ204165.1
NNW-KE3	160 – 792 (633 bp)	PVY^{NTN}	100	99	KM396648.1
		PVY^{Z}-NTN	100	99	KF850513.1
		$PVY^{N:O}$	100	99	HQ912871.1
		$PVY^{N:O}$	100	99	HQ912872.1
		$PVY^{N:O}$	100	99	AY745492.1
	5578– 6430 (853 bp)	PVY^{Z}	100	99	KP691322.1
		PVY^{N-W}	100	99	JQ924286.1
		$PVY^{N:O}$	100	99	HQ912871.1
		$PVY^{N:O}$	100	99	AY745492.1
		PVY^{O}-O5	100	99	FJ643479.1
	7582 – 8391 (809 bp)	PVY^{NTN}	100	99	JQ969033.2
		PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN-NW}	100	99	AB461452.1
		PVY^{NTN-NW}	100	99	AB461451.1
		PVY^{Z}-NTN	100	99	FJ204165.1
NNW-KE266	7582- 8888 (1307 bp)	PVY^{NTN-NW}	100	99	AB461453.1
		PVY^{NTN}	100	99	KM396648.1
		PVY^{NTN}	100	99	AJ890343.1
		PVY^{NTN}	100	99	AB711146.1
		PVY^{Z}-NTN	100	99	KF850513.1
NNW-KE278	7582- 8888 (1307 bp)	PVY^{Z}-NTN	100	99	FJ204165.1
		PVY^{NTN}	100	99	AJ890343.1
		PVY^{NTN}	100	99	AB711146.1
		PVY^{Z}-NTN	100	99	KF850513.1
		PVY^{NTN}	100	99	KM396648.1
NTN-S170	7582- 8888 (1307 bp)	PVY^{Z}-NTN	100	99	FJ204165.1
		PVY^{NTN}	100	99	KM396648.1
		PVY^{NTN}	100	99	AJ890343.1
		PVY^{NTN}	100	99	AB711146.1
		PVY^{NTN}	100	99	JQ969033.2

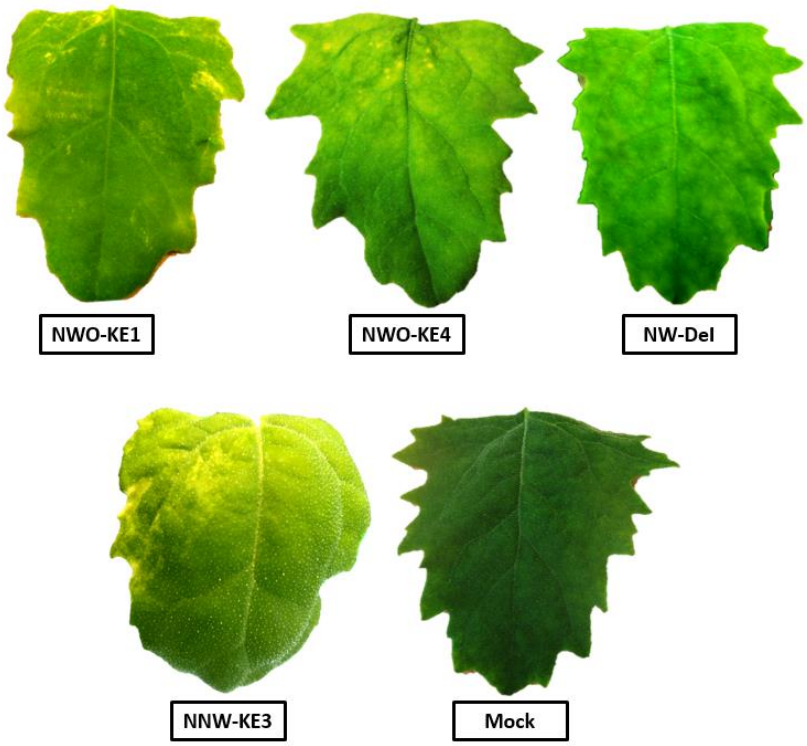

Figure S4. Symptoms induced by inoculation with extracts of samples NWO-KE1, NWO-KE4, NW-DeI, and NNW-KE3 on quinoa leaves compared with the mock treatment.

Table S4. Foliage symptoms recorded for potato cv. Désirée after inoculation with extract of ten different potato samples

Potato cultivar/Virus sample		NWO-Bdlm	NWO-C	NWO-F3	NWO-KE9	NNW-KE278	NWO-L	NWO-MB	NWO-Mt	NTN-S170	NWO-V
Désirée	15 dpi	M	MM	M	M	MM, Ch	M	M, Mo	MM	M	MM
	20 dpi	M	MM, M	MM, M	M	MM, Ch	MM, M, SM	M, Mo	MM	M	MM
	30 dpi	SM, DLL	MM	MM, LD	M, Mo, VN, LD	ChLL, ChYL	MM, Mo, DLL	MM, Mo, DLL	MM, M	MM, Mo, DLL	MM, M, LD
	45 dpi	SeM, DLL	MM, M, ChLL	MM, M, ChLL	Mo, VN, NL, LN, PN, LCr, SeSt	Ch	SeM	SeM, Mo, VN, LN, LD	MM, M, ChLL, MLCr	M, Mo, MLCr	MM, M, VN, PN, LCr

Ch = chlorosis, ChLL = chlorosis of lower leaves, ChYL = chlorosis in young leaves, DLL = death of lower leaves, dpi = days post inoculation, LCr = leaf crinkling, LD = leaf death, LN = leaf necrosis, M = mosaic, MLCr = mild leaf crinkling, MM = mild mosaic, Mo = Mottling, NL = necrotic lesions, PN = petiole necrosis, SeM = severe mosaic, SeSt = severe stunting, VN = veinal necrosis.

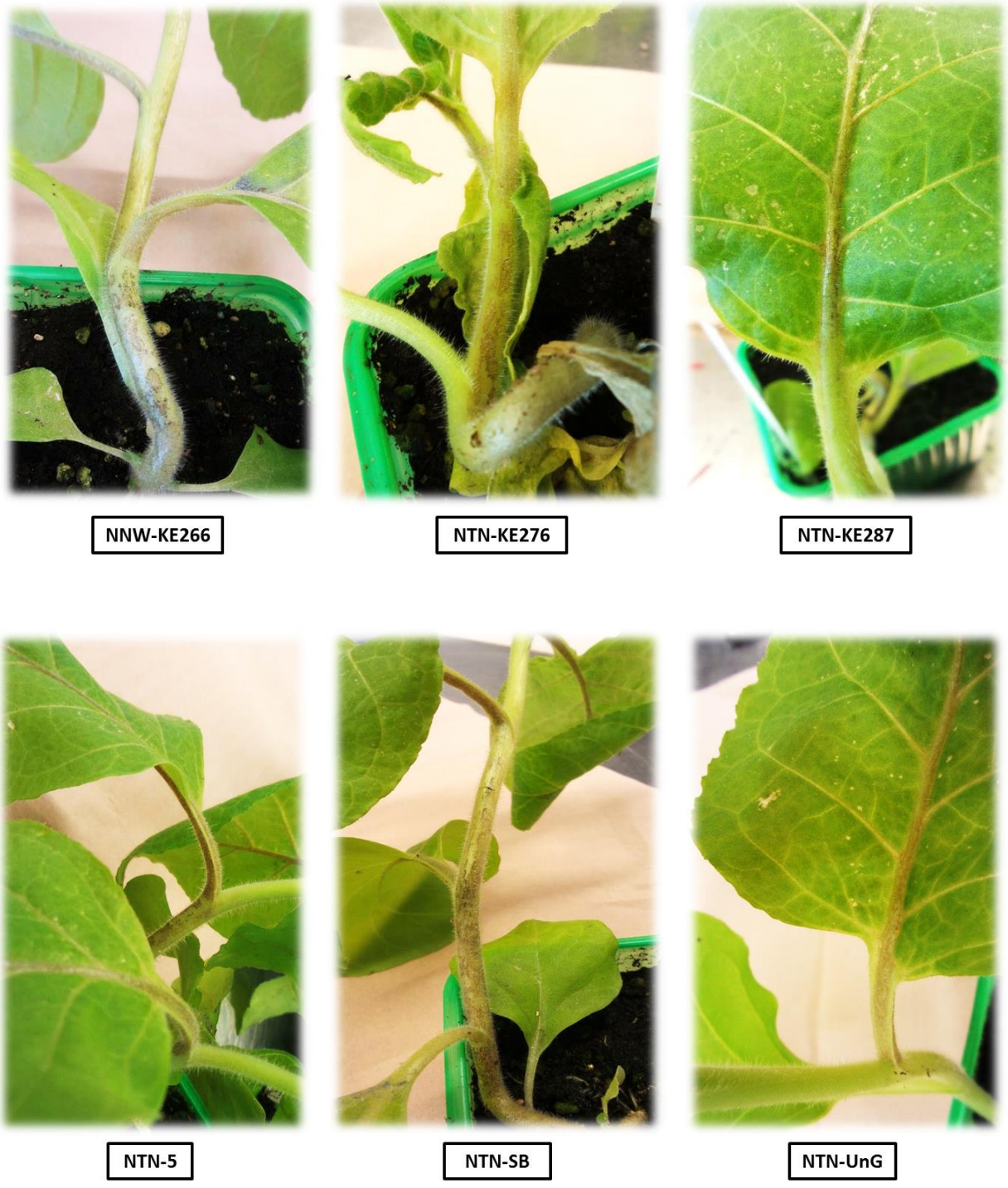

Figure S5. Examples of tobacco veinal and stem necrosis induced by inoculation with extracts of samples NNW-KE266, NTN-276, NTN-KE287, NTN-5, NTN-SB, and NTN-UnG on stems of tobacco cv. Samsun.

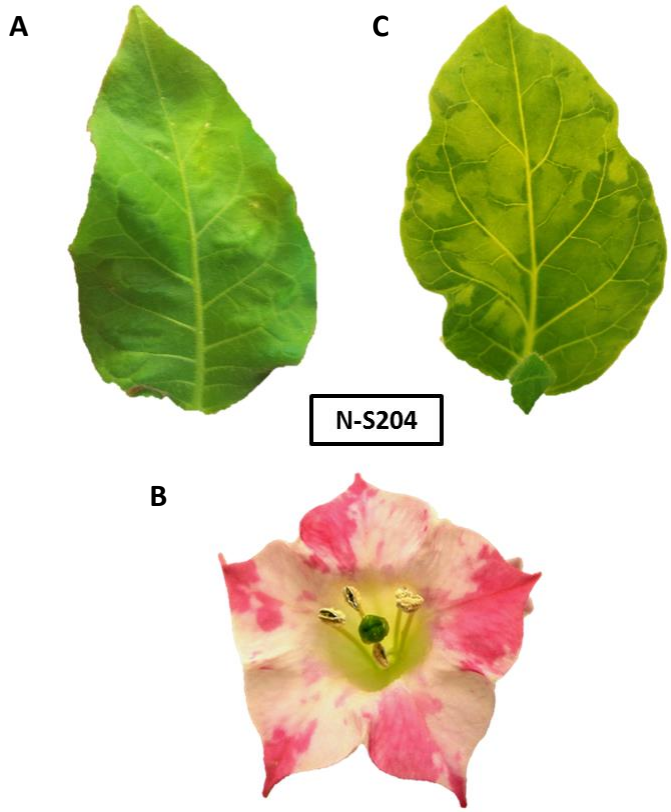

Figure S6. Symptoms induced by inoculation with extract of sample N-S204 on systemic leaves and flowers of tobacco cv. Samsun. (A) 30 dpi. (B) 90 dpi. (C) 130 dpi.

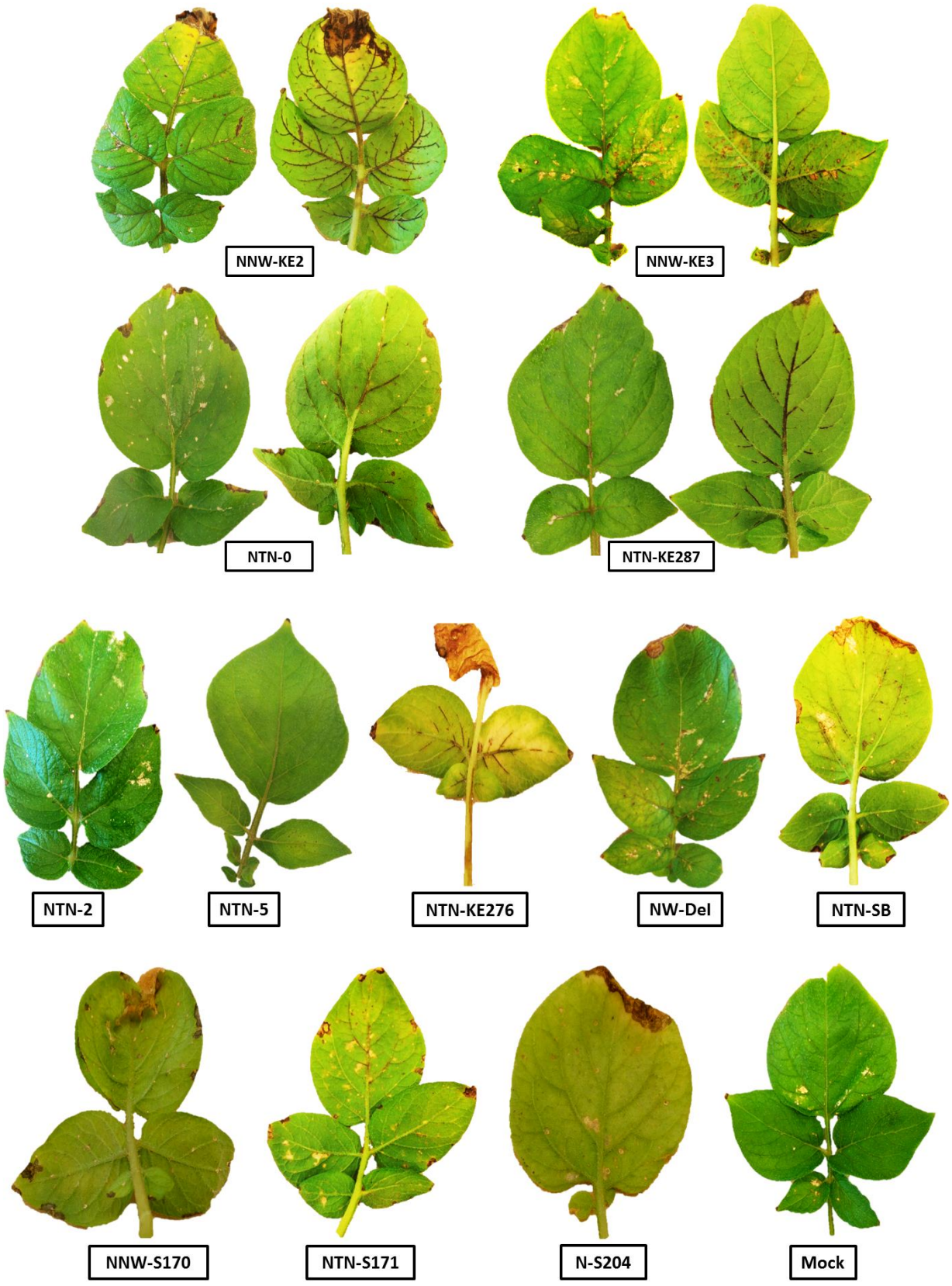

Figure S7. Screening potato cv. Désirée for hypersensitive response after mechanical inoculation with extract from 12 PVY-infected potato samples, which did not generate the diagnostic fragments for the non-recombinant PVY^{O} strain in multiplex-PCR assay. A mock-inoculated plant was included as a negative control.

Table S5. Screening for potato tuber necrotic ringspot disease (PTNRD) for 36 PVY samples after eight weeks of storage in the dark at room temperature

Sample ID	Potato Cultivar					
	Désirée		King Edward		Others	
	At harvest	During storage	At harvest	During storage	At harvest	During storage
NTN-0	None[a]	None	-[b]	-	cv. Unknown/ None	cv. Unknown/ PTNRD[c]
NTN-2	None	None	-	-	cv. Unknown/ None	cv. Unknown/ PTNRD
NTN-5	None	None	-	-	cv. Unknown/ PTNRD	cv. Unknown/ PTNRD
NNW-A	-	-	-	-	cv. Asterix/ None	cv. Asterix/ PTNRD
NWO-Bdlm	None	None	-	-	cv. Blue de la marche/ None	cv. Blue de la marche/ None
NWO-C	PTNRD	PTNRD	-	-	-	-
NW-DeI (Dutch)	None	None	-	-	-	-
N-DeIII (Dutch)	None	None	-	-	-	-
NTN-F1	-	-	-	-	cv. Folva/ PTNRD	cv. Folva/ PTNRD
NTN-F2	-	-	-	-	cv. Folva/ None	cv. Folva/ None
NWO-F3	None	None	-	-	-	-
NTN-G	None	None	-	-	cv. Unknown/ None	cv. Unknown/ None
NWO-KE1	None	None	None	None	-	-
NNW-KE2	None	None	None	None	-	-
NNW-KE3	None	None	None	None	-	-
NWO-KE4	None	None	None	None	-	-
NNW-KE6	-	-	None	None	-	-
NTN-KE7	-	-	None	None	-	-
NNW-KE8	-	-	None	None	-	-

[a]None = no symptoms, [b](-) = non-available, [c]PTNRD = potato tuber necrotic ringspot disease.

Cont. Table S5. Screening for potato tuber necrotic ringspot disease (PTNRD) for 36 PVY samples after eight weeks of storage in the dark at room temperature

Samples ID	Potato Cultivar					
	Désirée		King Edward		Others	
	At harvest	During storage	At harvest	During storage	At harvest	During storage
NWO-KE9	None	None	None	None	-[b]	-
E-KE10	-	-	None	None	-	-
NNW-KE266	None	None	None	None	-	-
NTN-KE276	None	None	None	None	-	-
NTN-KE287	None	None	-	-	-	-
NTN-KU	-	-	-	-	cv. King Utsäde / None	cv. King Utsäde / None
NWO-L	None	None	-	-	cv. Lurö/ None	cv. Lurö/ None
NWO-MB	None	None	-	-	cv. Magnum Bonum/ None	cv. Magnum Bonum/ None
NTN-Mn	-	-	-	-	cv. Mandel/ None	cv. Mandel/ None
NWO-Mt	None	PTNRD [c]	-	-	cv. Maritema/ None	cv. Maritema/ None
NNW-SA	-	-	-	-	cv. Solist/ None	cv. Solist/ None
NTN-SB	None	None	-	-	cv. Solist/ None	cv. Solist/ None
NNW-S9	-	-	-	-	cv. Solist/ None	cv. Solist/ PTNRD
NTN-S170	None	PTNRD	-	-	cv. Solist/ None	cv. Solist/ PTNRD
NTN-S171	None	PTNRD	-	-	-	-
N-S204	None	None	-	-	-	-
NWO-V	None	None	-	-	-	-

[a]None = no symptoms, [b](-) = non-available, [c]PTNRD = potato tuber necrotic ringspot disease.

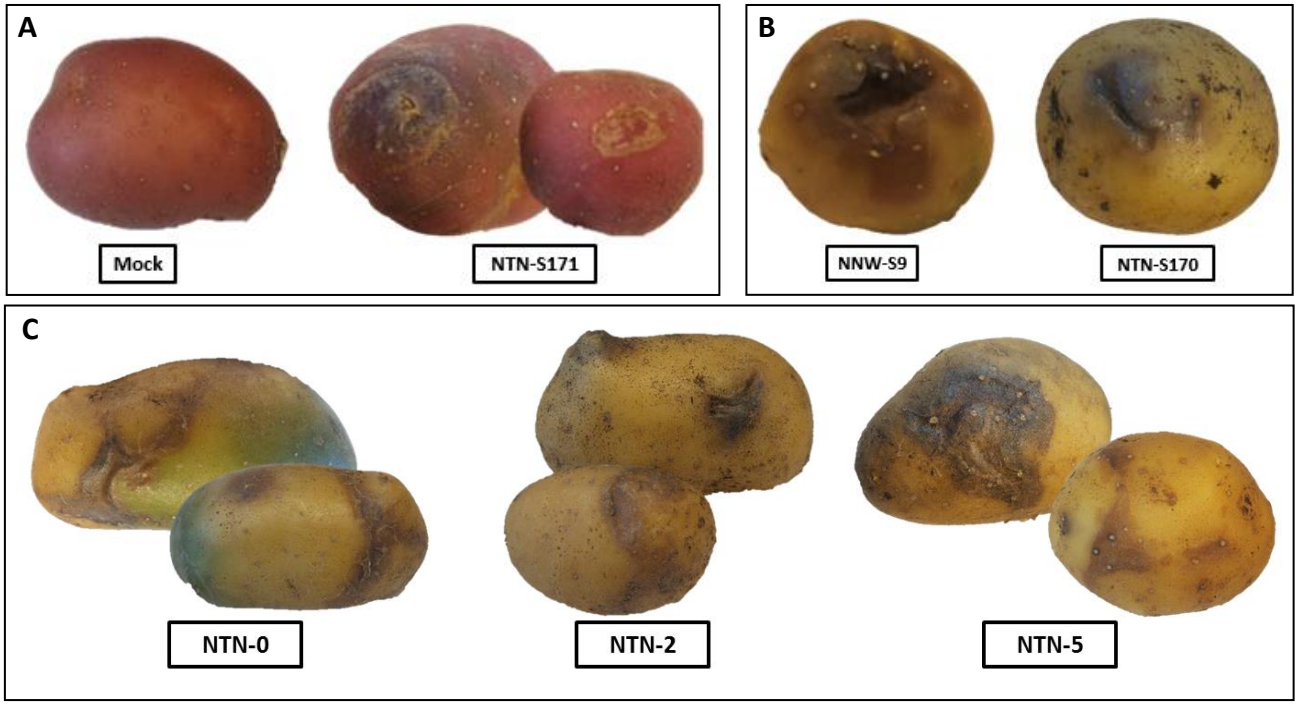

Figure S8. Examples of potato tuber necrotic ringspot disease symptoms on stored tubers. (A) Tubers of cv. Désirée plants inoculated with extract of NTN-S171 compared with mock treatment (virus-free). (B) Tubers of cv. Solist plants grown from samples NNW-S9 and NTN-S170. (C) Tubers of potato plants (cv. Unknown) grown from samples NTN-0, NTN-2 and NTN-5.

Nr. 157
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Department of Plant Biology
SLU
Box 7080
75007 Uppsala, Sweden